Animal Reproduction Science xxx (2015) xxx–xxx

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Paradoxical effects of bovine somatotropin treatment on the ovarian follicular population and *in vitro* embryo production of lactating buffalo donors submitted to ovum pick-up

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ARTICLE INFO

Article history:
Received 10 April 2014
Received in revised form
19 December 2014
Accepted 27 December 2014
Available online xxx

Keywords:
OPU
bST
Embryo
IGF
Oocyte quality

ABSTRACT

The aim of the present study was to evaluate the effect of bovine somatotropin (bST; 500 mg) administration on lactating buffalo donors submitted to two different ovum pick-up (OPU) and *in vitro* embryo production schemes with a 7 or 14 d intersession OPU interval. A total of 16 lactating buffalo cows were randomly assigned into one of four experimental groups according to the bST treatment (bST or No-bST) and the OPU intersession interval (7 or 14 d) in a 2 × 2 factorial design (16 weeks of OPU sessions). The females submitted to OPU every 14 d had a larger ($P < 0.001$) number of ovarian follicles suitable for puncture (15.6 ± 0.7 vs. 12.8 ± 0.4) and an increased ($P = 0.004$) number of cumulus–oocyte complexes (COCs) recovered (10.0 ± 0.5 vs. 8.5 ± 0.3) compared to the 7 d interval group. However, a 7 or 14 d interval between OPU sessions had no effect ($P = 0.34$) on the number of blastocysts produced per OPU (1.0 ± 0.1 vs. 1.3 ± 0.2, respectively). In addition, bST treatment increased ($P < 0.001$) the number of ovarian follicles suitable for puncture (15.3 ± 0.5 vs. 12.1 ± 0.4) but reduced the percentage (18.9% vs. 10.9%; $P = 0.009$) and the number (1.4 ± 0.2 vs. 0.8 ± 0.1; $P = 0.003$) of blastocysts produced per OPU session compared with the non-bST-treated buffaloes. In conclusion, the 14 d interval between OPU sessions and bST treatment efficiently increased the number of ovarian follicles suitable for puncture. However, the OPU session interval had no effect on embryo production, and bST treatment reduced the *in vitro* blastocyst outcomes in lactating buffalo donors.

1. Introduction

Reproductive technologies have enhanced the genetic stocks in the buffalo dairy industry. Although *in vivo* embryo technology has been used in buffaloes for over 30 years (Drost et al., 1983), multiple ovulation and embryo transfer programs still suffer from low efficiency and reduced commercial application in this species (Carvalho et al., 2002; Drost, 2007; Baruselli et al., 2013). In contrast, ovum pick-up (OPU) associated with the *in vitro* production of embryos (IVP) is an important alternative to rapidly increase the genetic gain, especially through the female lineage in buffalo (Boni et al., 1996; Gasparrini, 2002; Neglia et al., 2003, 2011; Sá Filho et al., 2009).

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http://dx.doi.org/10.1016/j.anireprosci.2014.12.017
0378-4320/

In spite of the continuous incorporation of IVP into embryo production operations worldwide (IETS, 2012), there are still several limiting factors for the commercial use of IVP in buffaloes (Gasparrini, 2002; Neglia et al., 2003, 2011; Sá Filho et al., 2009; Campanile et al., 2010; Di Francesco et al., 2012; Gasparrini et al., 2014). Buffalo females have lower numbers of primordial (Van Ty et al., 1989) and antral ovarian follicles (Baruselli et al., 1997; Baldrighi et al., 2014; Gimenes et al., 2015), reduced numbers of recovered cumulus–oocyte complexes (COCs) per OPU procedure (Neglia et al., 2003; Gupta et al., 2006), a greater incidence of follicular atresia (Van Ty et al., 1989) and decreased *in vitro* cleavage and blastocyst rates (Gasparrini et al., 2006; Sá Filho et al., 2009; Gimenes et al., 2015) compared to cattle (Merton et al., 2003; Pontes et al., 2010, 2011; Gimenes et al., 2015). Thus, strategies to enhance the ovarian follicular population, the percentage of non-atretic follicles and COCs quality are essential to improve OPU-IVP efficiency in buffaloes.

