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Antral Follicle Populations and Embryo Production – *In Vitro* and *In Vivo* – of *Bos indicus–taurus* Donors from Weaning to Yearling Ages

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Contents

Interest in *indicus–taurus* cattle has been increasing, as these animals are likely to present the best characteristics of Zebu and European bovine breeds. The aim of this study was to compare the embryo production of *indicus–taurus* donors with high vs low antral follicle counts obtained by *ovum pickup*/*in vitro* production (OPU/IVP) and superovulation (SOV)/embryo collection. Braford females at weaning age (3/8 Nelore × 5/8 Hereford, n = 137, 9 ± 1 month old) were subjected to six serial ovarian ultrasonographs and were assigned to two groups according to the number of antral follicles ≥3 mm as follows: G-High antral follicular count (AFC, n = 20, mean ≥40 follicles) and G-Low AFC (n = 20, mean ≤10 follicles). When the females (n = 40) reached 24 months of age, they were subjected to both OPU/IVP and SOV/embryo collection. The average number of follicles remained highly stable throughout all of the ultrasound evaluations (range 0.90–0.92). The mean number of COCs recovered (36.90 ± 13.68 vs 5.80 ± 3.40) was higher ($p < 0.05$) for females with high AFC, resulting in higher ($p < 0.05$) numbers of total embryos among females with high vs low AFC (6.10 ± 4.51 vs 0.55 ± 0.83). The mean number of embryos per collection was also higher ($p < 0.05$) for G-High vs G-Low (6.95 ± 5.34 vs 1.9 ± 2.13). We conclude that a single ultrasound performed at pre-pubertal ages to count antral follicles can be used as a predictor of embryo production following IVP and SOV/embryo collection in *indicus–taurus* females.

Introduction

Interest in *indicus–taurus* bovine breeds has been increasing due to their adaptability to produce meat and milk under stressful conditions, such as high temperature, parasites and poor pastures (Pontes et al. 2011). These characteristics are maintained in Nelore or Bhraman – Hereford cross-bred animals, usually referred to as 'Braford', which are popular beef cattle in Central and South America and other tropical and non-tropical areas.

Considering the importance of embryo production for genetic improvement, it is important to note the high variability in embryo production per donor following *ovum pickup* (OPU)/IVP and superovulation (SOV)/embryo collection (Pontes et al. 2009). For IVP-derived embryos, variation in oocyte yield reportedly influences the final number of embryos produced (Pontes et al. 2011). Additionally, some females produce their highest embryo yield following OPU/IVP or SOV/embryo collection (Pontes et al. 2009).

Some studies have reported high variability in the number of pre-antral and antral follicles among individual adult cattle (Erickson 1966; Burns et al. 2005; Silva-Santos et al. 2011). However, the number of antral follicles is repeatable in individuals during follicular waves (Burns et al. 2005; Ireland et al. 2007). Therefore, it is possible to identify bovine females with low, intermediate or high numbers of follicles during waves by ultrasonography. It is unknown whether variation in the ovarian follicular reserve and the antral follicular count (AFC) can affect *in vitro* or *in vivo* embryo production in cattle. Comparisons between *in vitro* and *in vivo* embryo production have revealed higher numbers of recovered and transferable embryos with high AFC vs low AFC, lower proportions of transferable embryos per animal after SOV of beef cattle, higher numbers of oocytes and blastocysts following follicular aspiration of abattoir ovaries with high numbers of antral follicles and similar proportions of blastocysts between groups (Ireland et al. 2007). However, *in vitro* and *in vivo* embryo productions in the same cattle have not been assessed. The aim of this study was to compare the efficiency of OPU/IVP vs SOV/embryo collection on embryo production in the same donors with consistently high vs low AFC. This comparison was performed after counting antral follicles by ultrasonography in *indicus–taurus* cattle from weaning to yearling ages.

