



Updates on embryo production strategies

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Abstract

The embryo production technologies are used to enhance genetic progress through female and male lineages. Advances in the control of ovarian follicular wave emergence, superstimulation and ovulation with self-appointed treatments have facilitated donor and recipient management. However, these procedures can be influenced by several factors related to the animals and their management. Therefore, researchers continue to investigate the ideal reproductive environments and treatments to maintain the viability of the techniques and field applicability.

Keywords: ET, IVP, OPU, SOV, synchronization.

Introduction

Among reproductive technologies, *in vivo* and *in vitro* embryo productions have been robust tools used to enhance genetic progress through female and male lineages. However, there is an ongoing need to simplify bovine superovulation (SOV) for *in vivo* embryo production, specifically by reducing the number animal handlings, without compromising embryo yield. Advances in the control of ovarian follicular wave emergence and ovulation with self-appointed treatments have facilitated donor and recipient management. However, these procedures can be influenced by several factors related to the animals and their management (Mapletoft *et al.*, 2002; Baruselli *et al.*, 2006, 2010; Bó *et al.*, 2006; Vasconcelos *et al.*, 2006). Therefore, researchers continue to investigate the ideal reproductive environments and treatments to maintain the viability of the techniques and field applicability.

Nowadays, 40.6% of the total embryo production in the world (1,275,874 embryos) are *in vitro* derived embryos (International Embryo Transfer Society - IETS, 2014). The success of *in vitro* embryo production (IVP) is directly related to the number and quality of the cumulus-oocyte complexes (COC) harvested by the ovum pick-up (OPU) procedure. Therefore, several studies have been performed to obtain the expertise related to oocyte quality and consequently, increase the outcomes of OPU-IVP large scale programs.

In this context, the present review aims to discuss some key points relating to genetics, breed, antral follicle populations, manipulation of ovarian follicular growth, frequency of OPU procedures and extrinsic factors (nutrition and heat stress) which are associated with oocyte and embryo quality for *in vivo* and *in vitro* procedures. Lastly, with this awareness of factors related to the efficiency of OPU-IVP, strategies to optimize the technology and its outcome will be discussed.

Strategies for *in vivo* embryo production

Superovulation of Bos taurus and Bos indicus donors without estrus detection

Traditional SOV protocols have some limitations, including the necessity of numerous animal handling events and detecting estrus to establish the stage of the estrous cycle for initiating superstimulatory treatments and to determine the time of AI. However, recent protocols have been designed to control follicular wave emergence and ovulation, allowing the initiation of superstimulatory treatments and the AI of donors at a self-appointed time (Bó *et al.*, 2006). Protocols for SOV without estrus detection are especially important when working with *Bos indicus* donors and high-yielding dairy *Bos taurus* cows, due to the inherent difficulties with estrus detection with these animals (Lopez, 2005; Baruselli *et al.*, 2006).

Thus, three important aspects should be considered when developing SOV protocols: 1) control of ovarian follicular dynamics and follicular wave emergence to initiate gonadotropin treatments; 2) time of ovulation induction and AI in superstimulated donors; and 3) type (FSH or eCG), dosage, and frequency of gonadotropin treatments used for SOV.

Synchronization of follicular wave emergence to initiate gonadotropin treatments

Follicular wave emergence for SOV can be controlled mechanically (follicle ablation; Kohram *et al.*, 1998) or pharmacologically (GnRH; Kohram *et al.*, 1998), LH, hCG, or estradiol plus progesterone (Bo *et al.*, 1995). In general, the most common treatment to

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electively induce follicular wave emergence involves the use of estradiol and progesterone (P4), especially in *Bos indicus* cattle, because of their prolonged anestrus and poor response to GnRH treatment at random stages of the estrous cycle (Baruselli *et al.*, 2003). The efficacy of estradiol and P4 administration, followed by the initiation of FSH treatment at the expected time of follicular wave emergence (4 days later), has been demonstrated in several studies with *Bos taurus* (reviewed by Bó *et al.*, 2006) and *Bos indicus* (Nogueira *et al.*, 2002) cattle. Regardless of the stage of the estrous cycle, estradiol benzoate (EB) treatment at P4 administration (either a norgestomet ear implant or P4-releasing intravaginal device) induces synchronous follicular wave emergence approximately 3 to 4 days after treatment (reviewed by Baruselli *et al.*, 2006). Therefore, co-treatment with estradiol and P4 has been considered the most successful hormone therapy to synchronize follicular wave emergence in cattle (Bó *et al.*, 2002).