An alternative strategy to improve IVP program outcomes in cattle and buffalo donors is the use of bovine recombinant somatotropin (bST) to significantly alter the number of ovarian follicles suitable for puncture, as well as the yield and quality of COCs recovered and *in vitro* blastocyst production rates (Bols et al., 1998; Buratini Jr. et al., 2000; Tripp et al., 2000; Roth et al., 2002; Petyim et al., 2003; Viana et al., 2004; Sá Filho et al., 2009). In buffalo donors, the incorporation of bST into OPU–IVP programs improves ovarian follicular population of cyclic buffalo heifers (Sá Filho et al., 2009).

Another factor that must be optimized in IVP is the OPU intersession interval (Boni et al., 1996; Gupta et al., 2006; Sá Filho et al., 2009; Neglia et al., 2011). Some studies have demonstrated that short intersession OPU schemes (once or twice per week) did not adversely affect the ovarian antral follicle population or the COCs yield per OPU session in buffaloes (Boni et al., 1996; Gupta et al., 2006). However, other reports have demonstrated that short intervals between OPU sessions are associated with a gradual decrease in the ovarian antral follicle population in buffalo females (Sá Filho et al., 2009; Neglia et al., 2011). On the other hand, increasing the interval between two consecutive OPU sessions could increase the occurrence of large follicles (Boni et al., 1996), increasing the incidence of atresia and therefore reducing the overall OPU–IVP efficiency. These controversial IVP results highlight the need for further studies of the optimal OPU intersession interval in buffaloes.

Furthermore, because bST reduces the duration of the dominant follicle phase in the first follicular wave to approximately 2 d (Kirby et al., 1997; Lucy, 2000), bST treatment may lead to an earlier emergence of the second follicular wave (Lucy et al., 1994). Therefore, the positive effect of bST treatment in buffalo oocyte donors could be related to the OPU inter-session interval.

Considering the restricted information on the use of bST and the OPU intersession interval in buffaloes, the present study aimed to evaluate the influence of bST treatment (given every 14 d) and the OPU intersession interval (7 or 14 d) on COCs yield, quality and *in vitro* development into blastocyst. We hypothesized that bST treatment would enhance the number of COCs recovered and *in vitro* embryo development after OPU; however, we expected that the positive effect of bST depends on the OPU intersession interval in buffalo donors.

2. Material and methods

2.1. Animals and management

The study was performed at a commercial dairy farm located in Sao Paulo state, Brazil, during the breeding season (autumn to winter in the south hemisphere; May to September). A total of sixteen ($n = 16$) 3- to 9-year-old lactating buffalo cows (*Bubalus bubalis*) with body condition scores ≥3 on a 1–5 scale (Baruselli et al., 2001) and 213.1 ± 16.6 d in milk (DIM) were enrolled in the trial. The lactating buffalo cows were milked twice a day, with the daily milk yield ranging from 5.4 to 11.4 L during the experimental period. The milk yield and lactation length were 2612.2 ± 175.3 L and 325.2 ± 19.1 d, respectively. All females were maintained under pasture conditions with free access to water and were supplemented with 3–5 kg of concentrate plus mineral salt according to milk production.

All cows were previously selected by ultrasonography exams according to the following criteria: (1) the presence of a corpus luteum (cyclic) and (2) an ovarian follicular population ≥8 follicles per ovary.

2.2. Experimental design

Lactating buffalo donors were randomly assigned to one of four treatment groups, according to the bST treatment (every 14 d bST was administered or not) and the interval between each OPU session (either 7 or 14 d) (Fig. 1). Therefore, the experimental groups were: 7 d/No-bST ($n = 4$), 7 d/bST ($n = 4$), 14 d/No-bST ($n = 4$) and 14 d/bST ($n = 4$). The average of DIM was similar among treatments 7 d/No-bST (236 ± 33.9 d), 7 d/bST (204 ± 38.1 d), 14 d/No-bST (198.8 ± 36.7 d) and 14 d/bST (208 ± 34.9 d). Cows treated with bST received a subcutaneous injection of 500 mg of bST (Boostin, MSD Animal Health, São Paulo, Brazil) in the ischiorectal fossa 7 d before the first OPU session and every 14 d thereafter until the eighth injection (Fig. 1). The No-bST group did not receive any additional treatments. The entire experiment lasted 16 weeks, with each female receiving a total of eight bST injections and submitted to 16 or 8 OPU sessions, depending on the inter-OPU interval, either 7 or 14 d.

