Theriogenology 138 (2019) 164–168

Contents lists available at ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

Superstimulation prior to the ovum pick-up improves the *in vitro* embryo production in nulliparous, primiparous and multiparous buffalo (*Bubalus bubalis*) donors

Júlia Gleyci Soares de Carvalho [a, c, *], Nelcio Antonio Tonizza de Carvalho [b], Bernardo Marcozzi Bayeux [c], Yeda Fumie Watanabe [d], Osnir Yoshime Watanabe [e], Rodolfo Daniel Mingoti [c], Pietro Sampaio Baruselli [c, **]

[a] *Universidade Federal de São Paulo, Escola Paulista de Medicina, Centro de Pesquisa em Urologia, São Paulo, São Paulo, Brazil*
[b] *Unidade de Pesquisa e Desenvolvimento de Registro, Centro de Pesquisa de Zootecnia Diversificada, Instituto de Zootecnia, Registro, São Paulo, Brazil*
[c] *Departamento de Reprodução Animal, Universidade de São Paulo, São Paulo, São Paulo, Brazil*
[d] *Vitrogen – YVF Biotech, Cravinhos, São Paulo, Brazil*
[e] *WTA - Watanabe Tecnologia Aplicada, Cravinhos, São Paulo, Brazil*

A R T I C L E I N F O

Article history:
Received 29 April 2019
Received in revised form
2 July 2019
Accepted 4 July 2019
Available online 9 July 2019

Keywords:
Buffaloes
Follicle
FSH
Oocyte
OPU-IVEP

A B S T R A C T

The aim of this study was to evaluate the ovarian follicular population, the oocyte yield and the *in vitro* embryo production (IVEP) of nulliparous (NU), primiparous (PR) and multiparous (MU) buffalo donors submitted to the superstimulation with FSH prior to the *ovum pick-up* (OPU). A total of 54 buffalo donors (18 NU, 15 PR and 21MU) received an intravaginal progesterone device (1.0 g) plus estradiol benzoate [2.0 mg, intramuscular (im)] at random stage of the estrous cycle (Day 0) during the breeding season (autumn and winter). Buffaloes from different categories were then randomly allocated to one of two groups (Control or FSH), in a cross-over experimental design. Buffalo donors in the Control group received no further treatment, whereas buffalo donors in the FSH group received a total dosage of 200 mg im of FSH on Days 4 and 5, in four decreasing doses 12 h apart (57, 57, 43 and 43 mg). On Day 7, the progesterone device was removed and the OPU procedure was performed in both groups. The same semen was used across all replicates and donor category. Data were analyzed by the GLIMMIX procedure of SAS 9.4®. There was no interaction between FSH treatment and animal category for all analyzed variables. Furthermore, no differences between animal category (P = 0.73) and FSH treatment (P = 0.53) were observed regarding the total follicles aspirated. However, the FSH treatment increased (P < 0.001) the proportion of large (>10 mm; FSH = 16.2% and Control = 2.0%) and medium-sized follicles (6–10 mm; FSH = 36.3% and Control = 6.1%) available for the OPU procedure. The total of recovered oocytes was greater in NU than in MU, and PR were similar to NU and MU (P = 0.05). No effect of FSH treatment was observed (P = 0.85) for this variable. Buffalo donors treated with FSH had a greater viable oocytes rate (P = 0.03), blastocyst rate (P = 0.03) and embryo yield per OPU-IVEP session (P = 0.07), however, no category effects were observed for these variables. These results provided evidence that superstimulation with FSH increased the proportion of large and medium-sized follicles available for the OPU procedure. Consequently, the FSH treatment enhanced the proportion of viable oocytes for culture and resulted in greater blastocyst rates and embryo yield per OPU-IVEP session in all buffalo donors categories.