Time of ovulation induction and AI in superstimulated donors

Although the control of follicular wave emergence allows for self-appointed initiation of gonadotropin treatments for superstimulation, the need to detect estrus to perform AI in superstimulated donors remains an important challenge. Therefore, several studies have been conducted to investigate the pharmacological control of the time of ovulation in superstimulated cattle, thus enabling timed AI (TAI). The interval to ovulation can be controlled by delaying the removal of the progestin/progesterone implant and administration of GnRH/LH at the end of SOV protocol (Bó *et al.*, 2002). Moreover, postponing the LH surge in relation to PGF2 α treatment allows the development of more follicles that acquire the capacity of ovulation, thereby resulting in more embryos (Rieger *et al.*, 1990; Vos *et al.*, 1994).

Studies directed toward the development of SOV protocols that allow TAI in various breeds of cattle treated with P4-releasing devices and EB on the first day of the protocol (day 0) have been reported (Baruselli *et al.*, 2006; Bó *et al.*, 2006). Protocols are named according to the interval from the first PGF2 α treatment to the time of P4 source removal (i.e. P-24 and P-36), which occurs before the induction of ovulation to avoid the deleterious effect of high P4 concentration on oocyte quality and sperm transport during the ovulation period (Nogueira *et al.*, 2002; Barros *et al.*, 2010). No significant differences in the number and quality of transferable embryos have been detected between the P-24 and P-36 treatments (Baruselli *et al.*, 2006). Therefore, both treatments can be used to superovulate *Bos taurus* and *Bos indicus* cattle with TAI.

However, due to differences in the diameter of the dominant follicle at deviation and ovulation in Nelore (smaller) than Holstein (greater) cows (Sartori *et al.*, 2001; Gimenes *et al.*, 2008), it is understandable

that the appropriate time to induce ovulation may differ. Therefore, treatment with GnRH or pLH to induce ovulation for TAI in superstimulated *Bos indicus* and *Bos taurus* donor cows should be done at 12 and 24 h, respectively, after the last FSH treatment (Baruselli *et al.*, 2006; Bó *et al.*, 2006).

Type (FSH or eCG), dosage, and number of gonadotropin treatments used for superovulation

A series of studies were conducted aiming to reduce the number of animal handlings that were required to induce SOV in *Bos indicus* donors (Baruselli *et al.*, 2008). The use of 3 doses of pFSH resulted in similar embryo production to the traditional 8 dose protocol with pFSH (Martins *et al.*, 2008). Based on the studies reviewed above, it was possible to reduce the number of animal handlings to complete the SOV protocol by reducing the number of FSH treatments in *Bos indicus* donors. However, results may be different in *Bos taurus* cows, which are less sensitive to exogenous gonadotropins (Bó *et al.*, 2006).

Considering the need to develop a simplified superstimulation protocols, studies have focused on alternative methods to maintain FSH release during a prolonged period in *Bos taurus* breeds. An alternative that has been studied for *in vivo* embryo production is the use of a single injection of pFSH in a 2% hyaluronan solution (biodegradable polymer; Tríbulo *et al.*, 2011). Vieira *et al.* (2015; Department of Animal Reproduction, USP, São Paulo, SP, Brazil; unpublished data) also reported extended elevated FSH concentrations in Holstein heifers treated with pFSH combined in a 0.5% hyaluronan solution. Previous studies reported a similar number of transferable embryos when a single (in 2% hyaluronan; Tríbulo *et al.*, 2011) or two (in 0.5 or 1% hyaluronan; Tríbulo *et al.*, 2012) IM injections of pFSH was administered compared to the traditional twice-daily IM injections of pFSH.

In order to reduce donor handling, a series of studies were designed to evaluate superstimulatory response after a single intramuscular injection of equine chorionic gonadotropin (eCG) in the P-36 (36 h between the first PGF2 α treatment and P4 source removal) protocol with FTAI in Nelore and Holstein donors (reviewed by Baruselli *et al.*, 2008). Collectively, treatment with eCG (1,500, 2,000 or 2,500 IU) resulted in a similar number of transferable embryos compared with eight decreasing doses of pFSH. However, a dose of 2,500 IU of eCG resulted in several large anovulatory follicles observed on the day of ova/embryo recovery in Nelore cows. The eCG treatment effectively reduced donor handling, but lower doses of eCG to SOV *Bos indicus* cows are recommended to avoid excessive superstimulatory responses. In *Bos taurus* dairy cows (Holsteins; reviewed by Baruselli *et al.*, 2008), treatment with eCG (2,000 or 2,500 IU) resulted in a similar number of transferable embryos compared with eight decreasing doses of pFSH. However, successive treatments with eCG after the third session resulted in



reduced embryo production. Based on these data, we concluded that it was possible to SOV donors with a single dose of eCG but decreasing embryo production is likely to occur with repeated use.

