Theriogenology 82 (2014) 318–324

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Superstimulation prior to the ovum pick-up to improve *in vitro* embryo production in lactating and non-lactating Holstein cows

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ARTICLE INFO

Article history:
Received 10 January 2014
Received in revised form 11 April 2014
Accepted 11 April 2014

Keywords:
Bovine
Holstein
In vitro embryo production
Oocyte competence

ABSTRACT

The present study evaluated the efficacy of superstimulation with p-FSH (Folltropin) before the ovum pick-up (OPU) on IVP in lactating and nonlactating Holstein donors. A total of 30 Holstein cows (15 lactating and 15 nonlactating) were blocked by lactation status to one of two groups (control or p-FSH), in a cross-over design. On a random day of the estrous cycle, all cows received an intravaginal progesterone device and 2.0 mg IM of estradiol benzoate (Day 0). Cows in the control group received no further treatment, whereas cows in the p-FSH group received a total dosage of 200 mg of p-FSH on Days 4 and 5 in four decreasing doses 12 hours apart (57, 57, 43, and 43 mg). On Day 7, the progesterone device was removed, and OPU was conducted in both groups (40 hours after the last p-FSH injection in the p-FSH–treated group). There was no difference between groups ($P = 0.92$) in the numbers of follicles that were aspirated per OPU session (17.2 ± 1.3 vs. 17.1 ± 1.1 in control and p-FSH-treated cows, respectively); however, p-FSH-treated cows had a higher ($P < 0.001$) percentage of medium-sized follicles (6–10 mm) at the time of the OPU (55.1%; 285/517) than control cows (20.8%; 107/514). Although recovery rate was lower (60.0%, 310/517 vs. 69.8%, 359/514; $P = 0.002$), p-FSH-treated cows had a higher blastocyst production rate (34.5%, 89/258 vs. 19.8%, 55/278; $P < 0.001$) and more transferable embryos per OPU session were produced in the p-FSH group (3.0 ± 0.5 vs. 1.8 ± 0.4; $P = 0.02$). Regardless of treatment, non-lactating cows had a higher blastocyst rate (41.9%, 106/253 vs. 13.4%, 38/283; $P = 0.001$) and produced more transferable embryos per OPU session (3.5 ± 0.5 vs. 1.3 ± 0.3; $P = 0.003$) than lactating cows. Thus, superstimulation of Holstein donors with p-FSH before OPU increased the efficiency of IVP. In addition, non-lactating donors had higher percentage of *in vitro* blastocyst development and produced more embryos per OPU session than lactating cows.

1. Introduction

The success of dairy operations is related to increased genetic gain, reproductive efficiency and milk yield per cow.

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0093-691X/$ – see front matter © 2014 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2014.04.013

Among the reproductive biotechnologies, IVP has been considered an important alternative to rapidly enhance genetic progress through the female lineage in dairy cattle. However, oocyte quality has been considered an important factor [1–4] contributing to the low fertility reported for high producing lactating dairy cattle [5]. In addition, regardless the significant variation within animal [6,7], IVP efficiency has been reported to be lower in lactating Holstein cattle as compared with Holstein heifers [8] and beef cattle [9]. Therefore, further studies are required to evaluate alternative strategies to improve the efficiency of IVP in Holstein donors, especially those that are lactating.

The outcome of IVP programs has also been associated with the stage of follicular growth at which ovum pick-up (OPU) is performed [10–14]. The acquisition of developmental potential of oocytes (e.g., the ability of the oocyte to reach the blastocyst stage) has been associated with follicular growth, i.e., developmental competence continues to be enhanced as follicular diameter increases and approaches the LH surge [15–20]. It has been shown that during oocyte growth in cattle mRNA, and proteins are stored in the oocyte [21], and the composition of RNA is essential to sustain the first few cell cycles of early embryo development [22]. Therefore, there may be an ideal range during follicular development where IVP is optimized; and in that context, the use of protocols for follicular wave synchronization, and superstimulation before OPU may be a strategy to improve the efficiency of this technology in dairy cattle.

