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Plasma anti-mullerian hormone: an endocrine marker for in vitro embryo production from *Bos taurus* and *Bos indicus* donors

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ABSTRACT

The aim of this study was to evaluate the association between plasma anti-mullerian hormone (AMH) concentration and in vitro embryo production (IVP) from *Bos taurus* (Holstein) and *Bos indicus* (Nelore) donors. A total of 59 Holstein (15 prepubertal heifers aged 8–10 mo, 15 cyclic heifers aged 12–14 mo, 14 lactating cows, and 15 nonlactating cows) and 34 Nelore (12 prepubertal heifers aged 10–11 mo, 10 prepubertal heifers aged 21–23 mo, and 12 cyclic heifers aged 24–26 mo) females were enrolled. All females underwent an ovum pick-up (OPU), without previous synchronization of the follicular wave, and IVP procedure. Immediately before the OPU procedure, blood samples were collected for subsequent AMH determination. A positive correlation was observed between the plasma AMH and number of in vitro embryos produced from Holstein ($r = 0.36$, $P < 0.001$) and Nelore ($r = 0.50$, $P = 0.003$) donors. For additional analyses, donors within each genotype were classified into 1 of 2 AMH categories (low or high) according to the average AMH concentration for each genotype. The results revealed that females classified as having high AMH presented a greater number of visible aspirated follicles (Holstein: 20.9 ± 1.5 vs 13.6 ± 0.9, $P < 0.0001$; Nelore: 54.3 ± 6.1 vs 18.6 ± 2.1, $P < 0.0001$) and a greater number of recovered cumulus-oocyte complexes (Holstein: 17.3 ± 1.5 vs 9.0 ± 0.9, $P < 0.0001$; Nelore: 45.3 ± 6.4 vs 13.4 ± 1.7, $P < 0.0001$). However, there was no difference in the blastocyst production rate (Holstein: 20.6% ± 4.0% vs 19.8% ± 4.2%, $P = 0.60$; Nelore: 33.7% ± 6.5% vs 27.4% ± 5.5%, $P = 0.41$, high and low AMH, respectively). Moreover, donors classified as having high AMH yielded a greater number of embryos produced per OPU (Holstein: 3.0 ± 0.7; Nelore: 7.0 ± 1.7) compared with those classified as having low AMH (Holstein: 1.2 ± 0.3, $P = 0.04$; Nelore: 2.2 ± 0.5, $P = 0.007$). In conclusion, although the plasma AMH concentration did not alter the ability of the cumulus-oocyte complex to reach the blastocyst stage, the AMH concentration in plasma can be an accurate endocrine marker for the in vitro embryo yield from either *B. taurus* (Holstein) or *B. indicus* (Nelore)

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donors. Therefore, AMH is a promising tool to enhance the overall efficiency of OPU–IVP programs in the field as a selective criterion for high embryo producing donors.

1. Introduction

The profitability of the dairy and beef industries is highly correlated to the meat and milk yield, genetic selection, and reproductive efficiency. Reproductive technologies, such as in vitro embryo production (IVP), have been applied worldwide to rapidly enhance the genetics of dairy and beef cattle through the female lineage. However, the efficiency of this technique has been impaired by the large variability among donors in their response to IVP procedures [1–4]. Therefore, the success of in vitro embryo technologies has been associated primarily with physiological characteristics, such as ovarian antral follicle population (AFP) and oocyte competence (eg, the ability of the oocyte to reach the blastocyst stage) [5–8].

The AFP has been related to several substances, including the concentrations of circulating insulin, insulin-like growth factor I, and anti-mullerian hormone (AMH) [9–13]. However, the strong association between the follicle population and the AMH concentration provides a consistent method for predicting the AFP [14–16]. AMH is a glycoprotein member of the transforming growth factor beta superfamily [17]. In females, AMH exclusively expressed in the gonads is produced only postnatally [18] within the granulosa cells of the small antral growing follicles and is reduced during terminal follicular growth [15,19–24]. Furthermore, AMH has been shown to be a modulator of early follicular growth, acting as a factor to prevent the premature depletion of the follicle reserve in ovaries [25–27]. The circulating AMH concentrations have been positively associated with the total number of ovarian follicles in mice [27], women [28], and in *Bos taurus* and *Bos indicus* cattle [13]. Furthermore, the measurement of circulating AMH concentrations can help to predict superovulatory responses and in vivo embryo production [23,29].

Additionally, the oocyte competence has been related to several factors, including individual variation [4,11,30,31], environment [3,32–34], and the stage of the estrous cycle [35–42]. Regarding the ovarian follicular wave, healthy follicles with diameters from 3 to 7 mm expressed higher AMH messenger RNA levels and contained higher AMH concentrations in the follicular fluid compared with larger antral and atretic follicles [23]. Finally, the scarce research focused on the correlation between the AMH concentration and the oocyte competence in vitro implies that further studies are required, which aim to improve commercial IVP outcomes.

The new researches will reinforce the possibility of using AMH concentration as a reliable endocrine marker to select donors with high AFPs [43], an increased oocyte quantity and quality and, consequently, a greater IVP efficiency. Therefore, in response to the lack of information regarding the effects of AMH concentration on the efficiency of ovum pick-up (OPU)–IVP programs in cattle, the present study was designed to evaluate the use of the plasma AMH concentration as an endocrine marker for IVP from *B. taurus* (Holstein) and *B. indicus* (Nelore) donors. The hypothesis was that donors with greater plasma AMH concentrations would present increased retrieved cumulus-oocyte complexes (COCs), enhanced in vitro competence of the recovered oocytes, and consequently greater embryo yield per OPU–IVP procedure compared with donors with lower AMH concentrations, regardless genotype (*B. taurus* or *B. indicus*).

2. Materials and methods

2.1. Experiment 1

2.1.1. Farm and animals

This experiment was conducted at a commercial dairy farm in Descalvado, São Paulo, in southwestern Brazil. A total of 59 Holstein (*B. taurus*) donors were used in this experiment. Aiming to enroll most female categories typically available for IVP procedure in a commercial dairy farm 15 prepubertal heifers aged 8 to 10 mo, 15 cyclic heifers aged 12 to 14 mo, 14 lactating cows, and 15 nonlactating cows were used.

All females were fed a total mixed ration formulated to meet or exceed the minimum nutritional requirements for Holstein heifers, lactating cows, or nonlactating cows [44]. The main ingredients were corn silage and Tifton hay as forage, as well as corn, soybean, and cottonseed meal-based concentrate. The present study was approved by the Bioethics Commission of the School of Veterinary Medicine and Zootechny of the University of São Paulo.