Materials and Methods

Animals and AFC

Braford females (3/8 Nelore × 5/8 Hereford, n = 137) maintained in *Brachiaria brizantha* pasture supplemented with mineral salt *ad libitum* were serially examined by ultrasonography from weaning (9 ± 1 months of age) to yearling ages (20 ± 1 months of age). At 24 months of age, the mean body condition score was 3.0 ± 0.5 (scale, 1–5; Lowman et al. 1976), and the average live body weight was 360 ± 10 kg. Several of the females were selected based on the AFC. The ovaries from each animal were monitored with a 7.5-convex intravaginal array transducer (Áquila PRO, Pie Medical, Maastricht, the Netherlands) spaced 60 day apart (days 0, 60, 120, 180, 240 and 300), and antral follicles were counted as described previously (Burns et al. 2005; Ireland et al. 2008). After six ultrasound evaluations performed by the same operator, females were assigned to two groups according to the number of antral follicles ≥3 mm (AFC – antral follicle count) as follows: females with a consistently high (G-High, ≥40 follicles; n = 20)

or low AFC (G-Low, ≤10 follicles; n = 20) in all ultrasound scans. Animals with intermediate AFC (>10 and <40 follicles; n = 97) were not studied further. When these females (n = 40) reached 24 ± 1 months of age, they were submitted to OPU/IVP procedures and thereafter to SOV/embryo collection.

Preparation of donor females

Before each procedure, faeces were removed from the rectum, and the perineal area was cleaned with tap water and 70% ethanol. Prior to OPU or embryo collection, each cow received epidural anaesthesia (4 ml of 2% lidocaine; Anestésico L, Pearson, São Paulo, SP, Brazil) to decrease peristalsis and discomfort.

Follicle aspiration

Previously described procedures were used for follicular aspiration (Seneda et al. 2001). Briefly, each visible follicle was aspirated using a real-time B-mode ultrasound scanner (Áquila PRO, Pie Medical), a 7.5-MHz convex array transducer fitted into the intravaginal device (Pie Medical) and a stainless steel guide. Follicular puncture was performed using a disposable 19-gauge 1/2 hypodermic needle (Becton Dickinson, Curitiba, PR, Brazil) connected to a 50-ml conical tube (Corning, Acton, MA, USA) via silicon tubing (0.8 m; 2 mm id). Aspiration was performed using a vacuum pump (WTA, Watanabe, Brazil) with a negative pressure of 75 mm Hg. The collection medium was phosphate buffer solution (PBS; Nutricell, Campinas, SP, Brazil) with 10 000 IU/l sodium heparin (Sigma H-3149).

