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KATIA CRISTINA SILVA-SANTOS

**COMPARAÇÃO DA PRODUÇÃO DE EMBRIÃO,
POPULAÇÃO DE FOLÍCULOS PRÉ-ANTRAIOS OVARIANA E
DO TEOR DE LIPÍDIOS NOS OÓCITOS ENTRE FÊMEAS
BOVINAS COM ALTA E BAIXA CONTAGEM DE FOLÍCULOS
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Orientador: Prof. Dr. Marcelo Marcondes Seneda

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Dedico este trabalho aos meus pais, que investiram na minha formação, incentivaram meus estudos e me apoiaram na decisão de mudar pra Londrina.

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“Mas tu, ó Senhor, estás comigo e és forte e poderoso”. **Jeremias 20:11**

“Imaginação é mais importante do que conhecimento. O conhecimento é limitado, a imaginação circunda o mundo”. **Albert Einstein**

"Você nunca sabe que resultados virão da sua ação. Mas se você não fizer nada, não existirão resultados." **Mahatma Gandhi**

"Deus nos fez perfeitos e não escolhe os capacitados, capacita os escolhidos. Fazer ou não fazer algo só depende de nossa vontade e perseverança". **Albert Einstein**

SILVA-SANTOS, Katia Cristina. **Comparação da produção de embrião, população de folículos pré-antrais ovariana e do teor de lipídios nos oócitos entre fêmeas bovinas com alta e baixa contagem de folículos antrais**. 2013. 97f. Tese (Doutorado em Ciência Animal) – Universidade Estadual de Londrina, Londrina, 2013.

RESUMO

O objetivo deste trabalho foi comparar a produção de embriões, a população de folículos pré-antrais e o teor de lipídios de oócitos recuperados por OPU entre fêmeas bovinas com alta e baixa contagem de folículos antrais (CFA). Para comparar as produções *in vitro* e *in vivo* de embriões entre fêmeas *indicus-taurus* com alta e baixa CFA, fêmeas Braford (3/8 Nelore x 5/8 Hereford; n = 137) foram examinadas por ultrassonografia seriadas (D0, 60, 120, 180, 240 e 300) para contagem de folículos antrais (CFA) ≥ 3 mm e divididas em grupos de alta CFA (G-Alta, ≥ 40 folículos, n = 20) e baixa CFA (G-Baixa, ≤ 10 folículos, n = 20). Aos 24 meses de idades, todas novilhas foram submetidas a uma sessão de OPU/PIV para obtenção de oócitos e embriões. Depois de 7 d, as mesmas fêmeas foram submetidas a protocolo de SOV/ colheita de embrião, utilizando sêmen do mesmo touro e da mesma partida que foi utilizado na PIV. A população de folículos antrais se manteve constante durante as avaliações. O número médio de embriões produzidos após OPU/PIV foi maior ($P \leq 0,05$) para G-Alta ($6,10 \pm 4,51$) comparado a G-Baixa CFA ($0,55 \pm 0,83$). Da mesma forma, o número médio de embriões produzidos após SOV/ colheita de embrião foi maior ($P \leq 0,05$) para G-Alta ($6,95 \pm 5,34$) comparado a G-Baixa CFA ($1,9 \pm 2,13$). Dessa forma, fêmeas de alta CFA apresentaram melhor desempenho reprodutivo comparado a fêmeas de baixa CFA, o que permite considerar a população de folículos antrais para prever o sucesso em programas de OPU/PIV e a resposta à SOV/ colheita de embrião. Em seguida, comparamos a quantidade de folículos pré-antrais entre fêmeas bovinas. Ovários de vacas *Bos indicus* (Nelore, n = 100) e *Bos taurus* (Aberdeen Angus, n = 100) foram coletados em abatedouros e examinados por ultrassonografia para CFA ≥ 3 mm e separação dos grupos de alta (Nelore, n = 8; Angus, n = 10) e baixa CFA (Nelore, n = 8; Angus, n = 10). Houve grande variação na quantidade de folículos pré-antrais entre os grupos e entre indivíduos. Não houve diferença entre a população de folículos pré-antrais de fêmeas com alta e baixa CFA, independente da raça. O número médio de folículos pré-antrais em fêmeas Nelore foi 48349 ± 30149 (alta CFA) e 33037 ± 31710 folículos (baixa CFA), e, em Angus, 35050 ± 36060 (alta CFA) e 30481 ± 43360 folículos (baixa CFA). Por fim, avaliamos a quantidade de lipídios por Maldi-MS em oócitos recuperados por OPU de fêmeas *Bos indicus* (Nelore) e $1/2$ *indicus-taurus* (Nelore x Angus) divididas em grupos de alta e baixa CFA, após cinco avaliações ultrassonográficas. Fêmeas Nelore apresentaram maior quantidade dos íons lipídicos $[PC (38:5) + Na]^+e/ou [PC (P-36:2) + Na, [PC (38:2) + H]^+, [PC (38:5) + Na]^+$ e $[TAG (60:8) + NH_4]^+$, o que sugere diferentes taxas de metabolismo de membrana entre *Bos indicus* e $1/2$ *indicus-taurus* devido à influência do sangue *Bos taurus*. Em resumo, a população de folículos antrais permaneceu constante no mesmo indivíduo e influenciou a produção de embriões de fêmeas bovinas. No entanto, não observamos associação entre a população de folículos antrais e pré-antrais, sugerindo haver outro mecanismo que justifique a maior quantidade de oócitos obtida de fêmeas *Bos indicus*.

Palavras-chave: Folículos antrais. Folículos pré-antrais. Oócitos. Embriões. Bovinos.

SILVA-SANTOS, Katia Cristina. **Comparison of the embryo yield, the population of preantral follicles and the oocyte lipid content among bovine females with high and low antral follicle count.** 2013. 97f. Thesis (Doctorate in Animal Science) – State University of Londrina, Londrina 2013.