2.1.2. Ultrasonography examinations

The ovarian cyclicity of each heifer was defined by the presence of a corpus luteum during 2 consecutive ultrasound examinations performed 14 d apart before the OPU procedure. All females were submitted to an OPU–IVP procedure without previous synchronization of the follicular wave. Immediately before the OPU, all visible ovarian follicles were counted and recorded. All ovarian examinations were performed by transrectal ultrasonography using a portable scanner (Aloka SSDV 500, Aloka, Tokyo, Japan) with a 5-MHz convex array transducer. During each ultrasound evaluation, all visible antral follicles ($\geq$2 mm in diameter; AFP) on both ovaries were recorded.

2.1.3. OPU procedure

For the oocyte collection procedure, cattle were restrained in a chute and epidural anesthesia was administered using 2% lidocaine hydrochloride (Lidovet, Bravet, Brazil) to facilitate the handling of the ovaries through the rectum. The perineal area was cleaned using water, dried, and sprayed with alcohol before each session. All follicles $\geq$2 mm were aspirated using a portable scanner with a 5-MHz convex array transducer

(Mindray DP 2200 vet, China) housed in a plastic vaginal probe with a stainless steel needle guide (20 G; 0.9 × 50 mm; Terumo Europe NV, Belgium) connected to aspiration equipment and a vacuum system (68 mm Hg of negative pressure; V-MAR 5000, Cook Australia, Queensland, Australia). Follicular aspirates were recovered via a 1.1-mm inner diameter by a 120-cm length circuit (Watanabe Tecnologia Aplicada, WTA Ltda, Cravinhos, São Paulo, Brazil) connected directly to a 50-mL conical tube containing 15 mL of Dulbecco phosphate-buffered saline (DPBS; Nutricell Nutrientes Celulares, Campinas, São Paulo, Brazil) and 5,000 IU/mL sodium heparin (Parinex, Hipolabor, Belo Horizonte, Minas Gerais, Brazil) at 35°C to 37°C. The vacuum connected to the needle was set at 85 to 90 mm Hg. All retrieval procedures were performed by the same veterinarian.

The conical tube containing the follicular aspirates was transported to a field laboratory and COCs were recovered using a 75-μm filter (Watanabe Tecnologia Aplicada) and DPBS supplemented with 1% Fetal Calf Serum (FCS). The COCs were washed once in DPBS supplemented with 1% FCS at 37°C and morphologically evaluated under a stereomicroscope at 8 to 20× magnification. The COCs were morphologically classified based on the number of cumulus cell layers, as follows: grade 1, more than 3 layers of compact cumulus cells; grade 2, at least 1 layer of cumulus cells; grade 3, denuded; and grade 4, atretic with dark cumulus cells and signs of cytoplasmic degeneration [45]. After evaluation, only grade 4 COCs were considered nonsuitable for culturing and were discarded. The COCs considered suitable for culturing were transported to the IVP laboratory in 1.5-mL cryotubes containing 4 - (2-hydroxyethyl) 1 - piperazinethanesulfonic acid (HEPES)-buffered tissue culture medium 199 (TCM-199; Gibco Life Technologies), 10% FCS, 49.4 μg/mL sodium pyruvate (Sigma-Aldrich Chemical Co, St. Louis, MO), and 50 μg/mL gentamycin at 37°C to 39°C.

2.1.4. COC processing

Before in vitro maturation (IVM), COCs were washed 3 times in HEPES-buffered TCM-199 supplemented with 10% FCS and 50 μg/mL gentamycin and once in maturation medium, composed of bicarbonate-buffered TCM-199 (Gibco Life Technologies) supplemented with 10% FCS, 50 μg/mL LH (APL, Ayerst, Rouses Point, NY), 5 μg/mL FSH (Folltropin-V, Bioniche Animal Health, Canada), 0.1 μg/mL estradiol (Estradiol 17β, Sigma-Aldrich Chemical Co), 22 μg/mL sodium pyruvate, and 50 μg/mL gentamycin. The COCs from each cow were cultured separately for 24 h in 70-μL drops of maturation medium under mineral oil (D'Altomare, São Paulo, Brazil) at 39°C in humidified air with 5% CO_2.

2.1.5. IVP procedures

After IVM, the COCs were washed and subjected to in vitro fertilization (IVF) in 70-μL drops of IVF medium under mineral oil. The IVF medium was Tyrodes albumin lactate pyruvate (TALP) supplemented with 10 μg/mL heparin, 22 μg/mL sodium pyruvate, 50 μg/mL gentamycin, 6 mg/mL fatty-acid-free Bovine Serum albumin (BSA), and Penicillin, hypotaurine, epinephrine solution (2 μM penicillin, 1 μM hypotaurine, and 0.25 μM epinephrine).

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

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

2.2. Experiment 2

2.2.1. Farm and animals

This experiment was conducted at an experiment station (Instituto de Zootecnia de Sertãozinho) located in Sertãozinho, São Paulo, in southwestern Brazil. A total of 34 Nelore (*B. indicus*) donors were used in this experiment. Aiming to enroll most female categories typically available for IVP procedure in a commercial beef farm, 12 prepubertal heifers aged 10 to 11 mo, 10 prepubertal heifers aged 21 to 23 mo, and 12 cyclic heifers aged 24 to 26 mo were used. All females were maintained on *Brachiaria brizantha* pasture with free access to mineralized-salt and water. The present study was approved by the Bioethics Commission of the School of Veterinary Medicine and Zootechny of the University of São Paulo.

Females were submitted to the same ultrasonography (Section 2.1.2) and OPU (Section 2.1.3) procedures described in Section 2.1; however, COC processing and IVP production procedures were different than Experiment 1 and are described in the following.

2.2.2. COC processing

The COCs were washed in TCM-199 HEPES (Gibco, Invitrogen Co, Grand Island, NY) with 10% (vol/vol) fetal bovine serum (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) fetal bovine serum (FBS; Gibco, Invitrogen Co), 25 mg/mL sodium bicarbonate, 22 μg/mL sodium pyruvate, 50 μg/mL amikacin, 0.5 μg/mL FSH (Pluset, Hertape Calier, Juatuba, 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 to 24 h.

2.2.3. IVP procedures

The COCs were washed and submitted to IVF in 90 μL drops of IVF medium under mineral oil. Fertilization occurred in TALP, as described previously [46], supplemented with 10 μg/mL heparin, 22 μg/mL sodium pyruvate, 50 μg/mL amikacin, 6 mg/mL fatty acid-free BSA, and Penicillin, hypotaurine, epinephrine solution (2 μM penicillin, 1 μM hypotaurine, and 0.25 μM epinephrine).