Superstimulation with porcine FSH (p-FSH) before the OPU has been used successfully for IVP programs in nonlactating *Bos taurus* donors, resulting in increased total embryo yields per OPU session [23,24], possibly because of the greater follicular diameters of the aspirated follicles. Another important and positive aspect regarding the improved efficiency of IVP after superstimulation is associated with the "coasting" period (i.e., a period of FSH withdrawal/starvation) in Holstein heifers and lactating cows [25,26]. Similar to the effect of follicular stage, the effect of gonadotropin starvation on the efficiency of IVP has been reported to be a simulation of the physiological alterations observed immediately before ovulation, resulting in improved oocyte competence [25]. However, the efficacy of "coasting" after superstimulation used in IVP in lactating donor cows has yet limited data. Considering the concerns related to the oocyte developmental competence (e.g., the oocyte capacity to yield into blastocyst) and donor lactational status, adjustments were studied to ascertain a follicular synchronization protocol for OPU which encompasses the beneficial effects of superstimulation (higher proportion of medium-sized follicles) and gonadotropin withdrawal (coasting), with the overall objective of improving the use of this biotechnology, especially in lactating dairy cattle.

Thus, the challenge is to promote the growth of a homogeneous follicle population and to recover competent oocytes suitable for IVP procedures. The present study evaluated the effect of superstimulation with p-FSH in Holstein oocyte donors (lactating and nonlactating cows) submitted to an OPU-IVP program. The hypothesis was that superstimulation with p-FSH before OPU in lactating and nonlactating Holstein donors would alter the proportion of medium-sized follicles available for OPU and enhance the *in vitro* competence of the recovered oocytes, increasing the general efficiency of IVP programs (number of embryos produced per OPU). We also hypothesized that OPU-IVP procedures would result in a higher number of blastocysts per OPU session in nonlactating than in lactating donors.

2. Materials and methods

2.1. Farm and animals

The present experiment was conducted in a commercial dairy farm in southeast Brazil (22°01'27″S/47°53'19″W) during January to March 2013. The herd was composed of 1500 lactating Holstein cows housed in free stall facilities, milked three times daily and with an average milk production of 30.1 ± 0.3 kg/day. The nonlactating cows were maintained in dry lot pens.

Lactating cows enrolled in the experiment trial were normal cycling cows with 172.9 ± 24.9 days in milk (± standard error of the mean [SEM]), had a daily milk production of 30.0 ± 1.3 (±SEM) and were on lactation number 1.7 ± 0.2 (±SEM). Nonlactating cows were normal cycling and on lactation number 2.2 ± 0.4 (±SEM).

All animals were fed with a total mixed ration formulated to meet or exceed the minimum nutritional requirements for lactating and non-lactating Holstein cows [27]. Briefly, the main ingredients were corn silage and Tifton hay as forage and a corn, soybean, and cottonseed meal-based concentrate.

2.2. Experimental design

A total of 30 Holstein donors (15 lactating cows and 15 nonlactating cows) were enrolled in cross-over experimental design, so that all females were submitted to both treatments: control and p-FSH. On random days of the estrous cycle (Day 0; AM) all cows received an intravaginal progesterone device (P4, Primer; Tecnopec, São Paulo, Brazil) and 2.0 mg intramuscular (IM) of estradiol benzoate (RIC-BE; Tecnopec). The control group received no further treatments, whereas the p-FSH group received 200 mg NIH-FSH-P1 (Folltropin; Bioniche Animal Health, Belleville, ON, Canada) divided into four decreasing doses (57, 57, 43, and 43 mg, 12 hours apart) on Days 4 and 5. On Day 7 AM (40 hours of "coasting" period in the pFSH group), the P4 devices were withdrawal immediately before OPU (Fig. 1).