ABSTRACT

The aim of the present study was to compare the embryo yield, the population of preantral follicles and the lipid content of oocytes recovered by OPU among cattle with high and low antral follicle count (AFC). To compare the *in vitro* and *in vivo* embryo production between *indicus-taurus* females with high- and low-AFC. Braford cattle (3/8 Nelore x 5/8 Hereford; n = 137) were evaluated using ultrasonography (D0, 60, 120, 180, 240 e 300) to count follicles (AFC, antral follicle count) ≥ 3 mm and were assigned into groups with high AFC (G-High, ≥ 40 follicles, n = 20) and low AFC (G-Low, ≤ 10 follicles, n = 20). At 24 months-old, all cattle were submitted to OPU/IVP to obtain oocytes and embryos. After 7 d, the same females were submitted to SOV/ embryo collection, using semen from the same sire and ejaculate used in IVP procedure. The average number of follicles remained highly stable through all ultrasound evaluations. The number of embryos after OPU/IVP was greater ($P \geq 0.05$) for G-High (6.10 ± 4.51) compared to G-Low AFC (0.55 ± 0.83). Also, the mean number of embryos after SOV/ embryo collection was greater ($P \leq 0.05$) for G-High (6.95 ± 5.34) compared to G-Low AFC (1.9 ± 2.13). Females with low AFC had a greater performance ($P \leq 0.05$) after OPU/IVP than after SOV/ embryo collection. Thus, females with high AFC had a greater reproductive performance compared to females with low AFC and the population of antral follicles could be used to predict the success of OPU/IVP and the response to SOV/ embryo collection. Then, we compared the population of preantral follicles amongst cattle. Ovaries of *Bos indicus* (Nelore, n = 100) and *Bos taurus* (Aberdeen Angus, n = 100) cows were collected at abattoir and evaluated by ultrasound to count antral follicles ≥ 3 mm and to assign females into groups of high (Nelore, n = 8; Angus, n = 10) and low AFC (Nelore, n = 8; Angus, n = 10). There was variation in the numbers of preantral follicles between groups and among individuals. There was no difference between the population of preantral follicles among females with high and low AFC, regardless of breed. The mean number of preantral follicles in Nelore females was $48,349 \pm 30,149$ (high AFC) and $33,037 \pm 31,710$ follicles (low AFC), and, in Angus females, $35,050 \pm 36,060$ (high AFC) and $30,481 \pm 43,360$ follicles (low AFC). Finally, we evaluated by Maldi-MS the lipid content of oocytes recovered by OPU from *Bos indicus* (Nelore) and 1/2 *indicus-taurus* (Nelore x Angus) females assigned into high- and low-AFC groups, after five ultrasound scans. Nelore females had a greater amount of lipid ions $[\text{PC (38:5) + Na}]^+$ and/or $[\text{PC (P-36:2) + Na}]^+$, $[\text{PC (38:2) + H}]^+$, $[\text{PC (38:5) + Na}]^+$ and $[\text{TAG (60:8) + NH}_4]^+$, which suggests different rates of membrane metabolism between *Bos indicus* and 1/2 *indicus-taurus* due to the influence of *Bos indicus* cattle. In summary, the population of antral follicles was highly stable within the same individual and influenced embryo production in cattle. However, we did not observe association between the population of antral and preantral follicles, and there might be other mechanism to account for the greatest numbers of oocytes obtained from *Bos indicus* cattle.

Key words: Antral follicles. Preantral follicles. Oocytes. Embryos. Cattle.

LISTA DE ILUSTRAÇÕES

Revisão de Literatura

- Figura 1** – Esquema ilustrando a origem das células da pré-granulosa durante a formação dos folículos primordiais21
- Figura 2** – Representação esquemática do desenvolvimento dos folículos ovarianos (Adaptado de Rodgers et al., 1999). 1. Oócito primário; 2. Célula da Pré-granulosa; 3. Membrana basal; 4. Células da Granulosa; 5. Cavidade antral; 6. Célula da teca e 7. Oócito secundário23
-
- Artigo 1 – Population of antral follicles and embryo production – *in vitro* and *in vivo* – of *indicus-taurus* donors from weaning to yearling ages**
- Figure 1** – Treatment schedule for superovulation of donor females with high and low antral follicle numbers52
-
- Artigo 2 – Comparison among antral and preantral follicle population in *Bos indicus* and *Bos taurus* purebred cows**
- Figure 1** – Variation in the preantral follicle population among individuals of the same group (G-High vs. G-Low AFC) and between breeds (Nelore – *Bos indicus* vs. Aberdeen Angus – *Bos taurus*).....67
- Figure 2** – Histological classification of polyovular follicles and ovigerous cords. Ovigerous cords (A) and multioocyte follicle (B) in the ovary of a *Bos taurus* cow with low AFC and multioocyte follicle in the ovary of a *Bos indicus* cow with low AFC (C). Presumptive nucleus of oogonias (arrows) within the ovigerous-like cords and presumptive nucleus of oocytes (Nu) enclosed within a follicle-like cell with a single layer of granulosa cells (GC). Sections were stained with periodic acid Schiff (PAS) and hematoxylin. Bars = 50 µm. Original magnification X400.....68

Artigo 3 – MALDI-MS lipid profiles of oocytes recovered by ovum pick-up from *Bos indicus* and 1/2 *indicus*-taurus with high vs. low oocyte yield

- Figure 1** – Tridimensional PLS-DA score plot of the oocytes collected by OPU to be used in this work (n = 83, 31 spectra). The analysis considered the lipid profile according to the oocyte quality, independently of breed or oocyte yield. Expanded oocytes (n = 8, 3 spectra) are represented by red triangles; GI oocytes (n = 10, 4 mass spectra) by green triangles; GII oocytes (n = 12; 5 spectra) by dark blue triangles; GIII oocytes (n = 31, 14 spectra) by light blue triangles; and COCs from preantral follicles (n = 23; 5 spectra) by pink triangles. Each component indicates the percentage of variability of the data explained81
- Figure 2** – Representative MALDI-MS of (a) expanded oocytes; (b) GI; (c) GII; (d) GIII oocytes; (e) COCs from preantral follicles83
- Figure 3** – Tridimensional PLS-DA score plot of the representative oocytes for each of the four experimental groups. 1/2 Nelore x Angus cows with high AFC (AH-group, n = 12, 6 mass spectra) are represented by red triangles; 1/2 Nelore x Angus with low AFC (AL-group, n = 5, 4 mass spectra) by green triangles; Nelore cows with high AFC (NH-group; n = 31; 10 mass spectra) by dark blue triangles; and Nelore cows with low AFC (NL-group; n = 5, 3 mass spectra) by light blue triangles. Each component indicates the percentage of variability of the data explained84
- Figure 4** – Line graphic showing the relative ion abundances (y-axis) in the lipid mass spectra in each sample of 1/2 Nelore x Angus (AH-group samples: A1 to A6; AL-group samples: A7 to A10), and Nelore (NH-group: N1 to N10; NL-group: N11 to N13).....85
- Figure 5** – Representative MALDI-MS of (a) NH-group; (b) NL-group; (c) AH-group; and (d) AL-group86

LISTA DE TABELAS

Artigo 1 – Population of antral follicles and embryo production – *in vitro* and *in vivo* – of *indicus-taurus* donors from weaning to yearling ages

- Table 1** – Mean (\pm SD) data for reproductive performance of Braford females with high (G-High, \geq 40 follicles) and low (G-Low, \leq 10 follicles) antral follicle count (AFC), comparing embryo production following *in vitro* (OPU/IVP) vs. *in vivo* (SOV/ embryo collection) procedures.....54
- Table 2** – Variation in embryo production among Braford females with high (G-High, \geq 40 follicles) and low (G-Low, \leq 10 follicles) antral follicle count (AFC), comparing the six greatest donors for *in vitro* (OPU/IVF) or *in vivo* (SOV/ embryo collection) procedures.....55

Artigo 2 – Comparison among antral and preantral follicle population in *Bos indicus* and *Bos taurus* purebred cows

- Table 1** – Mean (\pm SD) number of preantral follicles per ovary and antral follicles per female of *Bos indicus* (Nelore) and *Bos taurus* (Aberdeen Angus) cows (72–96 mo) with high (G-High) vs. low (G-Low) AFC66

Artigo 3 – MALDI-MS lipid profiles of oocytes recovered by ovum pick-up from *Bos indicus* and 1/2 *indicus-taurus* with high vs. low oocyte yield

- Table 1** – Tentative attribution of lipid species presenting the highest PLS-DA scores in the MALDI-MS lipid profiles of bovine oocytes used in this work82