Strategies for *in vitro* embryo production

Manipulation of follicular dynamics

Different reproductive biotechnologies have been used to manipulate follicular dynamic and improve results of OPU-IVP. Ovarian follicular wave dynamics prior to the OPU can be manipulated mechanically (follicle ablation) or pharmacologically (GnRH, LH, hCG, or estradiol/P4). After follicular ablation, a new follicular wave will emerge 1 to 1.5 days later (Adams *et al.*, 1992). Following the E2/P4 treatment, a new follicular wave will emerge 3 to 4 days after treatment (Bó *et al.*, 2002; Sá Filho *et al.*, 2013). Martins *et al.* (2012) evaluated the effect of synchronizing follicular wave emergence in Nelore (*Bos indicus*), Brangus (crossbred) and Holstein (*Bos taurus*) cows on the success of OPU programs. Donors were assigned to four groups: control (OPU at a random day of the estrous cycle), D1 (OPU 1 day after follicular wave emergence), D1+bST (OPU 1 day after follicular wave emergence associated to bST administration on day -5) and D1+ eCG (OPU 1 day after follicular wave emergence in association with administration of 400 IU of eCG on day -3). Overall, the eCG treatment resulted in a greater number of viable oocytes in Brangus and Holstein donors. However, the eCG and bST treatments resulted in a greater total number of blastocysts per OPU session only in Holstein donors.

The effect of follicular ablation (aspiration of all >8 mm follicles) or EB+P4 to synchronize follicular wave emergence prior to the OPU was evaluated (Rodriguez *et al.*, 2011). In this study, the effect of eCG

or pFSH to superstimulate follicle growth, on the number and quality of COC obtained by OPU in Brangus and Angus donors was evaluated. No significant effects of follicular wave synchronization was detected in the total number of follicles suitable for OPU (14.8 ± 1.2 vs. 14.5 ± 1.4), number of retrieved COC (8.3 ± 0.9 vs. 7.8 ± 1.0) or number of cultured COC (5.3 ± 0.8 vs. 4.8 ± 0.8). However, a greater number of follicles suitable for OPU (18.2 ± 1.1 vs. 11.2 ± 1.0) and total number of COC retrieved (9.7 ± 1.0 vs. 6.3 ± 0.8) and cultured (6.8 ± 0.8 vs. 3.3 ± 0.5) was observed in cows treated with pFSH compared to eCG.

In a recent study, our research group evaluated the effect of superstimulation in lactating and non-lactating Holstein donors submitted to synchronization of follicular wave emergence (Vieira *et al.*, 2014a). Superstimulation with twice daily injections of pFSH over 2 days prior to OPU resulted in greater OPU-IVP efficacy, regardless donor category (Table 1). Regardless of superstimulation treatment, non-lactating Holstein donors had superior IVP outcomes compared to lactating donors (Table 1). A second experiment was performed to evaluate the efficacy of a single IM injection of pFSH combined with 0.5% hyaluronan (MAP-5) in non-lactating Holstein donors submitted to OPU-IVP procedures (Vieira *et al.*, 2015; Department of Animal Reproduction, USP, São Paulo, SP, Brazil; unpublished data). Regardless of superstimulation treatment protocol, greater numbers of blastocysts were produced per OPU session ($P = 0.06$) in donors receiving pFSH (Control: 2.4 ± 0.5 ; 200 pFSH: 3.7 ± 0.7 ; 200 pFSHHA: 4.7 ± 0.7 ; 300 pFSHHA: 3.1 ± 0.6). Results demonstrate that superstimulation protocols for OPU/IVP programs in Holstein donors will increase embryo yield, and that this can be accomplished with reduced numbers of animal handling (6 to 3) when pFSH is administered in a 0.5% hyaluronan solution.

Table 1. Summary of oocyte and embryo production (mean \pm SE) after OPU-IVP in Control and pFSH-treated donors (lactating and non-lactating Holstein cows).