2.3. Ultrasonography examinations

Immediately before the OPU session, both ovaries were examined by transrectal ultrasonography using a portable scanner (Aloka SSDV 500; Aloka, Tokyo, Japan) with 5 MHz convex array transducer housed in a plastic vaginal probe. All visible follicles were quantified and classified according to their diameters (small [SF $<$ 6 mm], medium [MF = 6–10 mm], and large [LF = $>$ 10 mm] follicles).

2.4. Ovum pick-up

All donors were subjected to OPU in the morning of Day 7 (40 hours after the last treatment in the pFSH group or

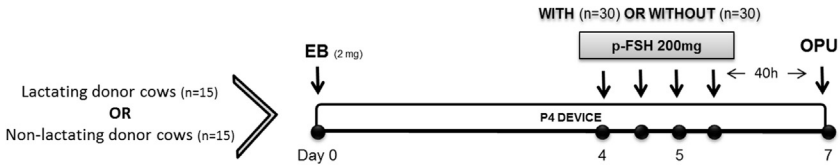

Fig. 1. Superstimulation protocol to evaluate the use of p-FSH before the ovum pick-up (OPU) in lactating (n = 15) and non-lactating (n = 15) Holstein donors in a cross-over experimental design. EB, 2.0 mg estradiol benzoate; P4 device, intravaginal device containing 1.0 mg of progesterone; p-FSH, 200 mg porcine FSH (57, 57, 43, and 43 mg; 12 hours apart).

"coasting" period). For the oocyte collection procedure, cattle were restrained in a chute, and epidural anesthesia was administered with lidocaine hydrochloride 2% (Lidovet; Bravet, Brazil) to facilitate the handling of the ovaries through the rectum. The perineal area was cleaned using water, dried and sprayed with alcohol before each session. All follicles of 2 mm or greater were aspirated using the portable scanner with a 5-MHz convex array transducer housed in a plastic vaginal probe with a stainless steel needle guide connected to aspiration equipment and a vacuum system. Follicular aspirates were recovered via a 1.1 mm i.d. by a 120 cm length circuit (Watanabe Tecnologia Aplicada, Cravinhos, SP, Brazil), connected directly to a disposable 20-ga × 2 inch hypodermic needle (0.9 × 50 mm; Terumo Europe NV, Belgium) and a 50-mL conical tube containing 15 mL of Dulbecco PBS (DPBS; Nutricell Nutrientes Celulares, Campinas, SP, Brazil) supplemented with 1% (vol/vol) fetal calf serum (FCS; Gibco Life Technologies, Grand Island, NY, USA) and 5000 IU/mL sodium heparin (Parinex; Hipolabor, Belo Horizonte, MG, Brazil) at 35 °C to 37 °C. The vacuum connected to the needle was set at 85 to 90 mm Hg. All retrieval procedures were performed by the same veterinarian. The conical tube containing the follicular aspirate was transported to a field laboratory and cumulus–oocyte complexes (COCs) were recovered using a 75 μm filter (Watanabe Tecnologia Aplicada) and DPBS supplemented with 1% FCS. The COCs were washed once in DPBS supplemented with 1% FCS at 37 °C and morphologically evaluated under a stereomicroscope at 8 to 20X magnification. The COCs were morphologically classified based upon the number of cumulus cell layers as follow: Grade 1, more than three layers of compact cumulus cells; Grade 2, at least one layer of cumulus cells; Grade 3, denuded; and Grade 4, atretic, with dark cumulus cells and signs of cytoplasmic degeneration [28]. After evaluation, only Grade 4 COCs were considered non-suitable to culture and discarded. The COCs considered suitable to culture were transported to the IVP laboratory in 1.5 mL cryotubes containing HEPES-buffered tissue culture medium 199 (TCM-199; Gibco Life Technologies, Grand Island, NY), 10% FCS, 49.4 mg/mL sodium pyruvate (Sigma–Aldrich Chemical Co.; St. Louis, MO), and 50 μg/mL gentamycin at 37 °C to 39 °C.