In vitro embryo production

Immediately after recovery, the aspirated material was washed and filtered through an Emcon embryo filter (Immuno Systems Inc., Spring Valley, WI, USA) with PBS (Nutricell). The cumulus oocyte complexes (COCs) were classified according to the presence of cumulus cells and the oocyte quality using the following criteria: 1) good, more than three layers of cumulus cells; 2) regular, at least one layer of cumulus cells; 3) denuded; and 4) atretic with dark cumulus oophorus and signs of cytoplasmic degeneration (Seneda et al., 2001). Both good and regular oocytes were considered viable and were used, whereas atretic oocytes were discarded. Prior to *in vitro* maturation (IVM), COCs were washed three times in TCM-199 HEPES (Gibco Life Technologies, Grand Island, NY, USA) supplemented with 10% foetal calf serum (FCS) and 50 μg gentamycin sulphate, and they were washed once in bicarbonate TCM-199 (Gibco Life Technologies) supplemented with 10% FCS, 5 μg luteinizing hormone (LH-Ayerst, Rouses Point, NY, USA), 0.5 μg follicle-stimulating hormone (FSH; Folltropin, Vetrepharm, Belleville, ON, Canada), 1 μg oestradiol (oestradiol-17β, Sigma E-8875), 2.2 μg pyruvate (Sigma P-4562) and 50 μg gentamicin/ml of medium. The COCs from each category were cultured separately for 24 h in 100 μL of maturation medium under mineral oil (D'Altomare, Santo Amaro, SP, Brazil) at 39°C and 5% CO_2 in air (Gordon 1994; Smith et al. 1996). Frozen–thawed sexed sperm (2×10^7/dose) were used from a Braford sire of known fertility based on the previous utilization for *in vitro* fertilization (IVF). For IVF, straws were thawed for 20 s in a 35°C water bath. The sperm were washed by centrifugation at 200 ***g*** for 30 min through a 90–45% Percoll gradient. The sperm were capacitated using heparin (30 μg/ml), and motility was stimulated by the addition of 40 μL/ml of penicillamine, hypotaurine and epinephrine (PHE; Parrish et al. 1986). After a visual assessment of motility, the sperm concentration was adjusted to 25×10^6 motile sperm/ml, and each fertilization drop (100 μL, 15–20 oocytes) received 4 μL of sperm (final concentration 1×10^5 sperm per drop; Seneda et al. 2001). After maturation, the COCs were washed three times in pre-fertilization TCM-199 medium supplemented with 25 mM HEPES (Gibco Life Technologies) and 0.3% BSA (Sigma A-9647). The COCs were then washed once in TALP fertilization medium supplemented with 10 g/ml heparin and 160 μL PHE solutions (Parrish et al. 1986; Bavister 1989). Presumptive zygotes had their cumulus cells removed and were transferred into 100 μL of culture medium for embryos [SOFaa BSA, containing 8 mg/ml BSA (free of fatty acid) and 1 mM glutamine] under the same temperature and CO_2 conditions used for IVF. The osmolarity was maintained at 270–280 mOsmol, and the pH was 7.4. The embryo rate was obtained from the total number of aspirated oocytes. The embryos were evaluated until Day 7 (Day 0 = day of IVF) according to IETS criteria (Wright 1998). Cleavage and blastocyst rates were recorded at days 3 and 7 of culture. Embryos graded as I, II and III were defined as viable. Expanded blastocysts of grade I quality were subjected to Cryotop vitrification according to the method described previously (Kuwayama et al. 2005).

Superovulation

The same females were subjected to a protocol of SOV and embryo recovery, which was started 7 day after the OPU procedure. All 40 donor females received the same treatment, which consisted of an auricular device (Crestar, Intervet-Schering Plough, Brazil) and 2.5 mg oestradiol benzoate IM (EB, Estrogin, Farmavet, São Paulo, São Paulo, Brazil) on Day 0 (D0). Between days 5 and 8, FSH (200 mg, IM, Foltropin-V, Bioniche, Canada) was administered twice daily in decreasing doses. On the morning of Day 7, the donors were given 500 μg cloprostenol IM (PGF2α, Ciosin, Intervet-Schering Plough, São Paulo, São Paulo Brazil), and they were given 200 IU eCG IM (Novormon, Syntex SA, Argentina) on the morning and the afternoon of Day 8. The device was removed at the time of the second dose of eCG (afternoon of Day 8). On the morning of Day 9, the females received 12.5 mg LH IM (Lutropin-V, Bioniche, Canada), and fixed-time artificial insemination (FTAI) was performed 12–24 h after the LH injection (Fig. 1).

Embryo recovery

Uterine flushing was performed 7 day after the LH injection, and the embryos were collected using a

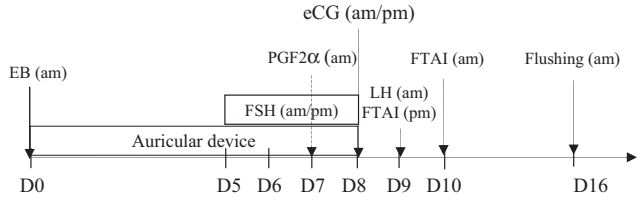

Fig. 1. Treatment schedule for the superovulation of *indicus–taurus* females with high and low antral follicle numbers

two-way Foley catheter passed through the cervix. The catheter tip was placed in the uterine body, caudally to the external bifurcation of the uterus, and both horns were flushed simultaneously. The uterus was flushed five or six times using 1 l total volume of Dulbecco's phosphate-buffered saline (DPBS, Nutricell, Campinas, São Paulo, Brazil). The embryos were collected on a filter, counted and evaluated according to IETS criteria (Wright 1998). Embryos graded as I, II and III were defined as viable, and embryos of grades I and II were frozen and stored at −196°C.