	Lactating cows		Non-lactating cows		P-value ⁵		
	Control	pFSH	Control	pFSH	Treatment	Categ	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, % ¹	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, % ²	76.9 (150/195)	82.6 (133/161)	78.0 (128/164)	83.9 (125/149)	0.05	0.77	0.88
Cleavage rate, % ³	65.3 (98/150)	63.2 (84/133)	72.7 (93/128)	72.8 (91/125)	0.81	0.16	0.69
Blastocyst rate, % ⁴	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

¹No. COCs /no. follicles aspirated; ²No. COCs cultured /no. total COCs retrieved; ³No. cleaved zygotes /no. oocytes cultured; ⁴No. blastocysts /no. oocytes cultured; ⁵Treatment = effect of treatment (Control vs. pFSH); Categ = effect of donor lactation status (lactating vs. non-lactating); Treat*Categ = interaction between treatment and donor lactation status. Adapted from Vieira *et al.* (2014a).



Factors that influence the efficiency of OPU-IVP programs

In cattle, factors such as genetics or breed, heat stress, nutrition and stage of the estrous cycle can significantly influence the response to different reproductive biotechnologies. For example, in relation to OPU-IVP, it has been reported that IVP is greater in *Bos*

indicus breeds than in *Bos taurus* breeds (Pontes *et al.*, 2010; Guerreiro *et al.*, 2014). The greater population of antral follicles found in *Bos indicus* cattle would appear to result in a greater number of suitable oocytes for *in vitro* culture (Batista *et al.*, 2014). In this context, *Bos indicus* females are reported to produce a greater number of total and cultured COC and greater blastocyst rates (Gimenes *et al.*, 2015; Table 2) than *Bos taurus* females.

Table 2. Effect of genetic groups on oocyte recovery and quality, and developmental competence in *Bos indicus* (Nelore) and *Bos taurus* (Holstein) heifers.

	Genetic group	
	Nelore (n = 9)	Holstein (n = 9)
Number of replicates	6	6
Number of OPU sessions	54	54
Oocyte recovery and quality		
Visualized follicles	41.0 ± 2.1 ^a	22.1 ± 1.3 ^b
Total oocytes	37.1 ± 2.6 ^a	15.4 ± 1.2 ^b
Recovery rate (%)	82.3 ^a	66.8 ^b
Oocytes submitted to IVC	25.6 ± 1.8 ^a	9.1 ± 0.9 ^b
Developmental competence		
Cleaved structures	21.1 ± 1.6 ^a	5.2 ± 0.5 ^b
Cleavage rate (%)	82.6 ^a	59.9 ^b
Blastocysts 7 day after IVF	7.3 ± 0.9 ^a	1.1 ± 0.2 ^b
Blastocyst rate (%)	28.3 ^a	14.1 ^b

^{a,b}P < 0.05. Adapted from Gimenes *et al.* (2015).

One factor related to the poor IVP yields in *Bos taurus* cattle can be partly attributed to the heat stress (HS; Al-Katanani and Hansen, 2002; Al-Katanani *et al.*, 2002; Ferreira *et al.*, 2011), mainly in tropical regions. However, previously reports have also shown that HS can exert a deleterious effect on ovarian follicular dynamics and oocyte competence *Bos indicus* cattle as well (Torres-Júnior *et al.*, 2008).

A previous seasonal experiment demonstrated that once the pool of ovarian oocytes is damaged by heat stress, two or three estrous cycles are required (after the end of heat stress) to restore the follicular pool and oocyte quality (Roth *et al.*, 2001). However, the study described above (Torres-Júnior *et al.*, 2008)

showed a carry-over effect on blastocyst production up to 105 days after the end of the heat stress (Fig. 1). Therefore, it seems that follicles and oocytes are damaged by heat stress during early stages of folliculogenesis, with a delayed deleterious effect on ovarian function. Nevertheless, *Bos indicus* breeds have been shown to be more resistant to tropical conditions (i.e. elevated temperature and humidity) than breeds that evolved in temperate climates (i.e. *Bos taurus*, as Holstein). Essentially, the adaptation of certain breeds to elevated heat and humidity is related to their ability to thermoregulate their body temperature (Bennett *et al.*, 1985; Hammond *et al.*, 1996; Gaughan *et al.*, 1999).