2.3. OPU procedure

In each OPU session, all antral follicles ≥2.0 mm in diameter (ovarian follicular population) were aspirated from each ovary. To perform the OPU procedures, a 5 MHz microconvex transvaginal transducer (Aloka® S-500, Aloka Co., Ltd., Tokyo, Japan) connected to a metal guide (WTA Ltda., Cravinhos, São Paulo, Brazil) that fit on top of an 18 G disposable needle (V-OPAA-1855- Cook Australia, Queensland, Australia) and was connected to a vacuum pump operating at a negative pressure of 68 mmHg (V-MAR 5000-Cook Australia, Queensland, Austrália) was used. The

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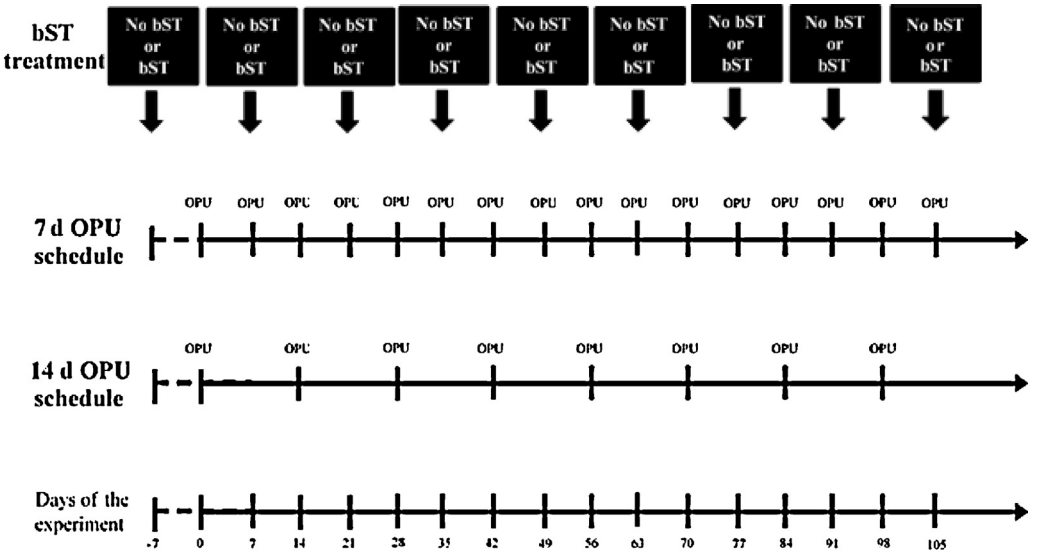

Fig. 1. Experimental design. bST treatment = buffalo cows received a subcutaneously injection of 500 mg of bST. OPU = ovum pick-up. Buffalo cows were subjected to OPU at 7 or 14 d intersession intervals.

aspiration line (VBOAS 18 L-Cook Australia, Queensland, Australia) was continuously rinsed with 1 L of DPBS (Dmpbs flush-Nutricell, São Paulo, Brazil), plus 5000 IU of sodium heparin (5 IU/mL; Liquemine®, Roche Brazil, São Paulo, Brazil) and 10 mL of fetal calf serum (FCS; Nutricell, São Paulo, Brazil) during the OPU. The tubes (50 mL, Corning Life Science Incorporated, MA, USA) for COCs collection were maintained at 37 °C. Prior each OPU session, cows had their ovaries scanned by ultrasonography to quantify the ovarian follicular population (the number of follicles suitable to be punctured). The recovery rate was defined as the number of COCs obtained per OPU divided by the total number of follicles suitable to be punctured immediately before the OPU procedure.