Semen

Frozen–thawed sperm (2×10^7/dose) from the same Braford sire and ejaculate was used for both IVP and SOV.

Statistical analysis

The results are presented as the means ± SD. All the statistical analyses were performed using the R software (R development core team 2013). Repeatability (proportion of the total variance that could be attributed to animal variance, range 0–1, 1 = perfect) was calculated (Boni et al. 1997). The means were not normally distributed and were analysed using the Kruskal–Wallis test with a Dunn's test for comparisons between groups. The embryo production per female with high AFC was normally distributed and was compared by ANOVA. Comparisons among the six greatest donors for the *in vitro* or *in vivo* procedures were performed using ANOVA with a t-test for comparisons between procedures. Cleavage, blastocyst rates, proportions of viable oocytes and freezable embryos were evaluated with a chi-square test. The proportions of vitrifiable embryos were compared using Fisher's exact test. For all of the analyses, $p \leq 0.05$ was considered to be significant.

Results

The average number of follicles remained highly stable throughout all of the ultrasound evaluations for both high and low AFC groups (G-High $r = 0.90$; G-Low $r = 0.92$). The embryo production by Braford females with high and low AFCs subjected to OPU/IVP and SOV/embryo collection is presented in Table 1. After follicular aspiration, both the mean number of COCs recovered (36.90 ± 13.68 vs 5.80 ± 3.40) and the viable oocytes (21.65 ± 10.05 vs 3.20 ± 2.44) were higher ($p < 0.05$) for females with high AFC, resulting in higher ($p < 0.05$) numbers of total embryos for the females with high AFC (6.10 ± 4.51 vs 0.55 ± 0.83). After embryo collection, the total number of recovered structures was higher for G-High (8.80 ± 6.78) compared with G-Low (2.25 ± 2.63) as well as for the mean number of embryos/collection ($p < 0.05$; 6.95 ± 5.34 vs 1.9 ± 2.13). Significant differences ($p < 0.05$) in average embryo production following OPU/IVP and SOV/embryo collection were found among females with low AFC (Table 1).

Table 1. Mean (±SD) reproductive performance of *indicus–taurus* females with high (G-High, ≥40 follicles) and low (G-Low, ≤10 follicles) antral follicle counts (AFCs), comparing embryo production following *in vitro* [*ovum pickup*/*in vitro* production (OPU/IVP)] and *in vivo* [superovulation (SOV)/embryo collection] procedures

	G-High (n = 20)	G-Low (n = 20)
Antral follicles (n)	47 ± 6	9 ± 3
Total oocytes recovered	738[a]	116[b]
Recovery oocyte rate (%)	78.51 (738/940)[a]	64.44 (116/180)[b]
Oocytes/OPU (n)	36.90 ± 13.68[a] (738/20)	5.80 ± 3.40[b] (116/20)
Viable oocytes/OPU (n)	21.65 ± 10.05[a] (435/20)	3.20 ± 2.44[b] (64/20)
Proportion of viable oocytes (%)	58.94 (435/738)	55.17 (64/116)
Cleavage rate (%)	61.25 (452/738)	56.03 (65/116)
Blastocyst rate (%)	16.53 (122/738)	9.48 (11/116)
Total embryos/OPU/IVP (n)	6.10 ± 4.51[aA] (122/20)	0.55 ± 0.83[bB] (11/20)
Vitrifiable embryos/OPU/IVP (n)	4.10 ± 3.24[a] (82/20)	0.20 ± 0.52[b] (4/20)
Proportion vitrifiable (%)	67.21[a] (82/122)	36.36[b] (4/11)
Total recovered structures/collection (n)	8.80 ± 6.78[a] (176/20)	2.25 ± 2.63[b] (45/20)
Total embryos/collection (n)	6.95 ± 5.34[aA] (139/20)	1.9 ± 2.13[bA] (38/20)
Freezable embryos/collection (n)	5.45 ± 5.77[a] (109/20)	1.7 ± 2.03[b] (34/20)
Proportion freezable (%)	78.42[a] (109/139)	89.47[a] (34/38)

[ab]Within a row, means without a common superscript differ ($p \leq 0.05$).
[AB]Within a column, means without a common superscript differ ($p \leq 0.05$).