For IVF, semen straws were thawed for 30 s in a 35°C water bath, and semen was deposited on top of a 400 μL 40:80% PureSperm gradient (Nidacon). The sperm was centrifuged for 6 min at 800 × *g*, supernatant was removed, and the pellet suspended with 1 mL fertilization medium. Sperm were centrifuged for 3 min at 200 × *g*, and 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 to 22 h incubation under the same conditions described for maturation. The same semen was used within each category.

After fertilization, presumptive zygotes were mechanically denuded. Culture took place in modified synthetic oviduct fluid as described previously [47] supplemented with 50 μg/mL amikacin, amino acids, citrate, myoinositol, 2.5% (vol/vol) FBS, and 6 mg/mL BSA (fatty acid free, Sigma A-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 post 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 seventh day of embryo culture.

2.3. Blood collection and AMH determination

In both experiments, blood samples were collected in vacuum tubes containing EDTA (Health Co, Canton, MA 02021) by coccygeal venipuncture immediately before the OPU session. The samples were immediately placed on ice and later centrifuged at 3000 × *g* for 15 min. Plasma samples were frozen at −25°C until subsequent analysis.

Plasma AMH concentrations were determined using the Bovine AMH enzyme-linked immunosorbent assay AL-114 kit (Ansh Labs, USA), previously validated for cattle [48]. For the AMH analysis only 1 assay was performed with 0.011 ng/mL of sensitivity and intra-assay coefficient of variation < 5%. The AMH assay was performed at the IgAc (Institute Genese of Scientific Analyses, Sao Paulo, Brazil).

For the purpose of analyzing the relationships between AMH concentration and the OPU–IVP efficiency, donors within each experiment were retrospectively classified into 1 of 2 AMH categories (low or high) according to the average plasma AMH in each experiment (the cut-off for low vs high AMH was 0.3 ng/mL in Holstein cattle in experiment 1 and 1.0 ng/mL for Nelore cattle in experiment 2).

2.4. Statistical analyses

Statistical analyses were performed using the GLIMMIX procedure of the Statistical Analysis System for Windows 9.3 (SAS 9.3). The variables evaluated were plasmatic AMH concentration, total number of follicles aspirated, total number of COCs retrieved, recovery rate (total number of COCs recovered per total number of follicles aspirated), number and percentage of cultured COCs (number of COCs cultured per total structures recovered), cleavage rate (number of cleaved zygotes per total number of COCs cultured), blastocyst rate (number of blastocysts produced per total number of COCs cultured), and number of blastocysts produced per OPU procedure.

Continuous data were tested for normality of the residues and homogeneity of variances using the Guided Data Analysis and transformed when necessary. The fixed effect included in the model was AMH categories (low and high) and animals within each animal category (experiment 1: Holstein prepubertal heifers aged 8–10 mo, cyclic heifers aged 12–14 mo, 14 lactating cows, and 15 nonlactating cows; experiment 2: Nelore prepubertal heifers aged 10–11 mo, prepubertal heifers aged 21–23 mo, and cyclic heifers aged 24–26 mo) were included as a random effect in the statistical model. It is important to highlight the relatively small number of animal per category within genotype. Therefore, aware of the limitation related to the analyses of the category effect (always maintained as random effect), results and discussion will only be presented as AMH concentration (continuous or categorized—high or low) within each genotype. And for these analyses, the power test were all above 80% estimated by power procedure of SAS, applying the 2-sample t test for mean difference with alpha equal to 0.05. For the correlation studies, significance was ascertained by Bravais–Pearson *r* critical values, as performed in PROC CORR and PROC REG of SAS 9.3 to obtain the regression functions. Means (±standard error of the mean) are used to describe all the response variables. And for all analysis, differences with $P \leq 0.05$ were considered statistically significant.

3. Results

The average AMH concentrations were 0.3 ± 0.02 ng/mL in *B. taurus* (Holstein) donors (prepubertal heifers aged 8–10 mo = 0.3 ± 0.04 ng/mL, cyclic heifers aged 12–14 mo = 0.3 ± 0.03 ng/mL, lactating cows = 0.2 ± 0.03 ng/mL, and nonlactating cows = 0.3 ± 0.03 ng/mL) and 1.0 ± 0.2 ng/mL in *B. indicus* (Nelore) donors (prepubertal heifers aged 10–11 mo = 0.7 ± 0.1 ng/mL, prepubertal heifers aged 21–23 mo = 1.4 ± 0.3 ng/mL, and cyclic heifers aged 24–26 mo = 1.4 ± 0.4 ng/mL).

A positive correlation was observed between the plasma AMH concentration and the number of follicles, the total COCs retrieved, the number of viable COCs, and the IVP from Holstein and Nelore donors (Figs. 1 and 2). In both genotypes, plasma AMH concentration was positively correlated with the number of punctured follicles, the total COCs retrieved the COC culture rate, and embryo production. However, there was no correlation with blastocyst rate (Figs. 1 and 2).

In both genotypes, females were classified into 1 of 2 AMH categories (low or high) according to the average AMH concentration in each genotype. The average AMH concentrations in the high and low categories were 0.4 ± 0.02 ng/mL versus 0.2 ± 0.01 ng/mL ($P < 0.0001$) among