2.4. Cumulus–oocyte complex (COC) processing

The COCs recovered by OPU were observed under a stereomicroscope (magnification of 50×) immediately after the aspiration of follicular fluid and graded into categories, as previous described by Di Francesco et al. (2011). Only COCs classified as grade I, II or III were considered suitable for *in vitro* embryo production (Neglia et al., 2003). After grading, COCs were washed four times in TCM-199 (GIBCO® Invitrogen Corporation, CA, USA) with 10% fetal calf serum (FCS) and then aliquoted into cryotubes (Corning Life Science Incorporated, MA, USA) containing Hepes-buffer (Nutricell, Campinas, Sao Paulo, Brazil) and TCM-199 with 10% FCS and maintained to 37 °C until transport to the lab. In the lab, the COCs were then transferred to TCM-199 medium supplemented with gentamicin (50 μg/mL), FSH (5 μg/mL), LH (50 μg/mL), estradiol (1 μg/mL) and pyruvate (22 μg/mL). For *in vitro* maturation (IVM), the COCs were placed into droplets of TCM-199 medium under mineral oil and incubated in 5% CO_2 in air at 38.5 °C and saturated humidity for 22–24 h.

2.5. In vitro fertilization (IVF) and embryo culture

The semen used in the trial was processed in a commercial AI Center (Central de Biotecnologia de Reprodução Animal–CEBRAN; Castanhal, PA, Brazil). The spermatozoa (sptz) were prepared from frozen-thawed buffalo semen derived from a single ejaculate of a commercial standard bull that was previously tested for IVP. Sperm was separated by Percoll gradient (45/90%). The pellet obtained after centrifugation was re-suspended to a final concentration of 2×10^6 sptz/mL in *in vitro* fertilization (IVF) medium, containing TALP supplemented with heparin (10 μg/mL), pyruvate (22 μg/mL), gentamicin (50 μg/mL), bovine serum albumin (6 mg/mL), and PHE solution (2 μM penicillin, 1 μM hypotaurine and 0.25 μM epinephrine). Fertilizing droplets (10–20COCs/100-μl droplet) were covered by mineral oil and were incubated under the same gas atmosphere as for IVM for 16–18 h.

After the IVF period, the presumptive zygotes were removed from the fertilization medium, stripped of cumulus cells by gentle pipetting and washed twice in a Hepes-buffered CR2 medium with essential and non-essential amino acids. The culture medium was composed of bicarbonate-buffered CR2 supplemented with 10% FCS and 30 mg/mL of bovine serum albumin. Presumptive zygotes were allocated into 50-μl droplets (20–25 presumptive zygotes per droplet) and the culture was carried out under the same atmosphere as for IVM and IVF.

On Day 2 of embryo development (Day 0 = day of IVF), the cleavage rate was evaluated. The embryo production evaluation was performed 7 d after IVF. On Day 6, 50% of the medium was removed from each droplet, and the same volume of fresh medium was added. Cleavage rates were defined as the number of zygotes with two or more cells on Day 2 divided by the total of COCs cultured, and blastocyst production rates were defined as the number of blastocysts on Day 7 divided by the total of COCs cultured.

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2.6. Statistical analysis

Data were analyzed by the SAS program, System for Windows (Statistical Analyses System, Version 9.3). When necessary, data were submitted to normalization using Guided Data Analysis. If normalization was not possible, the data were analyzed using Kruskal–Wallis non-parametric tests.

The number of aspirated follicles, the total number of COCs, COCs suitable for culture, COC quality, the number of cleaved embryos and number of blastocysts on Day 7 were analyzed by variance analysis (ANOVA) for multiple repetitions using a Mixed Procedure (SAS) followed by the Duncan test. Recovery, cleavage and blastocyst production rates were analyzed by a χ^2 test.

The data are presented as the means ± SEM. The level of significance to reject the null hypotheses (H0) was 5%, and a variable was considered significantly different when $P \leq 0.05$.

3. Results

There was no difference ($P = 0.77$) on the DIM at the first OPU procedure among treatments. The bST-treated cows presented a greater number of ovarian follicles available to be punctured per OPU session than the No-bST group ($P < 0.001$; Fig. 2). Buffalo cows subjected to the 14-d intersession OPU schedule presented a greater number of ovarian follicles suitable to be punctured ($P < 0.001$; Fig. 2) than buffalo cows submitted to OPU once a week. Furthermore, there was no interaction between OPU session interval and bST treatment ($P = 0.83$) for the size of the antral follicle population.

The number of COCs recovered was greater when buffalo cows were subjected to OPU at 14-d intervals (10.0 ± 0.5 vs. 8.5 ± 0.3; $P = 0.004$). However, there was no effect of bST treatment (9.6 ± 0.4 vs. 8.4 ± 0.4; $P = 0.07$) and no interaction between OPU session interval and bST treatment ($P = 0.07$) for the total number of COCs recovered.