The females with high and low AFCs that were selected for the highest embryo production following *in vitro* (G-High, n = 6; G-Low, n = 6) and *in vivo* procedures (G-High, n = 6; G-Low, n = 6) are shown in Table 2. For the females with high and low AFCs, the embryo yield from donors selected for the greatest embryo production following OPU/IVP did not differ from that following SOV/embryo collection (11.5 vs 7.2 for G-High; 1.7 vs 1.5 for G-Low). For the females selected based on the highest embryo production during the *in vivo* procedure, both donors with high and low AFCs produced higher ($p < 0.01$) average number of embryos following SOV/embryo collection compared with OPU/IVP (13.5 vs 6.2 for G-High; 4.5 vs 0.3 for G-Low).

Discussion

Comparisons between the *in vitro* and *in vivo* methods for bovine embryo production have been reported (Farin and Farin 1995; Gjørret et al. 2003; Hansen and Block 2004; Pontes et al. 2009). However, this is the first comparative study to show that counting antral follicles by ultrasound scan at weaning age can be predictive to assess the embryo yield from *indicus–taurus*

Table 2. Variation in embryo production among *indicus–taurus* females with high (G-High, ≥40 follicles) and low (G-Low, ≤10 follicles) antral follicle counts (AFCs), comparing the six greatest donors for *in vitro* [*ovum pickup* (OPU)/ *in vitro* fertilization (IVF)] or *in vivo* [superovulation (SOV)/embryo collection] procedures

	Donors of High AFC							Donors of Low AFC						
Greatest production following IVP (n = 6)	I	II	III	IV	V	VI	Mean	I	II	III	IV	V	VI	Mean
IVP	8	8	10	11	15	17	11.5	1	1	2	2	2	2	1.7
Embryo Collection	6	13	2	9	8	5	7.2	1	0	0	2	2	4	1.5
Greatest production following SOV/embryo collection (n = 6)	VII	VIII	IX	X	XI	XII	Mean	VII	VIII	IX	X	XI	XII	Mean
Embryo Collection	9	9	13	13	17	20	13.5[A]	2	3	4	4	7	7	4.5[A]
IVP	4	11	5	8	2	7	6.2[B]	0	0	0	2	0	0	0.3[B]

[ab]Within a column, means without a common superscript differ ($p \leq 0.05$).
[AB]Within a column, means without a common superscript differ ($p \leq 0.01$).

donors at yearling ages following IVP and SOV/embryo collection procedures.

The selection of cattle based on the antral follicle counts during follicular waves by ultrasound is possible due to strong reproducibility among individuals regardless of breed, age, season, stage of lactation or management conditions (Burns et al. 2005; Ireland et al. 2007, 2008). We also observed repeatability (range 0.90–0.92) in the numbers of antral follicles in Braford females with high (47 ± 7 follicles) vs low AFC (9 ± 2 follicles) during six ultrasound scans performed by the same operator in cattle from weaning to yearling ages (Table 1). This remarkable repeatability in follicular numbers enhanced the importance of the use of ultrasonography to count antral follicles at wave emergence; a single ultrasound scan could predict the superovulatory response in cattle (Singh et al. 2004). Beyond supporting the use of antral follicle counts to predict the superovulatory response, the present study shows that this count can be performed in cattle at yearling ages to predict the success of IVP programmes.