2.5. In vitro embryo production

Before IVM, COCs were washed three times in HEPES-buffered TCM-199, supplemented with 10% FCS and 50 μg/mL gentamycin, and once in maturation medium, composed of bicarbonate-buffered TCM-199 (Gibco Life Technologies) supplemented with 10% FCS, 50 μg/mL LH (APL, Ayerst, Rouses Point, NY), 5 μg/mL FSH (Folltropin-V, Bioniche Animal Health, Canada), 0.1 μg/mL estradiol (Estradiol 17β; Sigma–Aldrich Chemical Co.), 22 μg/mL sodium pyruvate, and 50 μg/mL gentamycin. The COCs of each cow were cultured separately for 24 hours in 70 μL drops of maturation medium under mineral oil (D'Altomare, São Paulo, SP, Brazil) at 39 °C in an atmosphere of 5% CO_2 in humidified air. After IVM, the COCs were washed and subjected to IVF in 70 μL drops of IVF medium under mineral oil. The IVF medium was Tyrodes albumin lactate pyruvate (TALP; Bavister and Yanagimachi, 1977) supplemented with 10 μg/mL heparin, 22 μg/mL sodium pyruvate, 50 μg/mL gentamycin, 6 mg/mL fatty acid-free BSA, and PHE solution (2 μM penicillin, 1 μM hypotaurine, and 0.25 μM epinephrine).

For IVF, semen straws were thawed for 30 seconds in a 35 °C water bath and semen was deposited on a 90% to 45% Percoll gradient prepared with sperm wash medium (modified Tyrode medium) and centrifuged at 320 × *g* for 30 minutes to separate the motile sperm and to remove the diluents and seminal plasma. Then, the sperm pellet was evaluated for motility and concentration. Each fertilization droplet received 5 μL of sperm, to achieve a final concentration of 1×10^6 live sperm/mL. Sperm and COCs were incubated at 38.5 °C in an atmosphere of 5% CO_2 in humidified air for 18 to 20 hours. The same sire was used with each donor during the cross-over.

Approximately 18 hours after insemination, presumptive zygotes were stripped of cumulus cells by mechanical pipetting in TALP medium. Groups of presumptive zygotes were cocultured on a monolayer of cumulus cells that had attached to the surface of the plate during IVM. Thus, to maintain the maximum amount of cumulus cells, the IVM medium was gently replaced with 50 μL of CR2aa medium (Watanabe et al., 1999) supplemented with 2% FCS and 30 mg/mL BSA for embryo culture at 39 °C in an atmosphere of 5% CO_2 in humidified air for 48 to 72 hours, at which time 30 μL of fresh culture medium was added (first feeding). Cleavage rate was recorded after 3 days of embryo culture. The second feeding was done on the sixth day of embryo culture, and the blastocyst rate (total number of blastocysts divided by total number of cultured oocytes) was recorded on the seventh day of embryo culture.

2.6. Statistical analysis

Statistical analyses were performed using the GLIMMIX procedure of the Statistical Analysis System for Windows 9.3 (SAS 9.3). The variables evaluated were the number of follicles in each size category at the time of OPU (small,

medium, and large), total number of follicles aspirated, total number of COCs recovered, recovery rate (total number of COCs recovered per total number of follicles aspirated), number and percentage of cultured COCs (number of COCs cultured per total structures recovered), cleavage rate (number of cleaved zygotes per total number of COCs cultured), blastocyst rate (number of blastocysts produced per total number of COCs cultured), and number of embryos produced per OPU procedure.

For the analysis, a binomial distribution was assumed for the categorical response variables. Continuous data were tested for normality of the residues and homogeneity of variances using the Guided Data Analysis, and transformed when necessary. The fixed effects included in the model were treatment (control vs. p-FSH), lactation status (lactating vs. nonlactating) and their interactions. The individual effect was included as a random effect.

Means ± SEM are used to describe all of the response variables. Differences with $P \leq 0.05$ were considered statistically significant, and $0.05 < P < 0.10$ were designated as a tendency.