LISTA DE ABREVIATURAS E SIGLAS

AFC/ CFA	<i>antral follicular counting</i> - contagem de folículos antrais
AMH	<i>anti-Müllerian hormone</i> - hormônio anti-Mülleriano
ANOVA	análise de variância
ART	<i>assisted reproductive therapy</i> - tratamento reprodutivo assistido
FGF	<i>fibroblast growth factor</i> - fator de crescimento de fibroblasto
CL	<i>corpus luteum</i> - corpo lúteo
COCs	complexos <i>cumulus oophorus</i>
DPBS	Dulbecco's Phosphate Buffered Saline - solução salina fosfato-tamponada Dulbecco
E2	estrógeno
EB	<i>estradiol benzoate</i> - benzoato de estradiol
eCG	<i>equine chorionic gonadotropin</i> - gonadotrofina coriônica eqüina
ET/ TE	<i>embryo transfer</i> - transferência de embriões
FOPA	folículos ovarianos pré-antrais
FSH	<i>follicle stimulant hormone</i> - hormônio foliculo estimulante
FTAI/ IATF	fixed-time artificial insemination - inseminação artificial em tempo fixo
GAP <i>junctions</i>	junções intercomunicantes tipo GAP
GDF-9	growth and differentiation factor - fator de crescimento e diferenciação
GFP	<i>green fluorescent protein</i> - proteína verde fluorescente
H3K4	hystone 3 at lysine 4 - histona 3 na lisina 4
IETS	<i>international embryo transfer society</i> - sociedade internacional
IGF-I	<i>insulin-like growth factor</i> - fator de crescimento semelhante à insulina
IVC	<i>in vitro</i> cultivate - cultivo <i>in vitro</i>
IVF/ FIV	<i>in vitro fertilization</i> - Fecundação <i>in vitro</i>
IVM	<i>in vitro maturation</i> - maturação <i>in vitro</i>
IVP/ PIV	<i>in vitro embryo production</i> - produção <i>in vitro</i> de embriões
LH	<i>luteinizing hormone</i> - hormônio luteinizante
LIF	<i>leukemia inhibitor factor</i> - fator inibidor de leucemia

MALDI	<i>matriz assisted laser desorption/ ionization</i> - dessorção/ ionização a laser assistida por matriz
MOET	<i>multiple ovulation embryo transfer</i> – múltipla ovulação e transferência de embrião
MS	<i>mass spectrometry</i> - espectrometria de massa
OPU	ovum pick up - aspiração folicular guiada por ultrassonografia
P4	progesterona
PAS	<i>periodic acid Schiff</i> - ácido periódico de Schiff
PC	<i>phosphatidylcholines</i> – fosfatidilcolinas
PGF2 α	prostaglandina
PHE	<i>epinephrine</i> – epinafrina
PL	<i>phospholipids</i> – fosfolipídeos
PLS-DA	partial least square- discriminant analysis - regressão parcial em mínimos quadrados- análise discriminante
SD	<i>standard deviation</i> - desvio padrão
SM	<i>sphigomyelin</i> – esfingomielina
SOV	superovulação
TGA	triacilglicerol
TGF	<i>transforming growth factor</i> - fator transformador do crescimento

SUMÁRIO

1 INTRODUÇÃO	18
2 REVISÃO DE LITERATURA	19
2.1 – O OVÁRIO MAMÍFERO.....	19
2.1.1 – Aspectos Morfológicos e Estruturais do Ovário	19
2.1.2 – População Folicular Ovariana.....	20
2.2 – OOGÊNESE E FOLICULOGÊNESE	20
2.3 – CARACTERIZAÇÃO LIPÍDICA DE OÓCITOS	22
2.4 – FOLÍCULOS OVARIANOS	22
2.4.1 – Classificação e Caracterização Estrutural e Ultraestrutural dos Folículos Ovarianos	24
2.4.1.1 – Folículos primordiais.....	24
2.4.1.2 – Folículos primários	25
2.4.1.3 – Folículos secundários	25
2.4.2 – Caracterização Estrutural e Ultraestrutural dos Folículos Antrais.....	26
2.5 – ATRESIA FOLICULAR.....	28
2.6 – RENOVAÇÃO FOLICULAR	29
2.7 – FISIOLÓGIA REPRODUTIVA DE FÊMEAS TAURINAS E ZEBUÍNAS	30
REFERÊNCIAS	32
3 HIPÓTESE	45
4 OBJETIVOS	46
4.1 – OBJETIVO GERAL	46
4.2 – OBJETIVOS ESPECÍFICOS	46
5 ARTIGOS PARA PUBLICAÇÃO	47
ARTIGO 1: Population of Antral Follicles and Embryo Production – <i>in vitro</i> and <i>in vivo</i> – of <i>Indicus-taurus</i> Donors from Weaning to Yearling Ages	47

Abstract	47
Introduction	48
Materials And Methods	48
<i>Animals</i>	48
<i>Antral follicular counting</i>	49
<i>Preparation of donor females</i>	49
<i>Follicles aspiration</i>	50
<i>In vitro embryo production</i>	50
<i>Superovulation</i>	51
<i>Embryo recovery</i>	52
<i>Semen</i>	52
<i>Statistical analysis</i>	53
Results	53
Discussion	55
Acknowledgments	58
References	58
ARTIGO 2: Comparison Among Antral and Preantral Follicle Population in <i>Bos indicus</i> and <i>Bos taurus</i> Purebred Cows	61
Abstract	61
Introduction	62
Materials and Methods	63
<i>Ovary collection and antrall follicular counting</i>	63
<i>Histological evaluation and follicle classification</i>	63
<i>Estimate of preantral follicles</i>	64
<i>Statistical analysis</i>	65
Results	65
Discussion	68
References	70
ARTIGO 3: MALDI-MS Lipid Profiles of Oocytes Recovered by Ovum Pick-up from <i>Bos Indicus</i> and 1/2 <i>Indicus-taurus</i> with High vs. Low Oocyte Yield	75
Abstract	75
Introduction	76
Materials and Methods	77
<i>Animals</i>	77

<i>Antral follicular counting</i>	78
<i>Follicle aspiration and oocyte recovery</i>	78
<i>Experimental design</i>	79
<i>Lipid analysis by MALDI-MS</i>	79
<i>Statistical analysis</i>	80
Results	80
Discussion	87
References	89
6 DISCUSSÃO GERAL	94
7 CONCLUSÕES	97
ANEXO	126
ANEXO A – Estimate of the Population of Preantral Follicles in the Ovaries of <i>Bos taurus-indicus</i> and <i>Bos taurus taurus cattle</i>	127
ANEXO B – Multioocyte Follicles in Adult Mammalian Ovaries	134
ANEXO C – Epigenética e Neo-Oogênese: Novos Conceitos em Foliculogênese	144

1 INTRODUÇÃO

Apesar dos trabalhos sugerindo continuidade da oogênese na vida adulta (JOHNSON et al., 2004; 2005; ZOU et al., 2009), o número de folículos pré-antrais nos ovários ao nascimento representa a reserva folicular da fêmea, que será utilizada ao longo da vida reprodutiva. Assim, os folículos pré-antrais são responsáveis pela renovação contínua de folículos antrais no ovário (GUILBAULT et al., 1986). Entretanto, aproximadamente 99,9% dos folículos pré-antrais sofrem atresia folicular e não chegam até a ovulação (CARROL et al., 1990).