Extreme variations in oocyte recovery and superovulatory responses remain serious problems for bovine embryo production (Pontes et al. 2009, 2011). The higher numbers of follicles and oocytes have been related to the success of IVP and SOV. (Kawamata 1994; Cushman et al. 1999; Taneja et al. 2000; Singh et al. 2004; Pontes et al. 2011). However, no studies have compared the efficiencies of *in vitro* and *in vivo* procedures performed in the same cattle. In the present study, females with high AFC subjected first to OPU/IVP and thereafter to SOV/embryo collection showed the best results compared with their low AFC counterparts. The embryo yield obtained by OPU/IVP from G-High vs G-Low females (6.10 ± 4.51 vs 0.55 ± 0.83) was lower compared with SOV/embryo collection (6.95 ± 5.34 vs 1.9 ± 2.13; Table 1). Differences in embryo production were found between procedures among G-Low females. Similarly, other authors reported a reduced number of IVP-derived embryos (1.3 vs 4.9) from abattoir ovaries of females with low antral follicle counts (<15 follicles). They further reported decreased embryo production following ovarian stimulation and embryo recovery (3.8 vs 5.4) for cattle with low vs high numbers of antral follicles (<15 vs >25 follicles; Ireland et al. 2007). Although differences in absolute numbers were found, we did not observe differences in the proportions of freezable embryos among females with high vs low AFC (78.42% vs 89.47%). Conversely, the aforementioned study (Ireland et al. 2007) reported a lower proportion of transferable embryos among females with high numbers of antral follicles (50.7% vs 79.8%).

Individual variation in oocyte production was reported in Nelore cows and was associated with embryo production and the number of pregnancies (Pontes et al. 2009, 2011). Donor cows producing large numbers of oocytes (59 oocytes/OPU) produced significantly more embryos and pregnancies (~ sixfold greater) than those with low oocyte production (10 oocytes/OPU; Pontes et al. 2011). We also observed a large variation in oocyte recovery (36.90 ± 13.68 vs 5.80 ± 3.40), although no differences were found in the proportions of viable oocytes (58.94% vs 55.17%) among donors with high and low AFCs. For both G-High and G-Low groups, there were no differences between cleavage (61.25 vs 56.03) and blastocyst (16.53 vs 9.48) rates. Similarly, Ireland et al. (2007) did not observe differences in cleavage (74.7% vs 74.0%) or blastocyst (30.9% vs 29.6%) rates, nor did they observe differences in the percentage of oocytes produced (60.3 vs 53.6) among females with high vs low follicle counts.

Individual variation in embryo yield also occurs with the *in vivo* technique. Only 30% of cows yield the majority of embryos (70%), and 25% of treated cows produce no embryos (Lerner et al. 1986; Looney 1986). Considering the embryo production, we compared the best donors after OPU/IVP and SOV/embryo collection. As shown in Table 2, it is clear that some donors present greater efficiency in embryo production following OPU/IVP, while others have great efficiency following SOV/embryo collection. No differences were found between the *in vitro* and *in vivo* procedures (11.5 vs 7.2 for G-High; 1.7 vs 1.5 for G-Low) comparing the embryo production of the six cows with higher embryo production following OPU/IVP. However, females with either high or low AFC selected based on increased embryos following the *in vivo* procedure produced higher numbers of embryos following SOV/embryo

collection compared with OPU/IVP (13.5 vs 6.2 for G-High; 4.5 vs 0.3 for G-Low).

In conclusion, this study reports the embryo yield from donors with high and low antral follicle numbers after almost 1 year of follicular growth as monitored by ultrasound, and *in vitro* and *in vivo* procedures were compared in the same donor cows. Although there remains individual variation in embryo yields, OPU/IVP and SOV/embryo collection procedures performed in the same cattle resulted in the greatest number of embryos from females with higher populations of antral follicles. Thus, the number of antral follicles present in the ovaries of cattle at weaning ages can reliably predict the success of IVP and the superovulatory response in cattle. In conclusion, this method can be used as an easy and cheap tool to identify potential efficient embryo producers.

Conflict of interest

This study does not present any conflict of interest.

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Author contributions

Katia Cristina Silva-Santos and Marcelo Seneda designed the study, analysed data and drafted the paper; Gustavo Santos designed the study and analysed data; Celso Koetz Júnior, Fábio Morotti, Letícia Siloto, Thiago Marcantonio, Reginaldo Luís Oliveira, and Danylo Lima analysed data; Mariana Urbano performed statistical analyses.

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