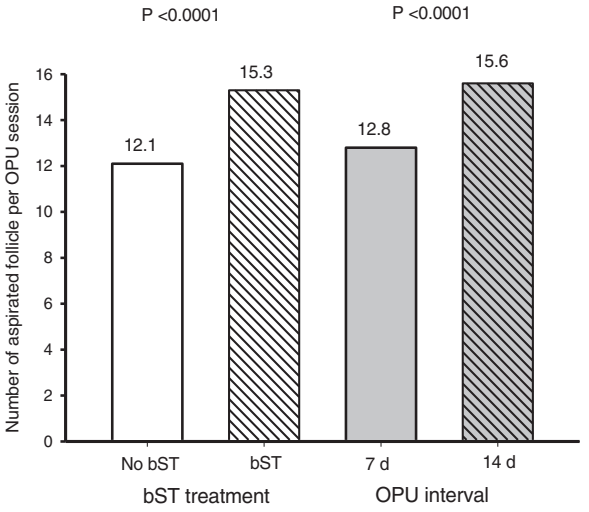

Fig. 2. Number of aspirated follicles per ovum pick-up (OPU) session in lactating buffalo donors. Donors were randomly assigned to one of four treatment groups; according to a treatment with bovine recombinant somatotropin (bST) administered every 14 d (bST or No-bST) and the interval between OPU sessions (every 7 or 14 d); No interaction between bST treatment and OPU inter-session interval ($P = 0.83$) was found.

An interaction was observed between bST treatment and OPU schedule on the recovery rate ($P = 0.03$). Cows receiving bST and submitted to OPU at 14-d intervals presented lower recovery rates than cows receiving bST and aspirated every 7 d (Table 1). However, the OPU session interval did not affect the recovery rate of buffalo cows in the No-bST group.

Another interaction was obtained between the OPU interval and the bST treatment for the number of COCs suitable to be cultured ($P = 0.01$; Table 1). Buffalo cows aspirated every 7 d without receiving bST presented a

Table 1
Effect of subcutaneous treatment with 500 mg of bST every 14 d on two different OPU schemes (7 and 14 d intersession interval) in lactating buffalo cows.

	Treatments[A]				*P values*[B]		
	No-bST		bST		bST	OPU interval	bST × OPU interval
	7 d	14 d	7 d	14 d			
No. of buffalo donors	4	4	4	4	–	–	–
No. OPU sessions	16	8	16	8	–	–	–
No. of aspirated follicles	11.3 ± 0.5	14.8 ± 0.8	14.3 ± 0.6	17.3 ± 1.0	<0.0001	<0.0001	0.83
No. of COCs recovered	7.6 ± 0.4	9.9 ± 0.6	9.3 ± 0.4	10.1 ± 0.7	0.07	0.004	0.07
COC recovery rate, %[C]	69.3[a]	73.6[a]	67.4[a]	58.5[b]	0.007	0.45	0.03
No. of COCs suitable for culture	5.5 ± 0.4[b]	7.6 ± 0.5[a]	6.8 ± 0.4[a]	6.8 ± 0.6[a]	0.57	0.01	0.01
No. of COCs degenerated	1.2 ± 0.1	1.1 ± 0.2	1.2 ± 0.1	1.3 ± 0.2	0.5	0.79	0.56
Cleavage rate, %[D]	33.4	32.7	35.1	26.0	0.49	0.18	0.24
No. of blastocysts produced	1.3 ± 0.2	1.7 ± 0.4	0.7 ± 0.1	0.8 ± 0.2	0.003	0.34	0.67
Blastocyst production, %[E]	18.6	19.5	9.6	13.4	0.009	0.42	0.60

[a,b] Within a row, means without a common letters differed ($P < 0.05$).

[A] Lactating buffalo donors were randomly assigned into one of four treatment groups according to treatment with bST administered every 14 d (bST or No-bST) and the interval between OPU sessions (every 7 or 14 d).

[B] bST = effect of treatment with bST every 14 d; OPU interval = effect of interval between OPU sessions (every 7 or 14 d) and bST × OPU interval = interaction between bST treatment and OPU session scheme.

[C] No. of COCs/No. of follicles aspirated.