Existe alta variação na população de folículos pré-antrais entre fêmeas da mesma raça e entre as raças *Bos indicus* e *Bos taurus* (SILVA-SANTOS et al., 2011; 2012). Da mesma forma, a população de folículos antrais em bovinos varia entre os indivíduos, mas é uma característica herdável e apresenta repetibilidade no indivíduo (BURNS et al., 2005; IRELAND et al., 2007; 2008). Estudos mostram que fêmeas com baixa contagem de folículos antrais (CFA) apresentam características usualmente associadas com envelhecimento e infertilidade, tais como ovários menores, redução da reserva folicular ovariana, menor responsividade à superovulação e menor quantidade de embriões transferíveis, menor quantidade de hormônio anti-Mülleriano (AMH) e concentração de progesterona durante o ciclo estral, menor espessura endometrial e maior quantidade de marcadores de células do *cumulus* indicativos de menor qualidade do oócito (IRELAND et al., 2011).

Centenas de oócitos podem ser obtidos em um único procedimento de aspiração folicular (OPU) em fêmeas *Bos indicus* (SANTOS et al., 2005), cuja média de oócitos (18-25) recuperada é três a quatro vezes superior a de fêmeas *Bos taurus* (RUBIN et al., 2005; MACHADO et al., 2003; PONTES et al., 2010). Entretanto, a quantidade de folículos pré-antrais nos ovários de fêmeas *Bos indicus* e *Bos taurus* de diferentes idades não justifica esta diferença na produção de oócitos (SILVA-SANTOS et al., 2011).

Neste contexto, o objetivo deste trabalho consistiu em comparar a produção de embriões durante as ondas de crescimento folicular, a quantidade de folículos ovarianos pré-antrais e o teor de lipídios de oócitos recuperados por OPU entre fêmeas puras (*Bos indicus* e *Bos taurus*) e cruzadas (*Bos indicus-taurus*) com alta e baixa CFA.

2 REVISÃO DE LITERATURA

2.1 – O OVÁRIO MAMÍFERO

2.1.1 – Aspectos Morfológicos e Estruturais do Ovário

O ovário é composto por uma região cortical e uma medular, circundado por epitélio superficial ou germinativo que repousa sobre uma membrana basal. Logo abaixo, observa-se a túnica albugínea e o estroma ovariano (MURDOCH, 1996). O ovário mamífero é um órgão complexo composto de vários tipos celulares: oócitos, células da granulosa, da teca, do estroma e do epitélio da superfície ovariana. Estes tipos celulares, presentes na região cortical do ovário, posteriormente se diferenciam em vários subtipos. Por exemplo, as células da granulosa diferenciam-se em células do cúmulus, murais ou luteais, enquanto que as células da teca desenvolvem-se em camadas internas e externas (ERICKSON; SHIMASAKI, 2003).

A região cortical é composta por colágenos dos tipos I e III, fibroblastos, vasos sanguíneos, linfáticos e terminações nervosas (Hafez; Hafez, 2004). No córtex ovariano, são encontrados folículos ovarianos quiescentes, em desenvolvimento ou atresia, corpos lúteos, corpos albicans e corpos hemorrágicos (MURDOCH, 1996). A região medular, localizada na porção mais interna do ovário, é constituída por tecido conjuntivo fibroblástico (fibroblastos, fibronectina e fibras colágenas do tipo I e III), nervos, vasos sangüíneos e linfáticos. É responsável pela nutrição e sustentação do ovário (HAFEZ; HAFEZ, 2004).

O ovário desempenha duas importantes funções, uma exócrina ou gametogênica (produção e liberação de oócitos) e uma endócrina ou esteroidogênica (produção e liberação de hormônios esteróides e de peptídeos; HAFEZ; HAFEZ, 2004).

A forma do ovário varia de acordo com a espécie e o estágio do ciclo estral (PINEDA, 1989; NUNEZ, 1993; HAFEZ; HAFEZ, 2004). Em bovinos, o ovário tem forma de amêndoa e o peso do ovário de vacas adultas varia de 10 a 20 g. O comprimento varia de 3,0 a 4,5 cm, e a largura, 1,5 a 2,0 cm, respectivamente (HAFEZ; HAFEZ, 2004).

2.1.2 – População Folicular Ovariana

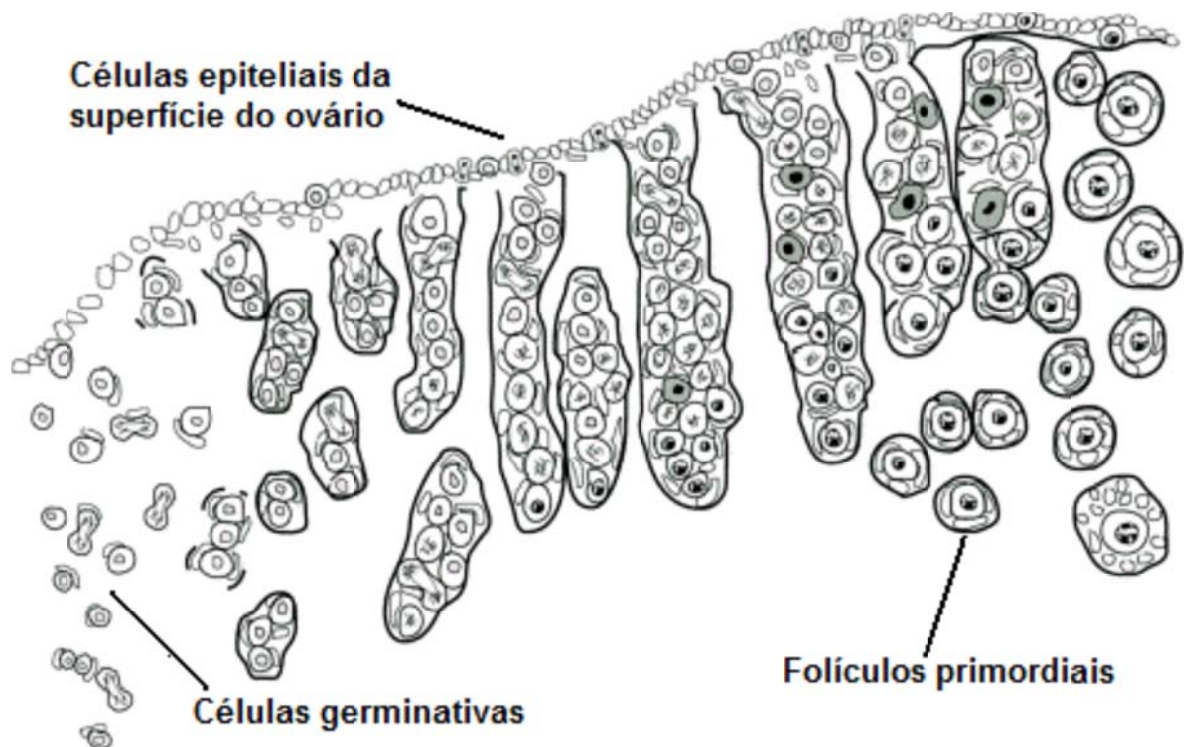
A população folicular ovariana é influenciada por diversos fatores como espécie, raça (CAHILL et al., 1979), genética (ERICKSON, 1966; SMITH et al., 1994), idade, níveis hormonais (PETERS, 1976; RÜSSE, 1983; ROY; TREACY, 1993) e estado reprodutivo do animal (ERICKSON et al., 1976). Além destes, deve-se ressaltar a variação individual na população folicular ovariana, com valores entre 0 e 720.000 folículos por ovário para fêmeas bovinas (ERICKSON, 1966). Estima-se que a população folicular ovariana ao nascimento seja de aproximadamente 235.000 folículos na vaca (BETTERIDGE et al., 1989), 160.000 folículos pré-antrais na ovelha (DRIANCOURT et al., 1991), e 2.000.000 na mulher (ERICKSON, 1986). A estimativa da população folicular pré-antral em ovários de fêmeas bovinas de diferentes idades e raças já foi relatada, com valores de 143.929 e 285.155 folículos para fetos, 76.851 e 109.673 folículos para novilhas, e 39.438 e 89.577 folículos para vacas, *Bos indicus* e *Bos taurus* respectivamente (SILVA-SANTOS et al., 2011).