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1. Introduction

The association of OPU with IVEP represents a biotechnique with great potential to improve the reproductive results and the dissemination of selected genetics in buffalo herds, contributing significantly to the increase in meat and milk production. The success of OPU-IVEP is directly related to oocyte quantity and

* Corresponding author. Universidade Federal de São Paulo, Escola Paulista de Medicina, Centro de Pesquisa em Urologia, São Paulo, São Paulo, Brazil.
** Corresponding author.
E-mail addresses: soaresjuliag@gmail.com (J.G.S. de Carvalho), barusell@usp.br (P.S. Baruselli).

https://doi.org/10.1016/j.theriogenology.2019.07.003
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quality [1–3]. However, the use of this biotechnology in buffalo has shown limited results due to certain characteristics inherent to the species, such as the low number of ovarian follicles and viable oocytes recovered per OPU [4–8], as well as the effect of the seasonality on oocytes quality [9,10].

The superstimulation with FSH prior to OPU has been successfully used in IVEP programs in cattle, resulting in the increase of total embryos produced per OPU session [11–13]. The FSH treatment for superstimulation prior to OPU can promote the growth of a homogenous follicular population and the recovery of competent oocytes suitable for the IVEP procedures [13,14]. Furthermore, follicles with larger diameters improved oocyte quality and the IVEP efficiency. 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]. A sequence of molecular and transcriptomal alterations during follicular (and oocyte) growth has been related to final oocyte development potential [19,21,22], indicating that oocyte development competence is acquired gradually during follicle growth. It is well established that oocyte quality is correlated to follicular diameter [23].

The follicle population quickly decreases with aging [24]. Therefore, heifers have more antral follicles in their ovaries [25], which could be associated with more efficient IVEP programs. However, there are some concerns that oocytes from young females have a lower developmental capacity than those from adult donors [26–29]. Consequently, the FSH treatment could be used as a superstimulation treatment to improve the OPU/IVEP efficiency in buffalo nulliparous heifers, as previously observed in cattle [30].

The aim of the present study was to evaluate the follicular population, the oocyte yield and the *in vitro* embryo production of buffalo donors from different categories submitted to FSH superstimulation before OPU. The hypothesis was that treatment with FSH prior to the ovum pick-up would improve the *in vitro* embryo production of buffalo donors, mainly in nulliparous.

2. Materials and methods

2.1. Animals and management

This experiment was conducted at Unidade de Pesquisa e Desenvolvimento de Registro (Centro de Pesquisa de Zootecnia Diversificada - Instituto de Zootecnia, Registro, São Paulo, Brazil, located at latitude 24° 26′ 15″ S and longitude 47° 48’ 45” W), during the breeding season which coincides with a decrease in day length (autumn and winter). Murrah buffalo (*Bubalus bubalis*) females (n = 54) of three categories [nulliparous (n = 18), primiparous (n = 15) and multiparous (n = 21)] were used. Nulliparous were normal cycling and presented 2.7 ± 0.1 [mean ± SEM (standard error of the mean)] years old and body condition score (BCS) of 3.8 ± 0.1 (scale 1–5, where 1 = very thin and 5 = very fat). Primiparous and multiparous cows were normal cycling and presented 236.5 ± 15.1 and 218.0 ± 22.1 days in milk, 4.4 ± 0.1 and 10.4 ± 0.7 years old and BCS of 2.9 ± 0.1 and 3.8 ± 0.1; respectively. The animals were maintained on a *Brachiaria brizantha* pasture with free access to water and mineralized salt.

2.2. Experimental design

All buffaloes (n = 54) received an intravaginal progesterone device (P4; 1.0 g; Sincrogest®, Ourofino Agronegócio, Brazil) plus 2.0 mg im EB (Sincrodiol®, Ourofino Agronegócio) at random stage of the estrus cycle (designated Day 0; D0 AM). Buffaloes were then randomly allocated into two groups: Control and treatment with FSH previously to OPU (FSH). The control group received no further treatments, whereas the FSH group received 200 mg im FSH (Folltropin®; Bioniche Animal Health, Belleville, ON, Canada) divided into four decreasing doses (57, 57, 43 and 43 mg, 12 h apart) on Days 4 and 5. On Day 7 a.m. (40 h of “coasting” period in the FSH group; [14]), the P4 device was removed and the OPU procedure was performed shortly thereafter (Fig. 1). The treatments were repeated twice in a crossover design performed with a 30-day interval between OPU, in two replicates.