[D] No. of cleaved zygotes/No. of oocytes cultured.

[E] No. of blastocysts produced/No. of oocytes cultured.

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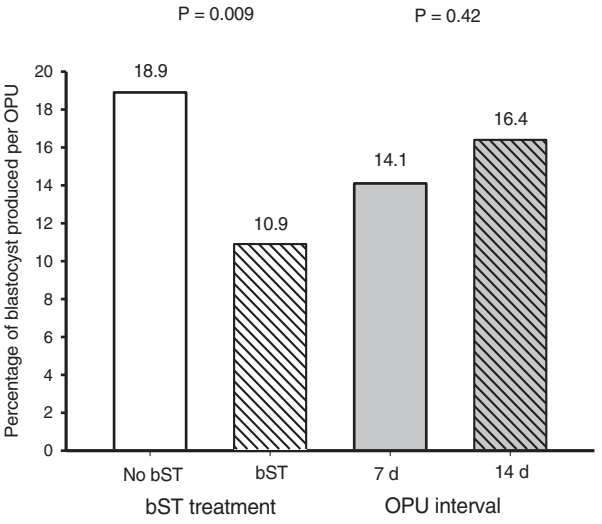

Fig. 3. Percentage of blastocyst produced per ovum pick-up (OPU) session in lactating buffalo donors. Donors were randomly assigned to one of four treatment groups; according to a treatment with bovine recombinant somatotropin (bST) administered every 14 d (bST or No-bST) and the interval between OPU sessions (every 7 or 14 d); No interaction between bST treatment and OPU inter-session interval ($P = 0.60$) was found.

lower number of COCs suitable to be cultured than the other experimental groups. Furthermore, the number of blastocysts produced per female per OPU session (No-bST = 1.4 ± 0.2 vs. bST = 0.8 ± 0.1, $P = 0.003$) and the blastocyst production rate ($P = 0.009$; Fig. 3) were lower in buffaloes treated with bST. However, there were no effects of OPU performed every 7 or 14 d on the number of blastocysts produced per OPU (1.0 ± 0.1 vs. 1.3 ± 0.2; $P = 0.34$) or the blastocyst production rate ($P = 0.42$; Fig. 3).

4. Discussion

In the present study, bST treatment increased the number of follicles suitable to be punctured, but reduced the number and the percentage of *in vitro* produced blastocysts, partially rejecting our original hypothesis. Additionally, a 7-d inter-session interval for OPU sessions reduced the number of follicles suitable to be punctured and the number of COCs recovered in lactating buffalo cows compared to a 14-d OPU interval.

The increase in the number of follicles suitable to be punctured among bST-treated buffalo cows is similar to a previous report in cycling buffalo heifers (Sá Filho et al., 2009). A positive effect of bST on the number of antral follicle recruited per follicular wave has been repeatedly described in *Bos taurus* cattle (Gong et al., 1991, 1993a,b; De La Sota et al., 1993; Lucy et al., 1993; Kirby et al., 1997), *Bos indicus* cattle (Buratini Jr. et al., 2000) and also in ewes (Joyce et al., 2000). The mechanism by which bST enhance the follicular population is not completely understood. It is thought that the effect of bST could be due to enhancement of the circulating concentration of IGF-I and insulin, which leads to an increase in the number of antral follicles recruited per follicular wave (Gong et al., 1994; Buratini Jr. et al., 2000; Bilby et al., 2004, 2006). Moreover, the use of bST is directly correlated with a beneficial effect of the growth hormone (GH) on follicles (Hull and Harvey, 2001). GH could act on its receptors in cumulus cells and oocytes via autocrine and/or paracrine mechanisms (Bevers and Izadyar, 2002). When cells from small follicles were cultured with insulin and GH, greater granulosa cells proliferation was observed (Langhout et al., 1991). Furthermore, supplementation with GH in the maturation media can accelerate events including nuclear maturation, expansion of cumulus cells and embryo development (Bevers and Izadyar, 2002).