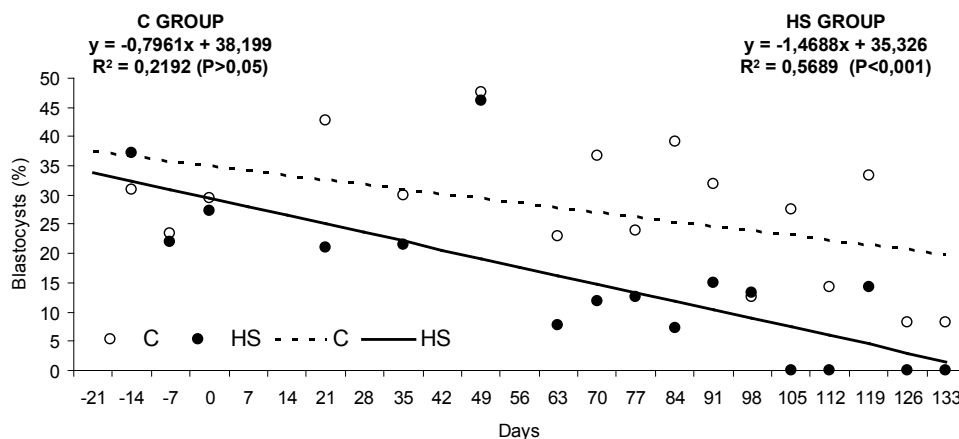


Figure 1. Percentage of blastocysts and regression equation's adjusted lines of oocytes recovered from Gyr (*Bos indicus*) cows exposed to thermoneutral (C) or heat-stress (HS) treatments. Adapted from Torres-Júnior *et al.* (2008).



Heat stress also has a deleterious effect on superovulatory response in Holstein donors. In a recent retrospective analysis, Vieira *et al.* (2014b) reported a negative effect of the warm season in Brasil on the number of *in vivo* produced embryos (2.8 ± 0.3 vs. 4.4 ± 0.4 ; $P = 0.03$) and percentage of embryos classified as grade I and II (21.4 vs. 32.8%, $P < 0.0001$) in Holstein donors. In addition, Ferreira *et al.* (2011) reported decreased COC numbers in Holstein cows when OPU was performed during the summer months. Yet, when blastocyst rates were evaluated, an interaction between group and season indicated that the effect of season was dependent on animal category. In the summer, blastocyst rate dropped in repeat breeder cows (usually females during late lactation) in comparison to winter, becoming lower than in cows at peak of lactation. However, regardless of season, blastocyst rates were lower in repeat breeder cow than in heifers. Additionally, repeat breeder blastocyst quality was compromised in comparison to heifers and cows at peak lactation during the summer,

A common aspect of commercial OPU-IVP programs is the use of non-lactating or late lactation cows as oocyte donors. In these animals, in addition to the effects of heat stress, diet may also influence IVP. In these animal categories, the negative effects of overfeeding (excessive energy intake) can compromise *in vitro* oocyte developmental competence, especially in over-conditioned (high body condition score) females

(Adamiak, 2005). The mechanisms that mediate these negative effects on oocyte competence may be related to endocrine alterations, such as hyperinsulinemia, peripheral resistance of insulin, and increased glucose and IGF-I, which may interfere with glucose transport in embryo cells and increased apoptosis.

The nutritional and metabolic state can interfere with follicular growth patterns, secretion of reproductive hormones, and oocyte quality in cattle (Leroy *et al.*, 2008; Ashworth *et al.*, 2009; Batista *et al.*, 2013; Sales *et al.*, 2015). Thus, metabolic imbalances may cause systemic alterations that can compromise the success of reproductive biotechnologies, such as OPU-IVP (Webb *et al.*, 2004; Adamiak, 2005). Our research group conducted a study to evaluate the impact of different energy intakes on metabolic profiles and oocyte quality of the non-lactating Gyr (*Bos indicus*) cows submitted to successive OPU sessions (Sales *et al.*, 2015). Diets were formulated to achieve maintenance (M) or 1.7% of maintenance (1.7M) for non-lactating cows. We observed that following 60 days of high energy feeding, cows had reduced *in vitro* oocyte competence (Fig. 2). All cows fed high energy diets had greater glucose and insulin concentrations and a greater level of insulin resistance as determined by the glucose tolerance test. Furthermore, cows receiving high energy diets, had a lower abundance of transcripts for GLUT 1, IGF 1R, IGF 2R and HSP70.1 genes in oocytes.