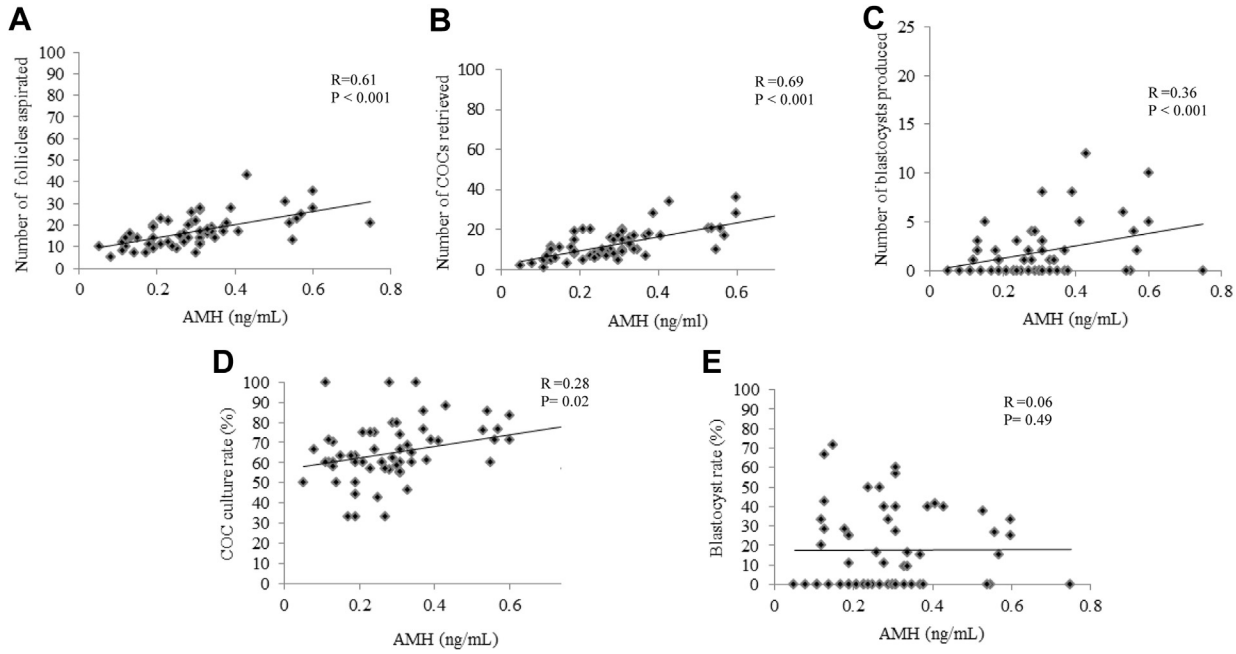

Fig. 1. Correlation between plasma anti-mullerian hormone (AMH) concentrations and variables related to ovum pick-up (OPU) and in vitro embryo production (IVP) in *Bos taurus* donors. Relationship between the number of follicles aspirated (A), the total COCs retrieved (B), the number of blastocysts produced (C), the COC culture rate (%, D), and the blastocyst rate (%, E) and the plasma AMH concentration in Holstein (*B. taurus*) donors. Blood samples for plasma AMH determination were collected by coccygeal venipuncture immediately before the OPU session. COCs, cumulus-oocyte complexes.

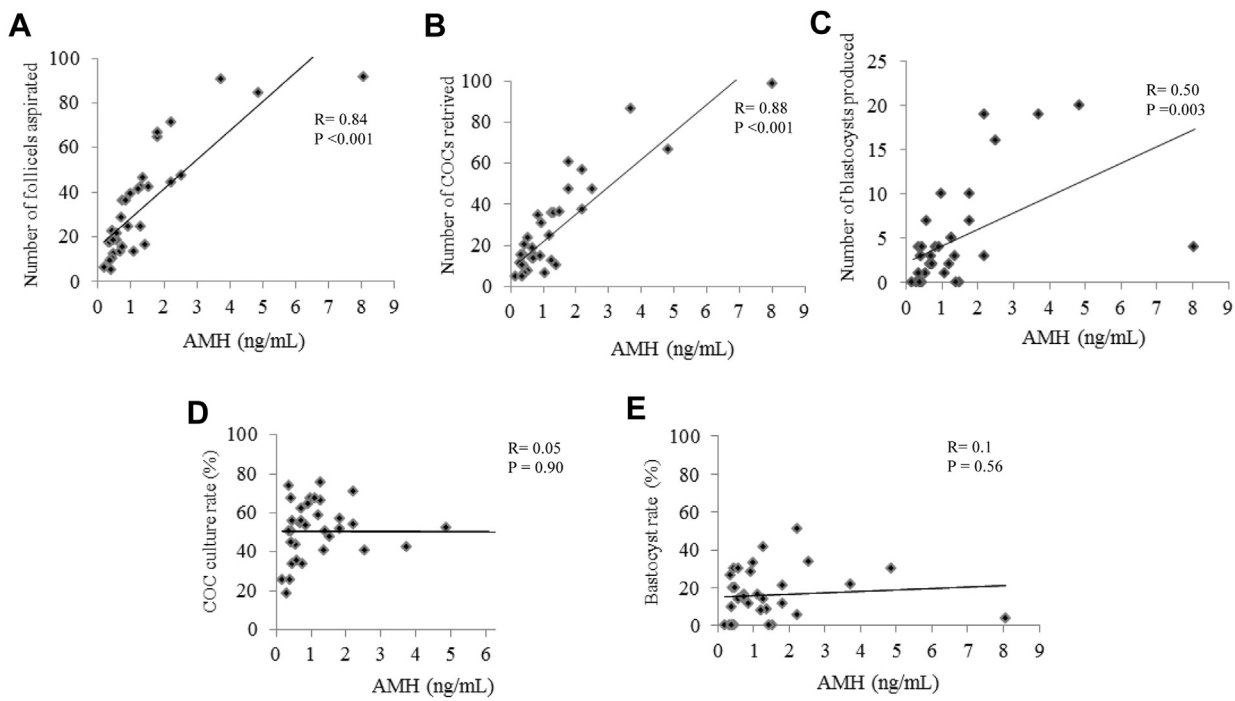

Fig. 2. Correlation between plasma anti-mullerian hormone (AMH) concentrations and variables related to ovum pick-up (OPU) and in vitro embryo production (IVP) in *Bos indicus* donors. Relationship between the number of follicles aspirated (A), the total COCs retrieved (B), the number of blastocysts produced (C), the COC culture rate (%, D), and the blastocyst rate (%, E) and the plasma AMH concentration in Nelore (*B. indicus*) donors. Blood samples for plasma AMH determination were collected by coccygeal venipuncture immediately before the OPU session. COCs, cumulus-oocyte complexes.

Table 1
Plasma AMH concentrations and cumulus-oocyte complex (COC) and embryo results (mean ± SEM) after OPU-IVP in *Bos taurus* (Holstein) donors classified into 2 AMH categories.

	AMH category[a]		*P*-values
	Low AMH	High AMH	
N	32	27	
Plasma AMH (ng/mL)	0.2 ± 0.01	0.4 ± 0.02	<0.0001
Total follicles aspirated	13.6 ± 0.9	20.9 ± 1.5	<0.0001
Total COCs retrieved	9.0 ± 0.9	17.3 ± 1.5	<0.0001
Recovery rate (%)[b]	64.7 ± 3.9	81.8 ± 3.2	0.01
COCs cultured	5.7 ± 0.7	12.3 ± 1.3	<0.0001
COC culture rate (%)[c]	61.2 ± 2.8	69.6 ± 2.4	0.02
Cleavage rate (%)[d]	54.5 ± 5.6	58.6 ± 5.3	0.98
Blastocysts produced per OPU	1.2 ± 0.3	3.0 ± 0.7	0.04
Blastocyst rate (%)[e]	19.8 ± 4.2	20.6 ± 4.0	0.60

Abbreviations: AMH, anti-mullerian hormone; COCs, cumulus-oocyte complexes; IVP, in vitro embryo production; OPU, ovum pick-up; SEM, standard error of the mean.