3. Results

There were no significant interactions between treatment and donor lactational status for any response variable (Table 1). There was no effect ($P = 0.92$) of treatment on the total number of follicles aspirated; however, p-FSH-treated donors had a lower proportion of small ($P < 0.001$) and large follicles ($P = 0.03$), and a higher proportion of medium follicles ($P < 0.001$) at the time of OPU (Fig. 2). Also, because of a tendency for a reduction in the number of COCs recovered from p-FSH-treated donors ($P = 0.10$), the lower recovery rate was significant ($P < 0.001$; Table 1).

Although there was no treatment effect on the number of COCs considered suitable to culture ($P = 0.52$), a higher percentage ($P = 0.05$) of COCs from p-FSH-treated donors was considered viable to use in IVP (Table 1). Although there were no differences in cleavage rates ($P = 0.81$), the p-FSH group had a higher blastocyst rate ($P < 0.001$; Table 1), and as a result, superstimulated Holstein donors produced more embryos per OPU session ($P = 0.01$; Table 1).

Regardless of treatment groups, donor lactational status did not affect any of the follicular, COCs or cleavage characteristics (Table 1). However, nonlactating cows produced a higher blastocyst rate ($P = 0.001$) and a higher number of transferable embryos ($P = 0.003$) than lactating Holsteins cows (Table 1).

4. Discussion

The results of the present study confirm the positive effect of superstimulation of bovine donors with p-FSH on the overall efficiency of the OPU-IVP technology [7,25,26]. Donors treated with p-FSH in the present study had a greater proportion of medium-sized follicles, an increased proportion of COCs suitable to culture and higher developmental rates of recovered COCs (higher blastocyst rate and numbers of embryos per OPU session); however, the lower recovery rate in p-FSH treated donors lessened the overall benefit to superstimulation. These data provide support for an alternative method to enhance the IVP production among lactating and non-lactating Holstein donors.

Previously, it was shown that 70% to 80% of the small follicle (<5 mm) population viewed at the beginning of the superstimulation treatments responded to exogenously administered [29,30] and in another study, became available for OPU (Durocher et al., 2006). Accordingly, the FSH treatments have been used to superstimulate donors for IVP [23,25,31]. Therefore, the reasoning for increasing the proportion of medium-sized follicles for aspiration, as reported previously [26,32], and as observed in the present study was based on the observation that oocyte development competence after OPU was influenced by the stage of follicular development [19].

The acquisition of oocyte developmental potential has been shown to be associated with follicular growth [15–17]. A sequence of molecular and transcriptomal alterations during follicular (and oocyte) growth has been related to final oocyte development potential [19,21,33], indicating that oocyte development competence is acquired gradually during follicle growth. Mourot, Dufort [34] have reported

Table 1
Summary of oocyte and embryo results (mean ± SEM) after OPU-IVP in control and p-FSH–treated donors (lactating and non-lactating Holstein cows).

Items	Lactating donors		Nonlactating donors		P-value[e]		
	Control	p-FSH	Control	p-FSH	Treatment	Lactation	Treat × Categ
No.	15	15	15	15	–	–	–
Total follicles aspirated	17.6 ± 1.6	18.2 ± 2.1	16.7 ± 1.5	16.3 ± 1.6	0.92	0.52	0.62
Total oocytes retrieved	13.0 ± 1.7	10.7 ± 1.5	10.9 ± 1.6	9.9 ± 1.5	0.10	0.51	0.54
Recovery rate, %[a]	73.9 (195/264)	59.0 (161/273)	65.6 (164/250)	61.1 (149/244)	<0.001	0.89	0.08
COCs cultured	10.0 ± 1.3	8.9 ± 1.3	8.5 ± 1.4	8.3 ± 1.3	0.52	0.58	0.57
COCs culture rate, %[b]	76.9 (150/195)	82.6 (133/161)	78.0 (128/164)	83.9 (125/149)	0.05	0.77	0.88
Cleavage rate, %[c]	65.3 (98/150)	63.2 (84/133)	72.7 (93/128)	72.8 (91/125)	0.81	0.16	0.69
Blastocyst rate, %[d]	10.8 (15/150)	17.3 (23/133)	31.3 (40/168)	52.8 (66/125)	<0.001	0.001	0.16
Embryos produced per OPU	1.0 ± 0.4	1.5 ± 0.5	2.7 ± 0.6	4.4 ± 0.8	0.01	0.003	0.17