2.2 – OOGÊNESE E FOLICULOGÊNESE

Nas espécies domésticas, as fêmeas nascem com um estoque de oócitos, formado ainda no decorrer da vida fetal, como consequência de dois processos: a oogênese e a foliculogênese (SAUMANDE, 1991). Em ruminantes, a oogênese pode ser definida como o desenvolvimento e a diferenciação das células germinativas primordiais da fêmea, culminado com a formação do oócito haplóide fecundado (RÜSSE, 1983). A foliculogênese é um processo fisiológico que se inicia com a formação do folículo primordial e culmina com o estágio de folículo maduro, também conhecido como folículo de *De Graaf* ou pré-ovulatório (SAUMANDE, 1981). O término da foliculogênese ocorre no momento da ovulação do folículo maduro, enquanto a oogênese se encerra somente após a fecundação (FIGUEIREDO et al., 2002). Durante o desenvolvimento fetal, as células germinativas primordiais migram do saco vitelínico para as cristas gonadais, sofrem sucessivas mitoses originando as oogônias. Nesta etapa, células somáticas do mesonefron circundam as oogônias, formando os cordões corticais, que são os precursores dos folículos primordiais. Os cordões corticais ou ovígeros são descritos como estruturas alongadas contendo células germinativas circundadas por células da pré-granulosa, que repousam sobre

a lâmina basal (Figura 1) (JUENGEL et al., 2002). As oogônias no seu interior sofrem sucessivas mitoses e diferenciam-se em oócitos, que iniciam o processo de divisão meiótica, o qual é interrompido em prófase da meiose I no estágio de diplóteno (SOTO-SUAZO; ZORN, 2005; VAN DEN HURK; ZHAO, 2005). A formação dos folículos primordiais ocorre quando os oócitos são individualizados a partir da separação dos cordões de células germinativas (BRISTOL-GOULD et al., 2006).

Figura 1 – Esquema ilustrando a origem das células da pré-granulosa durante a formação dos folículos primordiais.



Fonte: Adaptado de Juengel et al. (2002).

Em fêmeas bovinas, o oócito primário ou imaturo permanece no estágio de prófase I até imediatamente antes da ovulação. O processo de meiose é retomado em resposta à estimulação pelo hormônio folículo estimulante (FSH) e hormônio luteinizante (LH) (BUCCIONE et al., 1990), passando em seguida pelas fases de metáfase I, anáfase I e telófase I, ocorrendo a liberação do 1º corpúsculo polar e formação do oócito secundário (BETTERIDGE et al., 1989). O processo de maturação meiótica, *in vivo*, pode ocorrer apenas no oócito do folículo pré-ovulatório dominante e resulta, dentre outros fatores, da estimulação específica pelo pico pré-ovulatório de LH e FSH (ERICKSON, 1986).

No estágio de metáfase II, ocorre uma segunda interrupção da meiose (BETTERIDGE et al.,1989). Na maioria das espécies domésticas, o oócito permanece em metáfase II até ser ovulado e transportado para o oviduto, onde poderá ser fecundado. Caso a fecundação ocorra, o oócito retoma a meiose (BETTERIDGE et al.,1989; BUCCIONE et al.,1990) e culmina com a extrusão do segundo corpúsculo polar (GORDON, 1994), marcando assim o fim da oogênese.

2.3 – CARACTERIZAÇÃO LIPÍDICA DE OÓCITOS

O conteúdo lipídico de oócitos é variável entre espécies. Oócitos bovinos contêm aproximadamente 63 ng de fosfolípidos, o que corresponde a um quarto do conteúdo lipídico total (MCEVOY et al., 2000). O teor lipídico e a atividade mitocondrial são considerados marcadores celulares, devido ao seu papel na capacidade de desenvolvimento do oócito (MCEVOY et al., 2000; CUMMINS, 2004). Ambos podem contribuir para a maturação, fecundação e clivagem inicial. Durante a maturação, por exemplo, os lipídeos podem ser usados como fonte de energia; os estoques de triglicerídeos e colesterol diminuem (FERGUSON; LEESE, 1999; KIM et al., 2001) e a atividade mitocondrial aumenta possivelmente devido ao metabolismo oxidativo (RIEGER; LOSKUTOFF, 1994; TARAZONA et al., 2006). Interações entre lipídeos e organelas parecem ocorrer neste período devido à aglomeração da mitocondria, lipídeos e retículo endoplasmático liso (FLEMING; SAACKE, 1972; HYTTEL et al., 1997).

2.4 – FOLÍCULOS OVARIANOS

Os folículos ovarianos pré-antrais (FOPA) representam 90% da população folicular (SAUMANDE, 1991) e são responsáveis pela renovação contínua de folículos antrais no ovário (GUILBAULT et al., 1986). No entanto, aproximadamente 99,9% dos folículos pré-antrais presentes nos ovários não chegam até a ovulação (CARROL et al., 1990), pois sofrem um processo degenerativo ou apoptótico conhecido por atresia. Dessa forma, o ovário pode ser considerado um órgão de baixíssima produtividade (IRELAND, 1987).

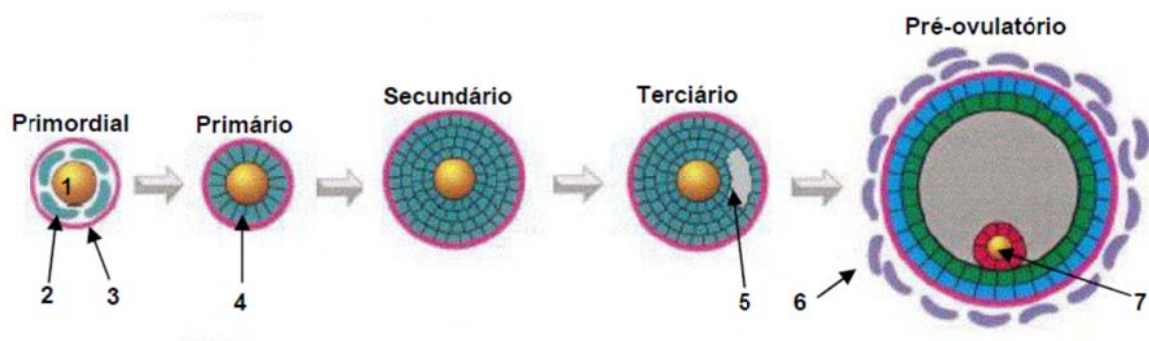
O folículo é a unidade morfofuncional do ovário, constituído por um oócito circundado por células somáticas da granulosa e tecais. O folículo apresenta

funções endócrina (produção e liberação de hormônios esteróides e outros peptídeos) e exócrina ou gametogênica, apresentando-se como elemento essencial para a manutenção da viabilidade oocitária. Dessa forma, o folículo proporciona um ambiente ideal para o crescimento e a maturação do oócito imaturo e permite que o oócito maduro alcance a ovulação (FIGUEIREDO et al., 2002).

Existe controvérsia sobre o aparecimento de folículos primordiais em ovários de fetos bovinos, com relatos ao redor 74 (TANAKA et al., 2001), 90 (RÜSSE, 1983; YANG; FORTUNE, 2008) e 150 dias de gestação (MOUSTAFA; HAFEZ, 1971).