[a] AMH category: the plasma anti-mullerian hormone (AMH) category (low or high) was retrospectively established according the average AMH concentration (the cut-off for low vs high AMH was 0.3 ng/mL) observed for *B. taurus* (Holstein) donors, as determined at the time of the OPU procedure.

[b] Number of COCs/number of follicles aspirated.

[c] Number of COCs cultured/number of total COCs retrieved.

[d] Number of cleaved zygotes/number of oocytes cultured.

[e] Number of blastocysts/number of oocytes cultured.

Holstein donors and 2.3 ± 0.5 ng/mL versus 0.5 ± 0.05 ng/mL in Nelore donors ($P < 0.0001$), respectively.

When donors within each genotype (Holstein or Nelore) were classified according to their AMH class (high or low), females classified as having high AMH had a greater number of recovered COCs and a greater number of viable COCs (Tables 1 and 2). Moreover, donors in the high AMH category yielded a greater number of blastocysts produced compared with donors in the low AMH category (Tables 1 and 2). However, the AMH class did not distinguish donors that produced COCs with greater in vitro ability to reach the blastocyst stage, with each AMH class having a similar embryo-producing rate (Tables 1 and 2).

Table 2
Plasma AMH concentrations and cumulus-oocyte complex (COC) and embryo results (mean ± SEM) after OPU–IVP in *Bos indicus* (Nelore) donors classified into 2 AMH categories.

	AMH category[a]		*P*-values
	Low AMH	High AMH	
N	18	16	
Plasma AMH (ng/mL)	0.5 ± 0.05	2.0 ± 0.3	<0.0001
Total follicles aspirated	18.6 ± 2.1	54.3 ± 6.1	<0.0001
Total COCs retrieved	13.4 ± 1.7	45.3 ± 6.4	<0.0001
Recovery rate (%)[b]	74.6 ± 5.3	77.5 ± 4.1	0.57
COCs cultured	6.7 ± 1.0	23.0 ± 2.7	<0.0001
COC culture rate (%)[c]	46.7 ± 3.7	54.7 ± 2.9	0.09
Cleavage rate (%)[d]	76.0 ± 8.2	89.8 ± 4.0	0.14
Blastocysts produced per OPU	2.2 ± 0.5	7.0 ± 1.7	0.0067
Blastocyst rate (%)[e]	27.4 ± 5.5	33.7 ± 6.5	0.41

Abbreviations: AMH, anti-mullerian hormone; COCs, cumulus-oocyte complexes; IVP, in vitro embryo production; OPU, ovum pick up; SEM, standard error of the mean.

[a] AMH category: the plasma anti-mullerian hormone (AMH) category (low or high) was retrospectively established according the average AMH concentration (the cutoff for low vs high AMH was 1.0 ng/mL) observed for *B. indicus* (Nelore) donors, as determined at the time of the OPU procedure.

[b] Number of COCs/number of follicles aspirated.

[c] Number of COCs cultured/number of total COCs retrieved.

[d] Number of cleaved zygotes/number of oocytes cultured.

[e] Number of blastocysts/number of oocytes cultured.

4. Discussion

The results of this research indicated that plasma AMH concentration might be a useful marker for predicting the IVP performance of *B. taurus* (Holstein) and *B. indicus* (Nelore) donors. A positive correlation was observed among all the quantitative parameters (eg, total follicles aspirated, total COCs retrieved, number of COCs cultured, and number of embryos produced per OPU) analyzed during the OPU–IVP procedures and the plasma AMH concentration, except for the variables related to in vitro development competence (ie, cleavage and blastocyst rates). Therefore, the authors confirmed the hypothesis that plasma AMH concentration is closely related to the amount of in vitro embryo production; however, the hypothesis that AMH concentration could be related to oocyte competence was rejected because no effect was observed in the proportion of embryos produced based on the total COCs cultured.

Among the potential factors that influence the oocyte development competence in cattle, the lactating status [49], and the sexual maturity of the donor [50] should be considered. Previous report indicates that oocytes from calves are less competent in developing to the blastocyst stage compared with oocytes retrieved from cows [51]. Additionally, Zaraza, et al [50] demonstrated that the expression profile of the 3 genes (GLUT3, GLUT8, and AKT1) was affected by animal category, and apoptosis was increased in blastocysts derived from prepubertal heifers. Therefore, considering the present data, it is important to highlight the relatively small number of animal per category within genotype. It is worth noting that probably the maximum potential effect of AMH was not shown because of the possible variability between the different animal categories. However, these specific impacts certainly require further studies.

The physiological function of AMH has been mentioned as an important foliculogenesis modulator. Previous studies have shown that AMH regulates both primordial follicle recruitment and FSH responsiveness of growing follicles [18,26,27,52]. Additionally, higher AMH concentrations were associated with an increased number of ovarian antral follicles regardless of genotype [13], similarly to the results presented herein. Previous report [53] have suggested that the population of antral follicles is sustained by a dynamic balance between mechanisms (eg, bone morphogenetic protein, inhibin/activin, and IGF systems) related to the primordial follicle recruitment and antral follicle atresia rates. Therefore, factors related to this dynamic balance are likely accounted for the physiological differences between genotypes [54]. Thus, it remains unclear the reasoning of retrieving more oocytes from *B. indicus* than *B. taurus* donors.

In this study, donors classified as having high AMH concentrations had more AFP, more recovered COC, more cultured COC, and more embryos produced, regardless

genotype. Similar results were observed when the measurement of AMH concentration before hormonal treatment was positively correlated to superovulatory responses and in vivo embryo production [55]. In humans, greater plasma AMH concentration is associated with increased FSH-stimulation responses and enhanced in vitro embryo production [56–59]. Therefore, although counting AFPs can be a predictor of embryo production following IVP, measuring the circulating AMH concentration may be a simple, rapid, and standardized method that could be applied worldwide in the cattle embryo industry.

In contrast, the results demonstrated no correlation between AMH concentration and oocyte competence (eg, blastocyst rates). This finding is similar to those in previous reports, in which no effect of AMH concentration was found in the proportion of viable oocytes, the cleavage rate or the blastocyst rate among donors [7,60]. In contrast, previous studies in humans have found an association between AMH concentration and fertilization rate, blastocyst development, embryo quality, and pregnancy outcome [56–59]. Nevertheless, other studies in humans found no association between the basal serum AMH concentration and embryo quality [61–63]. Therefore, using AMH as a predictor for oocyte quality is still controversial and requires more research.