Regarding the COCs recovery rate, an interaction was observed demonstrating that cows that received bST and were submitted to OPU 14 d apart presented lower recovery rates than the other experimental groups. This result can be related to the reduced duration of the first wave of the dominant follicle following treatment with bST (Kirby et al., 1997; Lucy, 2000). Consequently, an increased OPU intersession interval could lead to an enhanced occurrence of large follicles (Boni et al., 1996), which are associated with a reduced recovery rate in bovine females (Seneda et al., 2001). In addition, due to the inconsistent results between the number of follicles suitable for puncture and the recovery rate, the total number of COCs recovered was not different between the bST and No-bST treatment groups in the present study. Similar results were described in another study, where no improvement in the number of recovered COCs was observed in buffalo heifers treated with bST (Sá Filho et al., 2009). Also corroborating the present results, bST treatment did not affect the number of COCs recovered following the OPU procedure in cattle (Bols et al., 1998; Tripp et al., 2000). Therefore, although bST treatment increases the follicular population, the absence of a bST effect on COC yield can be explained by the morphological alterations of the follicular wall or of COCs induced by bST administration (Bols et al., 1998). These alterations could create difficulties in removing the oocytes from the follicle during the puncture procedure (Tripp et al., 2000). Additionally, the lack of a bST effect on the number of COCs could be even more evident when combined with the greater intersession interval, resulting in an impaired recovery rate.

It is important to emphasize that the blastocyst production rate and the number of blastocysts produced per buffalo cow per session were reduced when the animals received bST treatment. In cattle, previous reports demonstrated an absence of a bST positive effect on the *in vitro* embryo production rates (Bols et al., 1998; Tripp et al., 2000). A positive effect on blastocyst production has only been described when GH was added in culture to the *in vitro* medium (Mtango et al., 2003). One possible explanation of this negative result on *in vitro* oocyte competence could be related to the detrimental effects of superstimulation with IGF-I and insulin on oocyte development (Armstrong et al., 2001). High concentrations of IGF-I impair glucose uptake and could increase apoptosis in the inner cells mass of embryos (Chi et al., 2000).

It has been suggested that there is an optimal level of plasma IGF-I following bST treatment that improves the fertility of lactating Holstein cows (Bilby et al., 1999, 2004; Thatcher et al., 2006). In addition, discordant results have

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been reported depending on the donorsí lactation status. Several studies have demonstrated that bST treatment of lactating dairy cows enhances conceptus development and fertility (Bilby et al., 2004; Santos et al., 2004; Thatcher et al., 2006; Ribeiro et al., 2014); however, in non-lactating dairy cows, bST treatment decreases the pregnancy rate (Bilby et al., 2004). Therefore, the lower blastocyst production rate found in the present study might be the buffalo donorsí response to experiencing a high level of plasma IGF-I induced by 500 mg of bST treatment every 14 d.

Extending the interval between OPU sessions from 7 to 14 d resulted in a positive effect on the number of follicles available to puncture. This result demonstrated that the adoption of less intensive aspiration intervals could result in fewer alterations to follicular recruitment and consequently improve the number of follicles suitable for OPU. However, there are no data in literature demonstrating the ideal frequency of OPU in buffalos or cows to enhance IVP efficiency. Sá Filho et al. (2009) demonstrated that when buffalo heifers were aspirated at a short interval (twice a week), there were approximately 27% fewer follicles available to be punctured after the fifth OPU session. Similarly, greater results were obtained when bovine females underwent OPU a once a week instead of twice a week (Viana et al., 2004). However, earlier studies have also reported beneficial effects of the twice a week OPU scheme on the number of ovarian follicles and COCs recovery compared to a once a week scheme in cows (Garcia and Salaheddine, 1998) and in buffalos (Boni et al., 1996). Despite the reduced number of COCs suitable to culture, the 7 d intersession interval OPU scheme could be an important strategy to intensify *in vitro* embryo production during over a short period of time, especially in large-scale commercial programs.

In conclusion, OPU performed every 14 d and bST treatment efficiently increased the number of ovarian follicles suitable to puncture. However, the OPU inter-session interval had no positive effect on blastocyst production, and bST treatment reduced *in vitro* embryo production in lactating buffalo donors.

Conflict of interest

The authors declare that there are no conflict of interest.

Acknowledgements

The authors would like to thank the staff of the Santa Eliza Farm (Dourado-SP) for allowing the use of their animals and facilities during this study. This research was supported by the Vida Reprodutiva Consultoria - Serviços em Reprodução Animal (Cravinhos, SP, Brazil) and Vitrogen (Cravinhos, Brazil).

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