A população de folículos ovarianos localiza-se no córtex ovariano. Os folículos são classificados em pré-antrais ou não cavitários (primordiais, em transição, primários e secundários) e folículos antrais ou cavitários (terciários e préovulatórios; Figura 2), de acordo com o grau de evolução folicular.

Figura 2 – Representação esquemática do desenvolvimento dos folículos ovarianos.



1. Oócito primário; 2. Célula da Pré-granulosa; 3. Membrana basal; 4. Células da Granulosa; 5. Caverna antral; 6. Célula da teca e 7. Oócito secundário.

Fonte: Adaptado de Rodgers et al. (1999).

Na fase inicial do crescimento folicular, admite-se uma ação predominantemente local e vários fatores de crescimento foram identificados nas primeiras modificações foliculares. Dentre os mais estudados, encontram-se o *Kit Ligand* (PARROT; SKINNER, 1999), fator de crescimento e diferenciação - GDF-9 (VITT et al., 2000), fator de crescimento de fibroblasto - bFGF (NILSSON et al., 2001) e fator inibidor de leucemia - LIF (NILSSON et al., 2002).

2.4.1 – Classificação e Caracterização Estrutural e Ultraestrutural dos Folículos Préantrais

Os folículos ovarianos pré-antrais são classificados de acordo com a forma e o número de camadas de células que circundam o oócito imaturo em primordiais, primários e secundários (FIGUEIREDO et al., 2002). Os folículos primordiais e primários não podem ser distinguidos pelo diâmetro, mas sim, por diferenças morfológicas (HULSHOF et al., 1994). Os folículos primordiais apresentam um oócito rodeado por uma camada de 4 a 8 células da granulosa achatadas, os primários mostram um oócito rodeado por uma camada de 11 a 12 células da granulosa cuboidais e os secundários, mais de uma camada de células da granulosa cuboidais. Os folículos pré-antrais podem ser classificados também de acordo com o grau de viabilidade em folículos saudáveis (com lâmina basal intacta, oócito com não mais de três vacúolos citoplasmáticos, vesícula germinativa e nucléolos intactos), folículos em atresia inicial (estágio I: oócito com mais de três vacúolos citoplasmáticos e início de descondensação da cromatina), folículos em atresia moderada (estágio II: oócito com nucléolo e citoplasma em fragmentação e alta condensação da cromatina) ou folículos com atresia acentuada (estágio III: oócito completamente fragmentado ou ausente) (BUTLER, 1970; WANDJI et al., 1996).

2.4.1.1 – Folículos primordiais

Na espécie bovina, ao redor de 130 dias de gestação, uma camada de células somáticas planas ou achatadas, conhecidas como células da pré-granulosa, circundam o oócito primário ou imaturo (em prófase I), formando o primeiro e mais primitivo dos estádios foliculares, o folículo primordial. Após sua formação, as células da pré-granulosa param de se multiplicar e o folículo primordial entra no período de dormência ou quiescência. A proliferação celular é retomada somente quando o folículo primordial (quiescente) começa a crescer, meses ou anos após a sua formação (HIRSHFIELD, 1991). Os folículos primordiais possuem 30-40 µm de diâmetro. O oócito possui 20-25 µm de diâmetro (BECKERS et al., 1996) e o núcleo do oócito ocupa posição central com nucléolo evidente. As organelas estão uniformemente distribuídas no citoplasma ou bem próximas ao núcleo. A mitocôndria

é a organela mais evidente e é predominantemente arredondada. O retículo endoplasmático liso e o Complexo de Golgi são pouco desenvolvidos e várias vesículas estão espalhadas pelo citoplasma (LUCCHI et al., 2001). A grande maioria dos oócitos no ovário é armazenada nos folículos primordiais. Os mecanismos envolvidos no recrutamento e na ativação desses folículos ainda não são bem estabelecidos. Presume-se que em 100 dias o folículo primordial bovino atinja o estágio pré-ovulatório (BRITT, 1991).

2.4.1.2 – Folículos primários

Uma vez recrutado, o folículo primordial evolui para folículo primário, cujas células granulosas apresentam formato cuboide (HULSHOF et al., 1994), presentes em maior quantidade e mais volumosas (VAN DER HURK et al., 1997). Em bovinos, o folículo e o oócito nele contido medem, respectivamente, 40-60 μm e 30-40 μm de diâmetro (BECKERS et al., 1996). Nessa fase, há o surgimento da zona pelúcida, estrutura ao redor do oócito mantida por todo o desenvolvimento folicular (FIGUEIREDO et al., 2002).

Assim como nos folículos primordiais, o citoplasma dos oócitos dos folículos primários também contém numerosas mitocôndrias arredondadas. Com o desenvolvimento do folículo, a mitocôndria torna-se alongada (LUCCHI et al., 2001).

2.4.1.3 – Folículos secundários

Com o aumento do oócito, a caracterização da zona pelúcida, as primeiras células da teca (VAN DER HURK et al., 1997) e pelo menos duas camadas da granulosa, o folículo secundário encontra-se constituído (HULSHOF et al., 1994). O folículo secundário atinge 60-200 μm de diâmetro em vacas (FIGUEIREDO et al., 2002). No folículo secundário, o núcleo do oócito passa de uma posição central no oolema dos folículos primordiais para uma região excêntrica, situando-se na região entre a zona pelúcida e o centro do oócito. As organelas também se movem e ficam mais próximas à periferia (HYTTEL et al., 1997). O retículo endoplasmático liso aumenta de tamanho e a grande maioria das mitocôndrias são alongadas (LUCCHI et al., 2001). Nos folículos secundários e

estágios subsequentes, a comunicação entre as células da granulosa e o oócito é feita por junções intercomunicantes (*GAP junctions*) (HYTTEL et al., 1997).

2.4.2 – Caracterização Estrutural e Ultraestrutural dos Folículos Antrais

A categoria de folículos antrais compreende os folículos terciários e os folículos *De Graaf* ou também conhecidos como maduros ou pré-ovulatórios. Com a intensa proliferação das células da granulosa, há o surgimento do antro folicular, uma área preenchida por fluido folicular, característica do folículo antral (FIGUEIREDO et al., 2002). O surgimento dos primeiros folículos terciários em bovinos é observado aos 230 dias de gestação (ERICKSON, 1966; RÜSSE, 1983). Nesta espécie, a cavidade antral pode se desenvolver em folículos cujos diâmetros variam de 0,14-0,28 mm (LUSSIER et al., 1987). Os folículos crescem em tamanho e o diâmetro dos folículos primordiais aumenta de 0,020-0,040 mm (bovinos) para mais de 10 mm antes da ovulação (IRELAND, 1987). São necessários dois ciclos estrais para um folículo crescer do início da formação do antro (0,13 mm) ao tamanho pré-ovulatório (LUSSIER et al., 1987).