Finally, although ultrasound examination for counting AFPs has been reported as being a predictor for assessing the embryo yield following IVP or the in vivo embryo production in *B. taurus* [23,48,60,64–67] and *B. indicus* [6,64] cattle, it is important to highlight that variations in AFPs within individuals could occur depending on the phase of the estrous cycle in which the counting is being performed. This potential variation justifies performing AFP counts at the early follicular emergence phase instead of during the dominance phase, where the large dominant follicle could impair the visualization of the small follicles. Therefore, because all donors in this study underwent OPU without prior synchronization of the follicular wave emergence and because the AMH concentration remained positively correlated to embryo yield regardless of the stage of the estrous cycle, the evaluation of AMH concentration could be a more accurate method.

In conclusion, the data presented in this manuscript reports the relationship between plasma AMH concentrations and IVP in cattle. In both *B. taurus* (Holstein) and *B. indicus* (Nelore) cattle, the AMH concentration was positively correlated with the donor's embryo yield in an OPU–IVP program. The results presented here suggest that the AMH concentration could be used as an endocrine marker and a possible predictor of in vitro embryo production in cattle.

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References

[1] Pontes JHF, Nonato-Junior I, Sanches BV, Ereno-Junior JC, Uvo S, Barreiros TRR, Oliveira JA, Hasler JF, Seneda MM. Comparison of embryo yield and pregnancy rate between in vivo and in vitro methods in the same Nelore (bos indicus) donor cows. Theriogenology 2009;71:690–7.
[2] Merton JS, de Roos AP, Mullaart E, de Ruigh L, Kaal L, Vos PL, Dieleman SJ. Factors affecting oocyte quality and quantity in commercial application of embryo technologies in the cattle breeding industry. Theriogenology 2003;59:651–74.
[3] Ferreira RM, Ayres H, Chiaratti MR, Ferraz ML, Araújo AB, Rodrigues CA, Watanabe YF, Vireque AA, Joaquim DC, Smith LC, Meirelles FV, Baruselli PS. The low fertility of repeat-breeder cows during summer heat stress is related to a low oocyte competence to develop into blastocysts. J Dairy Sci 2011;94:2383–92.
[4] Ratto MH, Peralta OA, Mogollon G, Strobel P, Correa J. Transvaginal ultrasound-guided cumulus oocyte complexes aspiration and in vitro embryo production in suckled beef and lactating dairy cattle on pasture-based management conditions. Anim Reprod Sci 2011;129:1–6.
[5] Taneja M, Bols PEJ, de Velde AV, Ju J-C, Schreiber D, Tripp MW, Levine H, Echelard Y, Riesen J, Yang X. Developmental competence of juvenile calf oocytes in vitro and in vivo: influence of donor animal variation and repeated gonadotropin stimulation. Biol Reprod 2000;62:206–13.
[6] Pontes JHF, Melo Sterza FA, Basso AC, Ferreira CR, Sanches BV, Rubin KCP, Seneda MM. Ovum pick up, in vitro embryo production, and pregnancy rates from a large-scale commercial program using Nelore cattle (bos indicus) donors. Theriogenology 2011;75:1640–6.
[7] Silva-Santos KC, Santos GMG, Koetz Júnior C, Morotti F, Siloto LS, Marcantonio TN, Urbano MR, Oliveira RL, Lima DCM, Seneda MM. Antral follicle populations and embryo production – in vitro and in vivo – of Bos indicus–taurus donors from weaning to yearling ages. Reprod Domest Anim 2014;49:228–32.
[8] Bó GA, Peres LC, Cutaia LE, Pincinato D, Baruselli PS, Mapletoft RJ. Treatments for the synchronisation of bovine recipients for fixed-time embryo transfer and improvement of pregnancy rates. Reprod Fertil Development 2012;24:272.
[9] Alvarez P, Spicer LJ, Chase Jr CC, Payton ME, Hamilton TD, Stewart RE, Hammond AC, Olson TA, Wettemann RP. Ovarian and endocrine characteristics during an estrous cycle in Angus, Brahman, and Senepol cows in a subtropical environment. J Anim Sci 2000;78:1291–302.
[10] Fortune JE, Yang MY, Muruvi W. In vitro and in vivo regulation of follicular formation and activation in cattle. Reprod Fertil Development 2010;23:15–22.
[11] Sales JNS. Efeito da dieta com alta energia nos parâmetros metabólicos, endócrinos e reprodutivos de vacas Bos indicus e Bos taurus. In: Reprodução animal, USP; 2011.
[12] Satrapa RA, Castilho AS, Razza EM, Pegorer MF, Puelker R, Barros CM. Differential expression of members of the IGF system in OPU-derived oocytes from Nelore (Bos indicus) and Holstein (Bos taurus) cows. Anim Reprod Sci 2013;138:155–8.
[13] Batista EOS, Macedo GG, Sala RV, Ortolan MDDV, Sá Filho MF, Del Valle TA, Jesus EF, Lopes RNVR, Rennó FP, Baruselli PS. Plasma anti-mullerian hormone as a predictor of ovarian antral follicular population in *Bos indicus* (Nelore) and *Bos taurus* (Holstein) heifers. Reprod Domest Anim 2014. in press.
[14] Ireland JJ, Smith GW, Scheetz D, Jimenez-Krassel F, Folger JK, Ireland JLH, Mossa F, Lonergan P, Evans ACO. Does size matter in females? An overview of the impact of the high variation in the ovarian reserve on ovarian function and fertility, utility of anti-mullerian hormone as a diagnostic marker for fertility and causes of variation in the ovarian reserve in cattle. Reprod Fertil Development 2011;23:1–14.
[15] Monniaux D, Drouilhet L, Rico C, Estienne A, Jarrier P, Touze J-L, Sapa J, Phocas F, Dupont J, Dalbies-Tran R, Fabre S. Regulation of anti-mullerian hormone production in domestic animals. Reprod Fertil Development 2013;25:1–16.
[16] Rico C, Médigue C, Fabre S, Jarrier P, Bontoux M, Clément F, Monniaux D. Regulation of anti-müllerian hormone production in the cow: a multiscale study at endocrine, ovarian, follicular, and granulosa cell levels. Biol Reprod 2011;84:560–71.
[17] Cate RL, Mattaliano RJ, Hession C, Tizard R, Farber NM, Cheung A, Ninfa EG, Frey AZ, Gash DJ, Chow EP, Fisher RA, Bertonis JM, Torres G, Wallner BP, Ramachandran KL, Ragin RC, Manganaro TF, MacLaughlin DT, Donahoe PK. Isolation of the bovine and human