Treat × Categ interaction between treatment and donor lactation status.
[a] No. COCs/no. follicles aspirated.
[b] No. COCs cultured/no. total COCs retrieved.
[c] No. cleaved zygotes/no. oocytes cultured.
[d] No. blastocysts/no. oocytes cultured.
[e] Treatment, effect of treatment (control vs. p-FSH); lactation, effect of donor lactation status (lactating vs. nonlactating).

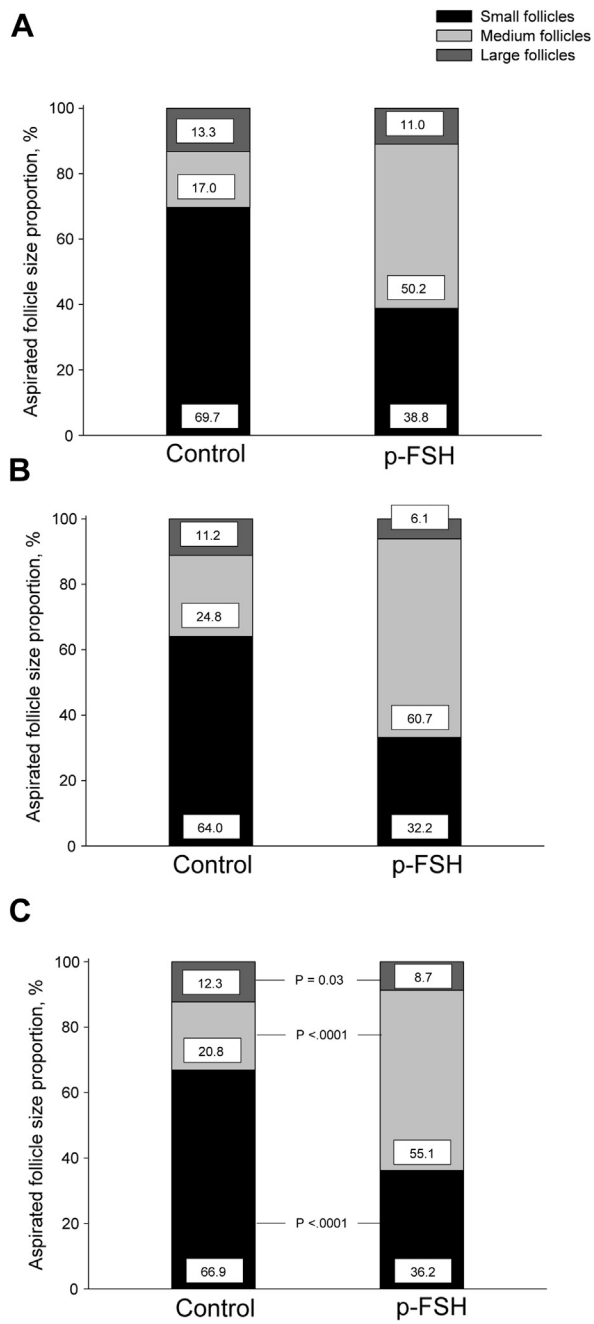

Fig. 2. Proportion of small (<6 mm), medium (6–10 mm), and large follicles (>10 mm) immediately before OPU (Day 7) in lactating (A; n = 30), non-lactating (B; n = 30), and both lactating and non-lactating Holstein donors (C; n = 60) submitted to OPU with and without p-FSH superstimulation. P-values within follicle size of 0.05 or less differ significantly. No donor lactation status or interaction between treatment and lactation status was observed (P > 0.05).