2.3. Ultrasonographic examinations

Ovaries were scanned by ultrasonography using a 7.5-MHz linear-array transrectal transducer (Mindray® DP-2200Vet; Shenzhen, Guangdong, China). Ovarian ultrasonographic examinations were performed on Day 7, immediately before the OPU session, in order to quantify and classify all the ovarian follicles according to their respective diameters: small [SF < 6 mm], medium [MF = 6–10 mm], and large [LF > 10 mm] follicles.

2.4. Ultrasound-guided ovum pick-up (OPU) procedure

All donors were restrained in a chute and epidural anesthesia was administered using 2% lidocaine hydrochloride (Lidovet, Bravet, Brazil). All follicles of 2 mm or more were aspirated using a portable scanner with a 5-MHz convex array transducer (Mindray DP - 2200 Vet, China). The latter was assembled into a vaginal-handle equipped with a stainless steel needle guide (20 G; 0.9 × 50 mm; Terume Europe NV, Belgium) connected to a vacuum pump system (85–90 mmHg of negative pressure; V-MAR 5000, Cook Australia, Queensland, Australia). Follicular contents were recovered using a 1.1 mm inner diameter by 120 cm length tubing (Watanabe Tecnologia Aplicada Ltda, Cravinhos, São Paulo, Brazil) and connected to a 50-mL conical tube containing 15 mL of Dulbecco phosphate – buffered saline (DPBS; Nutricell Nutrientes Celulares, Campinas, São Paulo, Brazil) and 5000 IU/mL sodium heparin (Parinex, Hipolabor, Belo Horizonte, Minas Gerais, Brazil) at 35 to 37 °C. All oocyte recovery procedures were performed by the same operator.

The conical tube containing follicular contents was immediately transported to a laboratory and cumulus-oocyte complexes (COCs) were recovered using a 75-µm filter (Watanabe Tecnologia Aplicada Ltda) and DPBS supplemented with 1% fetal bovine serum (FBS). The COCs were washed once in DPBS with 1% (FBS; Gibco, Invitrogen Co.) at 37 °C and morphologically evaluated under a stereomicroscope at × 8 to × 20 magnification. The COCs were

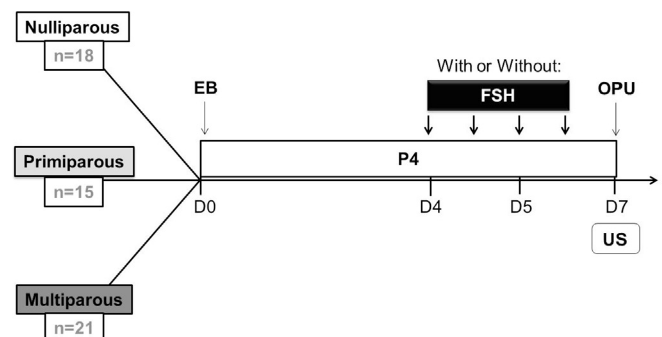

Fig. 1. Schematic diagram of the superstimulation protocol for OPU in three categories (Nulliparous, Primiparous and Multiparous) of buffalo donors. EB = 2.0 mg estradiol benzoate; P4 = 1.0 g intravaginal progesterone device; FSH = 200 mg follicle-stimulating hormone (57, 57, 43 and 43 mg; 12 h apart); US = ultrasonographic examination.

morphologically classified on the basis of the number of cumulus cell layers as follows: 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 cytoplasm degeneration [31]. After evaluation, only grade 4 COCs were considered nonsuitable for culture and were discarded. The remaining COCs were transported to the IVEP laboratory in 1.5-mL cryotubes containing HEPES buffered tissue culture medium 199 (TCM 199; Gibco Life Technologies), 10% FBS, 49.4 μg/mL sodium pyruvate (Sigma–Aldrich Chemical Co., St. Louis, MO, USA), and 50 μg/mL gentamycin at 37 °C to 39 °C.