genes for müllerian inhibiting substance and expression of the human gene in animal cells. Cell 1986;45:685–98.
[18] Durlinger ALL, Visser JA, Themmen APN. Regulation of ovarian function: the role of anti-mullerian hormone. Reproduction 2002; 124:601–9.
[19] Vigier B, Picard JY, Tran D, Legeai L, Josso N. Production of anti-mullerian hormone: another homology between sertoli and granulosa cells. Endocrinology 1984;114:1315–20.
[20] Takahashi M, Hayashi M, Manganaro TF, Donahoe PK. The ontogeny of mullerian inhibiting substance in granulosa cells of the bovine ovarian follicle. Biol Reprod 1986;35:447–53.
[21] Visser JA, Durlinger ALL, Peters IJJ, van den Heuvel ER, Rose UM, Kramer P, de Jong FH, Themmen APN. Increased oocyte degeneration and follicular atresia during the estrous cycle in anti-müllerian hormone null mice. Endocrinology 2007;148:2301–8.
[22] Monniaux D, di Clemente N, Touze J, Belville C, Rico C, Bontoux M, Picard J, Fabre S. Intrafollicular steroids and anti-mullerian hormone during normal and cystic ovarian follicular development in the cow. Biol Reprod 2008;79:387–96.
[23] Rico C, Fabre S, Médigue C, di Clemente N, Clément F, Bontoux M, Touzé J-L, Dupont M, Briant E, Rémy B, Beckers J-F, Monniaux D. Anti-müllerian hormone is an endocrine marker of ovarian gonadotropin-responsive follicles and can help to predict superovulatory responses in the cow. Biol Reprod 2009;80:50–9.
[24] Rico C, Medigue C, Fabre S, Jarrier P, Bontoux M, Clement F, Monniaux D. Regulation of anti-mullerian hormone production in the cow: a multiscale study at endocrine, ovarian, follicular and granulosa cell levels. Biol Reprod 2011;84:560–71.
[25] Monniaux D, Drouilhet L, Rico C, Estienne A, Jarrier P, Touzé J-L, Sapa J, Phocas F, Dupont J, Dalbiès-Tran R, Fabre S. Regulation of anti-müllerian hormone production in domestic animals. Reprod Fertil Development 2012;25:1–16.
[26] Durlinger ALL, Kramer P, Karels B, de Jong FH, Uilenbroek JTJ, Grootegoed JA, Themmen APN. Control of primordial follicle recruitment by anti-mullerian hormone in the mouse ovary. Endocrinology 1999;140:5789–96.
[27] Durlinger ALL, Gruijters MJG, Kramer P, Karels B, Ingraham HA, Nachtigal MW, Uilenbroek JTJ, Grootegoed JA, Themmen APN. Anti-müllerian hormone inhibits initiation of primordial follicle growth in the mouse ovary. Endocrinology 2002;143:1076–84.
[28] Fanchin R, Schonäuer LM, Righini C, Guibourdenche J, Frydman R, Taieb J. Serum anti-müllerian hormone is more strongly related to ovarian follicular status than serum inhibin B, estradiol, FSH and LH on day 3. Hum Reprod 2003;18:323–7.
[29] Monniaux D, Baril G, Laine A-L, Jarrier P, Poulin N, Cognié J, Fabre S. Anti-müllerian hormone as a predictive endocrine marker for embryo production in the goat. Reproduction 2011; 142:845–54.
[30] Brevini Gandolfi TAL, Gandolfi F. The maternal legacy to the embryo: cytoplasmic components and their effects on early development. Theriogenology 2001;55:1255–76.
[31] Leroy J, Van Soom A, Opsomer G, Goovaerts IGF, Bols PEJ. Reduced fertility in high-yielding dairy cows: are the oocyte and embryo in danger? Part II. Mechanisms linking nutrition and reduced oocyte and embryo quality in high-yielding dairy cows. Reprod Domest Anim 2008;43:623–32.
[32] Hansen PJ. Exploitation of genetic and physiological determinants of embryonic resistance to elevated temperature to improve embryonic survival in dairy cattle during heat stress. Theriogenology 2007;68:S242–9.
[33] Paula-Lopes F, CC C, Al-Katanani Y, CE K, Rivera R, Tekin S, Majewski A, Ocon O, Olson T, Hansen P. Genetic divergence in cellular resistance to heat shock in cattle: differences between breeds developed in temperate versus hot climates in responses of preimplantation embryos, reproductive tract tissues and lymphocytes to increased culture temperatures. Reproduction 2003;125:285–94.
[34] Roth Z. Disruption of nuclear maturation and rearrangement of cytoskeletal elements in bovine oocytes exposed to heat shock during maturation. Reproduction 2005;129:235–44.
[35] Machatkova M, Jokesova E, Horky F, Krepelova A. Utilization of the growth phase of the first follicular wave for bovine oocyte collection improves blastocyst production. Theriogenology 2000;54:543–50.
[36] Machatková M, Jokešová E, Petelíková J, Dvořáček V. Developmental competence of bovine embryos derived from oocytes collected at various stages of the estrous cycle. Theriogenology 1996;45:801–10.
[37] Hendriksen P, Vos PL, Steenweg W, Beevers M, Dieleman SJ. Bovine follicular development and its effect on the in vitro competence of oocytes. Theriogenology 2000;53:11–20.
[38] Merton JS, de Roos APW, Mullaart E, de Ruigh L, Kaal L, Vos PLAM, Dieleman SJ. Factors affecting oocyte quality and quantity in commercial application of embryo technologies in the cattle breeding industry. Theriogenology 2003;59:651–74.
[39] Vassena R, Mapletoft RJ, Allodi S, Singh J, Adams GP. Morphology and developmental competence of bovine oocytes relative to follicular status. Theriogenology 2003;60:923–32.
[40] Nagano M, Hishinuma M, Katagiri S, Takahashi Y. The relationship between oocyte morphology and ovarian status in cattle. J Reprod Development 2007;53:953–8.
[41] Gimenes LU. Taxa de recuperação in vivo e competência in vitro de oócitos bubalinos, zebuínos e taurinos aspirados em diferentes fases da onda de crescimento folicular. In: Reprodução animal, Universidade de São Paulo; 2010.
[42] Hagemann LJ. Influence of the dominant follicle on oocytes from subordinate follicles. Theriogenology 1999;51:449–59.
[43] Batista EOS, Macedo GG, Sala RV, Ortolan M, Sá Filho MF, Del Valle TA, Jesus EF, Lopes R, Rennó FP, Baruselli PS. Plasma anti-mullerian hormone as a predictor of ovarian antral follicular population in Bos indicus (Nelore) and Bos taurus (Holstein) heifers. Reprod Domest Anim 2014;49:448–52.
[44] NRC. Nutrient requirements of dairy cattle. 7th rev. Natl Acad Sci; 2001.
[45] Seneda MM, Esper CR, Garcia JM, JAd Oliveira, Vantini R. Relationship between follicle size and ultrasound-guided transvaginal oocyte recovery. Anim Reprod Sci 2001;67:37–43.
[46] Parrish JJ, Susko-Parrish JL, Winer MA, First NL. Capacitation of bovine sperm by heparin. Biol Reprod 1988;38:1171–80.
[47] Vajta G, Rindom N, Peura TT, Holm P, Greve T, Callesen H. The effect of media, serum and temperature on in vitro survival of bovine blastocysts after open pulled straw (OPS) vitrification. Theriogenology 1999;52:939–48.
[48] Ireland JLH, Scheetz D, Jimenez-Krassel F, Themmen APN, Ward F, Lonergan P, Smith GW, Perez GI, Evans ACO, Ireland JJ. Antral follicle count reliably predicts number of morphologically healthy oocytes and follicles in ovaries of young adult cattle. Biol Reprod 2008;79: 1219–25.
[49] Vieira LM, Rodrigues CA, Castro Netto A, Guerreiro BM, Silveira CRA, Moreira RJC, Sá Filho MF, Bó GA, Mapletoft RJ, Baruselli PS. Superstimulation prior to the ovum pick-up to improve in vitro embryo production in lactating and non-lactating Holstein cows. Theriogenology 2014;82:318–24.
[50] Zaraza J, Oropeza A, Velazquez MA, Korsawe K, Herrmann D, Carnwath JW, Niemann H. Developmental competence and mRNA expression of preimplantation in vitro–produced embryos from prepubertal and postpubertal cattle and their relationship with apoptosis after intraovarian administration of IGF-1. Theriogenology 2010;74:75–89.
[51] Camargo LSA, Viana JHM, Sá WF, Ferreira AM, Vale Filho VR. Developmental competence of oocytes from prepubertal Bos indicus crossbred cattle. Anim Reprod Sci 2005;85:53–9.
[52] Durlinger A, Visser J, Themmen A. Regulation of ovarian function: the role of anti-mullerian hormone. Reproduction 2002;124:601–9.
[53] Monniaux D, Clément F, Dalbiès-Tran R, Estienne A, Fabre S, Mansanet C, Monget P. The ovarian reserve of primordial follicles and the dynamic reserve of antral growing follicles: what is the link? Biol Reprod 2014;90:85.
[54] Silva-Santos KC, Santos GMG, Siloto LS, Hertel MF, Andrade ER, Rubin MIB, Sturion L, Melo-Sterza FA, Seneda MM. Estimate of the population of preantral follicles in the ovaries of Bos taurus indicus and Bos taurus taurus cattle. Theriogenology 2011;76:1051–7.
[55] Monniaux D, Barbey S, Rico C, Fabre S, Gallard Y, Larroque H. Anti-müllerian hormone: a predictive marker of embryo production in cattle? Reprod Fertil Development 2010;22:1083–91.
[56] Lin W-Q, Yao L-N, Zhang D-X, Zhang W, Yang X-J, Yu R. The predictive value of anti-mullerian hormone on embryo quality, blastocyst development, and pregnancy rate following in vitro fertilization-embryo transfer (IVF-ET). J Assisted Reprod Genet 2013;30:649–55.
[57] Hazout A, Bouchard P, Seifer DB, Aussage P, Junca AM, Cohen-Bacrie P. Serum antimüllerian hormone/müllerian-inhibiting substance appears to be a more discriminatory marker of assisted reproductive technology outcome than follicle-stimulating hormone, inhibin B, or estradiol. Fertil Sterility 2004;82:1323–9.
[58] Eldar-Geva T, Ben-Chetrit A, Spitz IM, Rabinowitz R, Markowitz E, Mimoni T, Gal M, Zylber-Haran E, Margalioth EJ. Dynamic assays of inhibin B, anti-mullerian hormone and estradiol following FSH stimulation and ovarian ultrasonography as predictors of IVF outcome. Hum Reprod 2005;20:3178–83.