Os folículos terciários são constituídos de um oócito circundado pela zona pelúcida, várias camadas de células da granulosa, uma pequena cavidade antral, uma membrana basal e duas camadas de células tecais (teca interna e teca externa) (GORDON, 1994). Caracterizam-se pela presença de numerosas microvilosidades dentro da zona pelúcida, bem como de numerosas partículas lipídicas e mitocôndrias arredondadas e alongadas. Um maior número de complexos de Golgi pode ser observado e os grânulos corticais estão distribuídos no ooplasma, podendo-se evidenciar ainda os microtúbulos (FAIR et al., 1997). Os folículos de *De Graaf* representam o estágio terminal do desenvolvimento folicular. Neles predominam mitocôndrias arredondadas, mas mitocôndrias encapuzadas, que caracterizam o completo crescimento do oócito em bovinos, também são encontradas. Retículo endoplasmático liso e rugoso são observados em grande quantidade. Podem ser identificados grânulos da cortical e microtúbulos no ooplasma do oócito. O espaço perivitelino é formado neste estágio de desenvolvimento e há um aumento no número de vesículas e de complexos de Golgi. É também no final deste estágio que a função do nucléolo é inativada, como indica a marginalização dos centros fibrilares, sinalizando uma presumível retração

dos genes rRNA do nucléolo. Concomitantemente, a atividade transcricional do oócito é diminuída. Foi demonstrado, entretanto, que a transcrição de mRNA não é completamente inativada com o crescimento total do oócito (HYTTEL et al., 1997).

A população de folículos antrais é altamente variável entre indivíduos, entretanto, mantém alta repetibilidade individual (BURNS et al., 2005; IRELAND et al., 2007; 2008). A variação na população de folículos antrais entre indivíduos durante as ondas de crescimento folicular está associada com a expressão de genes envolvidos na produção de estradiol pelas células da granulosa (CYP19A1), na regulação da atividade do FSH (hormônio anti-Mülleriano - AMH), na diferenciação e no funcionamento das células tecais (TBC1D1), na responsividade ao estradiol (ESR1, ESR2) e com determinantes da qualidade do oócito nas células do cumulus (CTSB) (IRELAND et al., 2009). O AMH é uma glicoproteína de 140 kDa que pertence à família do fator transformador do crescimento (TGF- β), produzido nas células da granulosa e que é expresso somente nas gônadas (VIGIER et al., 1984; CATE et al.; TAKAHASHI et al., 1986; LEE et al., 1996; MONNIAUX et al., 2008). O AMH é um ótimo marcador endócrino para o número de folículos em crescimento, já que inibe o recrutamento de folículos primordiais para o *pool* de folículos em crescimento, além de reduzir a responsividade dos folículos em crescimento ao FSH (DI CLEMENTE et al., 1996; DURLINGER et al., 1999; 2001; 2002). Atualmente, o AMH é o melhor marcador endócrino da reserva ovariana em humanos (VAN ROOIJ et al., 2002; GRUIJTERS et al., 2003; VISSER et al., 2005, 2006) e tem sido utilizado na reprodução assistida para prever a resposta a tratamentos estimulatórios em mulheres (MUTTUKRISHNA et al., 2004; 2005; ELDAR-GEVA et al., 2005; PENARRUBIA et al., 2005; EBNER et al., 2006; MCILVEEN et al.; SMEENK et al., 2007; ELGINDY et al.; KWEE et al., 2008). A contagem de folículos antrais (CFA) também pode ser utilizada em tratamentos superestimulatórios para prever baixa resposta ovariana em humanos (HENDRIKS et al., 2005; 2007), com a mesma acurácia e valor clínico do AMH (BROER et al., 2009).

2.5 – ATRESIA FOLICULAR

Como mencionado anteriormente, aproximadamente 99,9% dos folículos pré-antrais presentes nos ovários não chegam até a ovulação, sofrendo processo degenerativo ou apoptótico conhecido por atresia. A atresia folicular não é igualmente prevalente em todos os estádios de desenvolvimento folicular (FORTUNE, 1994). A atresia é um processo fisiológico, de duração desconhecida, que parece ser um dos elementos que controla o número de folículos selecionados até chegar à ovulação. A duração precisa, bem como o estádio no qual os folículos ovarianos são mais susceptíveis de sofrer atresia, não são conhecidos (HENDERSON et al., 1987). O processo de atresia usualmente difere entre folículos pré-antrais (primordiais, primários e secundários) e antrais. Em folículos pré-antrais, as primeiras alterações indicativas de atresia ocorrem no oócito, como por exemplo, retração da cromatina nuclear e fragmentação oocitária, o que desencadeia o processo de eliminação irreversível dos folículos ovarianos nesta fase de desenvolvimento (MORITA; TILLY, 1999). Em folículos pré-antrais, alterações nas células da granulosa são raramente observadas (JORIO et al., 1991). É importante ressaltar que após a formação da cavidade antral, ocorre uma alteração na sensibilidade do oócito e das células da granulosa. A partir deste estágio, o oócito torna-se altamente resistente e as primeiras alterações indicativas de atresia são observadas nas células da granulosa. O aparecimento de células da granulosa com núcleos picnóticos, onde se observa condensação da cromatina e retração nuclear, podem ser considerados como os primeiros sinais morfológicos de atresia, que são observados predominantemente em células da granulosa em proximidade da cavidade antral. Posteriormente, fragmentos de núcleos picnóticos ou corpos apoptóticos são observados na cavidade antral (HUGHES; GOROSPE, 1991; TILLY, 1996). Com a progressão da atresia, observa-se redução no número de camadas das células da granulosa e invasão do folículo por fibroblastos e macrófagos. Após estas drásticas mudanças na camada granulosa o oócito, frequentemente, sofre pseudomaturação, fragmenta-se e, finalmente, é eliminado durante os estágios finais de atresia (BYSKOV, 1974). Apesar de ser um fenômeno natural, a atresia reduz de maneira significativa o número de oócitos potencialmente ovuláveis, diminuindo, conseqüentemente, a produção de oócitos viáveis durante a vida reprodutiva de um animal (FIGUEIREDO et al., 2002).

2.6 – RENOVAÇÃO FOLICULAR

Apesar do conceito do estoque finito e não renovável de células germinativas (ZUCKERMAN, 1951) ser amplamente aceito, Johnson et al. (2004; 2005) surpreenderam a comunidade científica ao demonstrarem indícios de continuidade da oogênese e foliculogênese no período pós-natal, apontando as células-tronco da medula óssea como responsáveis pela renovação dos gametas femininos. Estes pesquisadores sugerem a ocorrência de células germinativas nos ovários, na medula óssea e no sangue periférico. Os trabalhos de Johnson et al. (2004; 2005) geraram muita controvérsia no meio científico. Um dos aspectos refere-se à ocorrência da menopausa e a inatividade ovariana em fêmeas senis. As críticas também estão relacionadas à ausência de sinais de início e término da primeira prófase meiótica e subsequente fase de diplóteno nos supostos oócitos derivados de células-tronco. Além disso, o curto período para crescimento dos novos folículos permitiu o questionamento da eficácia da esterilização química. A teoria de neo-oogênese/foliculogênese proposta por Johnson et al. (2004; 2005) é polêmica e desafia um conceito de mais de 100 anos. A polêmica não parece estar próxima do fim, principalmente depois do relato do nascimento de camundongos a partir de oócitos neoformados depois do cultivo de células germinativas de ovários de camundongos adultos transgênicos para proteína GFP e transferência para ovários de fêmeas esterilizadas quimicamente (ZOU et al., 2009). Depois dos primeiros relatos do grupo de Johnson e Tilly, diversos estudos mostraram-se a favor da neo-foliculogênese (ABBAN; JOHNSON; BUKOVSKY et al.; CELIK et al.; ZOU et al.; 2009; DE FELICI; PACCHIAROTTI et al.; PARTE et al.; VIRANT-KLUN; SKUTELLA, 2010; VIRANT-KLUN et al., 2011). E outros concluem não haver renovação folicular (BRISTOL-GOULD et al.; EGGAN et al., 2006; LIU et al., 2007; BEGUM et al., 2008; FADDY; GOSDEN, 2009; ZHANG et al., 2010; BYSKOV et al., 2011). Recentemente, este grupo relatou semelhante ao que ocorre em camundongas adultas, ovários provenientes de mulheres em idade para reprodução possuem células germinativas mitoticamente ativas que podem ser propagadas *in vitro* e gerar oócitos *in vitro* e *in vivo* (WHITE et al., 2012). Apesar da questão permanecer em discussão, os trabalhos sugerem revisão dos conceitos sobre foliculogênese.