2.5. COCs processing and in vitro embryo production (IVEP)

The COCs were washed in TCM-199 HEPES (Gibco, Invitrogen Co., Grand Island, NY, USA) with 10% (vol/vol) (FBS; Gibco, Invitrogen Co.) and 22 μg/mL sodium pyruvate. The oocytes of each donor were matured in 100 μL of TCM 199 (Gibco, Invitrogen Co.) supplemented with 10% (vol/vol) FBS, 25 mg/mL sodium bicarbonate, 22 μg/mL sodium pyruvate, 50 μg/mL amikacin, 0.5 μg/mL FSH (Pluset, Hertape Calier, Juatuaba, Minas Gerais, Brazil), and 100 IU/mL hCG (Vetecor, Hertape Calier), under mineral oil and incubated under 5% of CO_2 in air, at 38.8 °C and high humidity for 22–24 h.

The COCs were washed and submitted to IVF in 90 μL drops of IVF medium under mineral oil. Fertilization occurred in Tyrode's solution, as described previously [32], supplemented with 10 μg/mL heparin, 22 μg/mL sodium pyruvate, 50 μg/mL amikacin, 6 mg/mL fatty acid-free BSA, and Penicillamine, hypotaurine, epinephrine solution (2- mM penicillin, 1-mM hypotaurine, and 0.25-mM epinephrine).

For IVF, semen straws were thawed for 30 s in a 35 °C water bath, and semen was placed on a 400 μL 40:80% PureSperm gradient (Nidacon). The sperm were centrifuged for 6 min at 800×*g*, the supernatant was removed, and the pellet suspended with 1-mL fertilization medium. Sperm were centrifuged for 3 min at 200×*g*, and the supernatant was removed. Sperm motility and concentration were assessed and 1×10^6 motile sperm/mL was added to each fertilization drop. Fertilization took place over 18–22 h incubation under the same conditions described for maturation. The same semen from a single bull and production batch was used throughout the study.

After fertilization, presumptive zygotes were mechanically denuded. Culture took place in modified synthetic oviduct fluid as described previously [33] supplemented with 50 μg/mL amikacin, 0.1-mM amino acids, 340,02-μM citrate, 2775-mM myoinositol, 2.5% (vol/vol) FBS, and 6 mg/mL BSA (fatty acid free, SigmaA-8806) at 38.8 °C in humidified air with 5% CO_2, 7% O_2, and 88% N_2.

The cleavage rate was recorded 48 h after insemination, and at this time 50% of fresh culture medium was exchanged (first feeding). The second feeding was performed on the fifth day of embryo culture and the blastocyst rate (total number of blastocysts divided by total number of cultured oocytes) was recorded on the sixth day of embryo culture.

2.6. Statistical analysis

Statistical analyses were performed using Statistical Analysis System for Windows (SAS, Version 9.4 for Windows; SAS Inst., Cary, NC). Research was design and analysis in a crossover trial using 18 nulliparous, 15 primiparous and 21 multiparous buffalo females that were randomly divided into two groups: Control or FSH (treated with 200 mg of FSH). All data were tested for normality of residuals using *UNIVARIATE* procedure according by *Shapiro-Wilk* test, non-normally distributed data were transformed before analysis if improvement in *residual* distribution and *outliers* were removed when necessary. Effect was determined by one-way ANOVA using *Type III sums of squares*.

The variables evaluated on OPU-IVEP were the total number of aspirated follicles; follicle category (small, <6 mm; medium, 6–10 mm; large, > 10 mm); total number of recovered oocytes; oocytes recovery rate (number of recovered COCs per number of aspirated follicles); total number of viable oocytes; viable oocytes rate (number of total viable oocytes per total COCs recovered); blastocyst rate (number of blastocysts per total number of cultured COCs); and number of blastocysts per OPU procedure.

Categorical data were analyzed by logistic regression using the *Glimmix* procedure fitting a *Binomial* distribution. However, continuous data were analyzed using the same procedure fitting a *Poisson* distribution. The model included group (Control or FSH), Categories (nulliparous, primiparous and multiparous), time crossover (factor one and factor two), replicate (first and second) and meaningful *two-way interactions* as fixed effects. The option *residual* was input as random effect. Animal within *Crossover time* was used as the *error term*. Furthermore, were including the option, *ddfm* = *Satterthwaite*, to the model statement adjusted the degrees of freedom for variances. *Tukey* honest significant difference post hoc test was performed to determine differences. Values are presented as *means* ± *SEM*. Differences with $P \leq 0.05$ were considered significant, and $0.05 < P < 0.10$ were discussed as tendencies.