[59] Lekamge DN, Barry M, Koto M, Lane M, Gilchrist RB, Tremelle KP. Anti-mullerian hormone as predictor of IVF outcome. Reprod Biomed Online 2007;14:602–10.
[60] Ireland J, Ward F, Jimenez-Krassel F, Ireland JLH, Smith GW, Lonergan P, Evans ACO. Follicle numbers are highly repeatable within individual animals but are inversely correlated with FSH concentrations and the proportion of good-quality embryos after ovarian stimulation in cattle. Hum Reprod 2007;22:1687–95.
[61] Ebner T, Sommergruber M, Moser M, Shebl O, Schreier-Lechner E, Tews G. Basal level of anti-müllerian hormone is associated with oocyte quality in stimulated cycles. Hum Reprod 2006;21:2022–6.
[62] Smeenk JMJ, Sweep FCGJ, Zielhuis GA, Kremer JAM, Thomas CMG, Braat DDM. Antimüllerian hormone predicts ovarian responsiveness, but not embryo quality or pregnancy, after in vitro fertilization or intracyoplasmic sperm injection. Fertil Sterility 2007;87:223–6.
[63] Lie Fong S, Baart EB, Martini E, Schipper I, Visser JA, Themmen AP, de Jong FH, Fauser BJ, Leaven JS. Anti- mullerian hormone: a marker for oocyte quantity, oocyte quality? Reprod Biomed Online 2008;16:664–70.
[64] Pontes JHF, Silva KCF, Basso AC, Rigo AG, Ferreira CR, Santos GMG, Sanches BV, Porcionato JPF, Vieira PHS, Faifer FS, Sterza FAM, Schenk JL, Seneda MM. Large-scale in vitro embryo production and pregnancy rates from Bos taurus, Bos indicus, and indicus-taurus dairy cows using sexed sperm. Theriogenology 2010;74:1349–55.
[65] Kawamata M. Relationships between the number of small follicles prior to superovulatory treatment and superovulatory response in Holstein cows. J Veteterinay Med Sci 1994;56:965–7.
[66] Singh J, Domınguez M, Jaiswal R, Adams GP. A simple ultrasound test to predict the superstimulatory response in cattle. Theriogenology 2004;62:227–43.
[67] Cushman RA, DeSouza JC, Hedgpeth VS, Britt JH. Superovulatory response of one ovary is related to the micro- and macroscopic population of follicles in the contralateral ovary of the cow. Biol Reprod 1999;60:349–54.