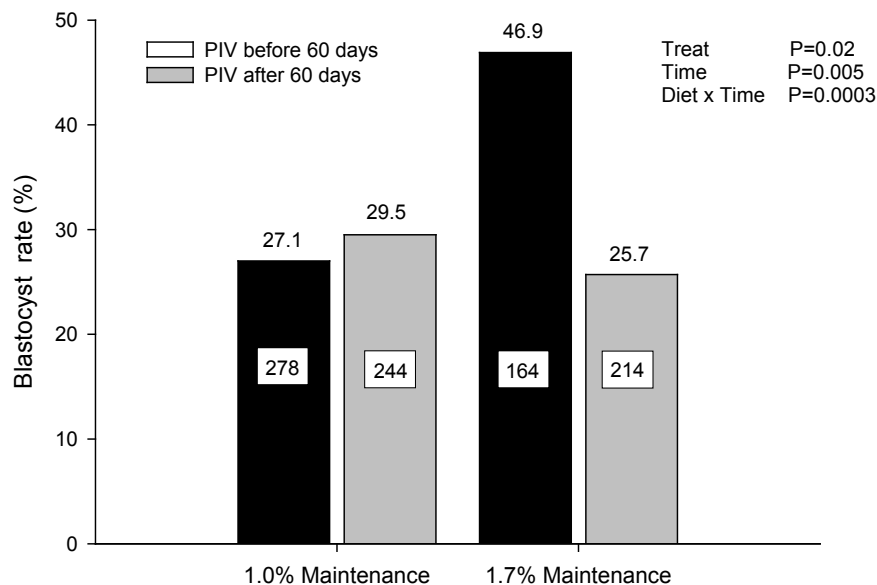


Figure 2. *In vitro* embryo production in non-lactating cows (n = 14) fed diets to meet 100 or 170% of energy of maintenance and submitted to nine OPU session at 14 day intervals. Adapted from Sales *et al.* (2015).

The stage of the estrous cycle at the time of OPU can also influence recovery rates, oocyte quality and *in vitro* embryo production (Hendriksen *et al.*,

2000; Merton *et al.*, 2003; Vassena *et al.*, 2003; Machatkova *et al.*, 2004). The number of COC retrieved was greater when the OPU is performed during the early



phase of the follicular wave (Machatkova *et al.*, 2004), probably due to the greater recovery rate after the aspiration of small follicles (4 mm; Seneda *et al.*, 2001). However, despite the lower recovery rates, higher *in vitro* competence was observed when oocytes were obtained during early dominance phase of the dominant follicle (Merton *et al.*, 2003; Vassena *et al.*, 2003; Hendriksen *et al.*, 2004). Similarly, stages of the follicular wave affected the numbers of oocytes recovered and *in vitro* competence following OPU in Nelore cows (Melo, 2007). Higher numbers of COC were recovered and *in vitro* embryo production was higher when Nelore cows were subjected to OPU on days 3 and 5 compared to days 7 and 9 after follicular wave emergence.

Based in the protocols described above, our research group designed a trial to evaluate different times relative to follicular wave emergence to perform the OPU-IVP in crossbred (*Bos taurus* x *Bos indicus*) heifers (Gimenes *et al.*, 2007). Follicular wave emergence was synchronized with EB and P4 in all donors and OPU was done 2, 4 and 6 days after follicular wave emergence. Higher blastocyst and hatched blastocysts rates and higher numbers of nuclei in hatched blastocysts were observed when OPU was done on day 2 of the follicular wave. However, no effect of follicular wave status was observed in either Holstein (*Bos taurus*) or Nelore (*Bos indicus*) donors in a subsequent study (Gimenes *et al.*, 2015).

The unexpected effects of day of the follicular wave on OPU-IVP variables may be due to the method of synchronization of follicular wave emergence (pharmacologic synchronization vs. follicular ablation). Although our purpose was to provide a more practical method of synchronization of follicular wave emergence, the pharmacologic induction of follicular wave emergence could result in a cumulative follicle population containing follicles undergoing regression together with the new follicle cohort. Therefore, more studies must be conducted to clarify this matter.

Conclusion

The success of *in vivo* and *in vitro* embryo production is closely associated to oocyte and embryo quality. Therefore, factors related to breed, heat stress and nutrition should be considered before applying SOV or OPU-IVP in the field. Adequate control of environmental and nutritional conditions should be one of the requisites to be accomplished before implementing any reproduction biotechnology. On the other hand, strategies established to manipulate follicular wave dynamics (synchronization of the follicular wave emergence and superstimulation) can optimize the efficiency of embryo production techniques. Once these biotechnologies can be efficiently applied on a large scale in the field, significant enhancements in livestock genetic gain can

be accomplished bringing productivity and economic return for the activity.

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