differences in oocyte gene expression profiles according to the size of the follicle from which the oocyte was retrieved e.g., higher mRNA levels for PSMB2, SKIIP, CDC5L, RGS16, and PRDX1 in oocytes of follicles more than 8 mm. In addition, Chu, Dufort [35] demonstrated increased expression of PTTG1, BTG4, PAPOLA and LEO1 genes in oocytes retrieved from superstimulated donors compared to untreated donors. Considering that these genes are related to transcription and cell cycle regulation, the authors suggested that stimulation with exogenous FSH could allow for the accumulation of more messenger, which would result in the preservation of oocyte quality. Collectively, these observations suggest that potential factors affecting oocyte developmental competence might be altered by superstimulation with exogenous FSH prior to the OPU.

The oocyte quality concept involves the competence to yield a viable blastocyst within an *in vitro* production system [7]. In this context, donors superstimulated with p-FSH in the present study had increased blastocyst production rates compared with the untreated donors. Similar results were reported by Blondin, Bousquet [25] in a study performed in *Bos taurus* beef cattle, also treated with exogenous FSH. However, recent report has shown that the oocyte quality gained after an optimal coasting period has limited lifespan, possibly because of an intensification of the transcript degradation mechanism [36]. Therefore, the present data suggest that the increased *in vitro* embryo production of donors superstimulated with p-FSH before OPU was related to the gonadotropin stimulus effect. However, it is important to highlight that this positive effect can be a response to an associative influence of the p-FSH treatment added to the fact that all p-FSH-treated donors had a 40 hours coasting period.

Although oocyte quality has been shown to improve with follicle growth [12], follicle size was negatively correlated with the recovery rate in *Bos taurus* cows [28] and heifers [23]. Similar results were observed in the present study with Holstein donor cows. Seneda, Esper [28] suggested that the reduced volume and viscosity of follicular fluid and the lower intrafollicular pressure of small follicles might favor the OPU procedure (i.e., increased COCs recovery rates following intrafollicular needle insertion) compared with the large follicles. Therefore, as a matter of necessity, the mechanism to retrieve the maximum proportion of COCs with optimal development potential and without adversely affecting recovery rate needs to be studied and established to enhance IVP systems.

Despite the improvement in the IVP in lactating donors after p-FSH treatment in the present study, the efficiency of the *in vitro* system was even greater in nonlactating Holstein donors. Superovulation and embryo production has also been reported to be higher in nonlactating cows or heifers compared with lactating Holstein cows [3,37,38]. Dairy cows present a peculiar metabolic system, linked to nutrition and disruption of endocrine profiles. Lactating dairy cows metabolic profile are commonly characterized by the lower concentrations of progesterone and estradiol [39] and increased concentrations of NEFA (nonesterified fatty acids) and BHBA (β-hydroxybutyrate [40]); and this peculiar metabolism has been associated with a suboptimal follicle microenvironment, compromising oocyte quality and resulting in a failure to conceive [1,5,39,41–43]. Therefore, the greater challenge of lactating cows to maintain an optimal reproductive efficiency might be part of the explanation to the lower results observed in the IVP. Although IVP was improved after treatment with p-FSH in lactating donors, lactation results in such highly modified metabolism that it will be difficult to completely

overcome treatment strategies. Accordingly, nonlactating donors may be considered the preferred donor to be enrolled in OPU-IVP programs, due to the higher yield of embryos per OPU session.

In conclusion, superstimulation with p-FSH increased the proportion of medium-sized follicles available for the OPU procedure. Consequently, the treatment also enhanced the proportion of COCs suitable for culture and resulted in greater blastocyst rates and embryo yield per OPU-IVP session. Regardless of gonadotropin treatment, nonlactating donors had higher *in vitro* oocyte competence and produced more embryos per OPU session, resulting in an overall higher OPU-IVP efficiency compared with lactating donors. It can be concluded that p-FSH superstimulation treatment can effectively improve embryo yield of *in vitro* production systems in lactating or nonlactating Holstein donors. Importantly, nonlactating donors should be the elective lactation status to be used in OPU-IVP programs, regardless superstimulation treatments.

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