3. Results

There was no interaction between treatments, animal category and replicate for all analyzed variables. The effects of FSH treatment and category are summarized on Table 1.

No differences between animal category (P = 0.73) and FSH treatment (P = 0.53) were observed regarding the total follicles aspirated. However, the FSH treatment increased (P < 0.001) the proportion of large (>10 mm; FSH = 16.2% and Control = 2.0%) and medium (6–10 mm; FSH = 36.3% and Control = 6.1%; Fig. 2) diameter follicles available for the OPU procedure.

The total of recovered oocytes was greater in NU than in MU, and PR were similar to NU and MU (NU = 12.0 ± 0.9^{a}; PR = 10.2 ± 1.2^{ab}; MU = 9.0 ± 0.8^{b}; P = 0.05). No effect of FSH treatment was observed (P = 0.85) for this variable. Buffalo donors treated with FSH had a greater viable oocytes rate (P = 0.03), blastocyst rate (P = 0.03) and embryo yield per OPU-IVEP session (P = 0.07), however, no category effect were observed for these variables.

4. Discussion

The present study evaluated the follicular population, the oocyte yield and embryo production of buffalo donors submitted to FSH superstimulation before to OPU. It was found that FSH treatment prior to OPU increased the proportion of medium and large diameter follicles available for the OPU procedure in all buffalo donors categories. Consequently, the treatment increased the proportion of viable oocytes for culture and resulted in greater blastocysts rates and embryos produced per OPU-IVEP session. The present research supports the hypotheses that FSH treatment prior to OPU increases the *in vitro* embryo production of buffalo donors from different categories.

Similarly, Vieira et al. (2014) found that superstimulation with FSH did not increase the number of follicles aspirated per OPU session, but increased the proportion of medium-sized follicles available for the OPU procedure and improved the blastocyst rates and embryo yield per OPU-IVEP session in Holstein cows. Furthermore, it has been reported that the use of FSH does not increase the number of ovarian follicles available for aspiration,

Table 1
Summary of OPU and IVEP responses (mean ± SEM) of nulliparous, primiparous and multiparous buffalo donors submitted or not to FSH treatment previously to OPU.

	Nulliparous		Primiparous		Multiparous		*P Value*		
	Control	FSH	Control	FSH	Control	FSH	Treat	Cat	Treat*Cat
Number of animals	18	18	15	15	21	21			
Total aspirated follicles, mean	20.3 ± 2.4	18.3 ± 1.6	21.3 ± 4.4	17.7 ± 2.9	18.1 ± 2.2	17.6 ± 1.7	0.53	0.73	0.85
Total recovered oocytes, mean	11.7 ± 1.6	12.3 ± 1.0	11.5 ± 2.0	9.0 ± 1.2	8.7 ± 1.0	9.3 ± 1.2	0.85	0.05	0.46
Oocytes recovery rate, %	67.5%	73.3%	65.9%	55.2%	52.6%	52.6%	0.92	0.71	0.92
Total viable oocytes, mean	6.6 ± 1.3	7.8 ± 0.9	5.9 ± 1.5	5.7 ± 1.1	4.3 ± 0.7	5.6 ± 0.9	0.26	0.08	0.72
Viable oocytes rate, %	50.5%	57.7%	47.4%	56.2%	50.4%	57.1%	0.03	0.46	0.95
Embryos per OPU, mean	1.8 ± 0.5	3.7 ± 0.7	2.4 ± 0.6	2.7 ± 0.8	2.0 ± 0.5	2.6 ± 0.7	0.07	0.25	0.22
Blastocyst rate, %	16.7%	34.4%	27.3%	27.8%	24.3%	31.6%	0.03	0.89	0.25

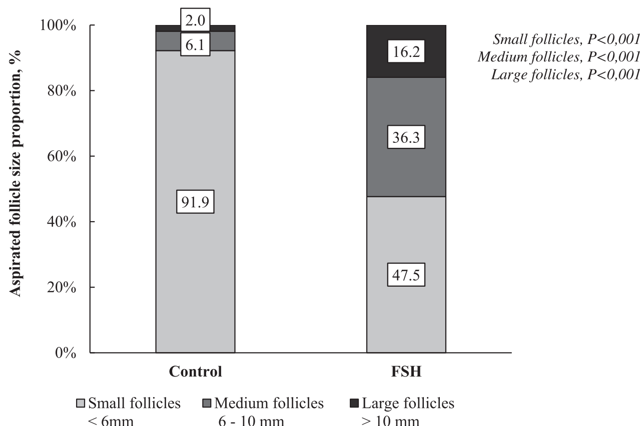

Fig. 2. Proportion of small (<6 mm), medium (6–10 mm) and large (>10 mm) follicles in buffalo donors submitted to OPU-IVEP with or without previous FSH superstimulation. P values for follicular diameter differ significantly.

however, it increases their diameter [27,34]. COCs recovered from follicles with larger diameters have greater developmental competence when compared to COCs from smaller follicles [35,36]. A meta-analysis of the results from different IVEP publications in buffalo, suggested that oocytes with more than three to five cumulus layers recovered from large-sized follicles resulted in maximum maturation rate and subsequent embryonic development [37]. These results support that the increase in follicular diameter caused by FSH treatment may result in improvement of oocyte quality, resulting in greater efficiency of IVEP.

The "coasting" period (period between the last FSH application and the OPU) is an important factor that may influence the result of the superstimulation, since it is at this moment that the prematuration process and acquisition of oocyte competence occurs [14,35]. The duration of the "coasting" period in cattle presents variable results, already being reported a greater blastocyst rate with a "coasting" period of 48 h [38] and also satisfactory results when using a "coasting" period of 20–24 h [39]. In the present study, 40 h of "coasting" period were used, and animals treated with FSH had increased blastocysts rates and embryos produced per OPU-IVEP session, similar to those reported in cattle [13,14]. In a recent study in buffalo, the FSH treatment followed by a coasting period of 40–44 h was effective in increasing the number of follicles, the total number of COCs, the number of good-quality COCs and the number of blastocysts in breeding and non-breeding seasons [40]. It has been suggested that the increase in the *in vitro* embryo production in donors superstimulated with FSH prior to OPU is related to the stimulatory effect of this gonadotrophin. However, it is important to highlight that this positive effect may be a response to an associative influence of FSH treatment added to the fact that all FSH treated donors had a 40 h "coasting" period [13].

Studies reported that superstimulation with gonadotrophins prior to OPU stimulates follicular growth, increasing the proportion of aspirable follicles, viable oocytes and the number of embryos produced from calves and heifers [34,36]. In a comparative study between Holstein calves treated or not with FSH prior to OPU, it was observed that gonadotrophin treatment increased the number of visualized follicles and the number and rate of cultured COCs [41].

In the present study, greater number of recovered oocytes and an increase in the number of viable oocytes in nulliparous buffalo compared to multiparous were observed. These results support other studies that verified higher number of recovered oocytes after follicular aspiration in young buffaloes [3,42]. However, no category effect or interaction category*FSH treatment were found for viable oocytes rate, blastocyst rate and number of embryos produced per OPU, rejecting the initial hypothesis of this study. These data indicate that the FSH treatment improved the OPU/IVEP efficiency regardless of the category.

The results of the present study lead to the conclusion that the superstimulation of buffalo donors with FSH prior to OPU increases the efficiency of IVEP, by increasing the proportion of medium and large diameters follicles available for the OPU procedure. Consequently, the treatment enhanced the proportion of viable oocytes for culture and resulted in greater blastocysts rates and embryos produced per OPU-IVEP session in buffalo donors from different categories.

Acknowledgements

The authors would like to acknowledge the support from CNPq (Process 306759/2016-0), FAPESP and CAPES of Brazil.

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