2.7 – FISILOGIA REPRODUTIVA DE FÊMEAS ZEBUÍNAS E TAURINAS

Existem diferenças na fisiologia reprodutiva entre *Bos taurus indicus* (*Bos indicus*) e *Bos taurus taurus* (*Bos taurus*) que devem ser consideradas, pois implicam em diferentes técnicas de manejo e respostas a tratamentos hormonais. O conhecimento dessas particularidades é fundamental para aumentar a eficiência reprodutiva dos rebanhos (BARUSELLI et al., 2007). Com relação ao estro comportamental, existem diferenças observadas entre raças (RAE et al., 1999), e embora ainda não completamente elucidadas, entre grupos genéticos (zebuínos e taurinos). Fêmeas *Bos indicus* geralmente apresentam estro de duração mais curta (aproximadamente 10 horas), o que dificulta sua detecção (BÓ et al., 2003). Além disso, mais de 50% dos animais desse grupo genético iniciam a manifestação de estro no período noturno (entre 18:00 e 6:00 horas) (PINHEIRO et al., 1998; MEMBRIVE, 2000), sendo que cerca de 30% iniciam e encerram o estro durante a noite (PINHEIRO et al., 1998), dificultando o manejo e a eficácia da detecção de estro. Em condições brasileiras de manejo, a avaliação do comportamento reprodutivo de vacas de corte com auxílio de radiotelemetria (*Heat-Watch*) mostrou que a duração do estro em *Bos indicus* é menor do que em *Bos taurus* ($12,9 \pm 2,9$ horas em Nelore vs. $16,3 \pm 4,8$ horas em Angus) (MIZUTA, 2003). Apesar disso, o intervalo entre o estro e a ovulação não apresentou diferenças entre estas duas raças (Nelore, $27,1 \pm 3,3$ horas vs. Angus, $26,1 \pm 6,3$ horas).

Considerando o número de ondas de crescimento folicular por ciclo estral, animais da raça Holandesa apresentam predominância de duas e três ondas de crescimento folicular por ciclo estral (SAVIO et al., 1988; SIROIS; FORTUNE, 1988; GINTHER et al., 1989; WOLFENSON et al., 2004). Entretanto, em zebuínos existem relatos que descrevem maior incidência de 3 ondas, sendo notificada a presença de até 4 ondas de crescimento folicular por ciclo estral (BRAHMAN – RHODES et al., 1995; NELORE – FIGUEIREDO et al., 1997; GIR – VIANA et al., 2000). Além da diferença no número de ondas, existem trabalhos que descrevem que fêmeas *Bos indicus* recrutam maior número de folículos por onda de crescimento folicular que fêmeas *Bos taurus* ($33,4 \pm 3,2$ vs $25,4 \pm 2,5$) (CARVALHO et al., 2008). Essa característica tem influência direta na eficiência da técnica de transferência de embriões e de OPU-PIV, indicando vantagem de fêmeas zebuínas sobre taurinas. Utilizando-se o método de lavagem uterina, a média de embriões

obtidos é similar entre esses grupos genéticos (CASTRO-NETO et al., 2005). No entanto, a média de oócitos recuperados por aspiração folicular é maior para fêmeas zebuínas (média de 18 a 25 oócitos) (WATANABE et al., 1999; THIBIER, 2004; RUBIN et al., 2004) do que para taurinas (média de 4 e 14 oócitos por sessão de aspiração folicular) (MACHADO et al., 2003; RUBIN et al., 2005; MARTINS JR et al., 2007). Existem relatos de que o número de folículos recrutados por onda de crescimento folicular apresenta diferenças entre indivíduos, e essa característica possui alta repetibilidade durante a vida reprodutiva da fêmea (BONI et al., 1997; BURNS et al., 2005; IRELAND et al., 2009). Alguns autores levantaram a hipótese de que a possibilidade de um maior número de folículos presentes no ovário de *Bos indicus* poderia ser devido à elevada concentração de IGF-I, mesmo na presença de baixos níveis de FSH (BÓ et al., 2003). Essa diferença nas concentrações de FSH e de IGF-I pode explicar a maior sensibilidade ao tratamento superovulatório em doadoras *Bos indicus* (BARROS; NOGUEIRA, 2001). Existem relatos que confirmam que é possível reduzir consideravelmente a dose de FSH para superovular fêmeas Nelore (BARUSELLI et al., 2003), empregando doses inferiores às usualmente utilizadas para *Bos taurus*. Há diferenças moderadas entre o tamanho do trato reprodutivo e a divergência folicular entre *Bos taurus* e *Bos indicus* (ADAYEMO; HEATH, 1980; SARTORELLI et al., 2005). Em bovinos da raça Holandesa (*Bos taurus*), a divergência tem início por volta do dia 2,8 após a emergência (GINTHER et al., 1996) e, em novilhas da raça Nelore (*Bos indicus*), 2,5 a 2,7 dias após a ovulação (GIMENES et al., 2005; SARTORELLI et al., 2005; CASTILHO et al., 2006). O diâmetro máximo alcançado pelo folículo dominante em cada onda de crescimento folicular também difere entre fêmeas *Bos taurus* (17,1 e 16,5 mm para a primeira e segunda onda) (GINTHER et al., 1989) e *Bos indicus* (11,3 e 12,1 mm, respectivamente) (FIGUEIREDO et al., 1997). Para animais com três ondas de crescimento folicular, os diâmetros máximos foram de 16,0; 12,9 e 13,9 mm para *Bos taurus* (GINTHER et al., 1989) e 10,4; 9,4 e 11,6 mm para *Bos indicus* (FIGUEIREDO et al., 1997). O diâmetro do corpo lúteo também parece ser menor em *Bos indicus* (17 a 21 mm de diâmetro) (RHODES et al., 1995; FIGUEIREDO et al., 1997) do que em *Bos taurus* (20 a 30 mm de diâmetro) (GINTHER et al., 1989; KASTELIC et al., 1990). Da mesma maneira, há relatos de que a concentração de progesterona produzida pelo CL também é inferior em zebuínos em relação aos taurinos (SEGERSON et al., 1984).

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3 HIPÓTESE

O número de folículos antrais presentes no ovário está diretamente relacionado com a reserva folicular ovariana e influencia o conteúdo lipídico de oócitos/ qualidade oocitária e a produção *in vivo* e *in vitro* de embriões em fêmeas bovinas.

4 OBJETIVOS

4.1 – OBJETIVO GERAL

Comparar a produção de embriões, o teor de lipídios de oócitos e a quantidade de folículos ovarianos pré-antrais de fêmeas bovinas com alta e baixa contagem de folículos antrais presentes nos ovários.

4.2 – OBJETIVOS ESPECÍFICOS

- Comparar a produção *in vitro* e *in vivo* de embriões entre fêmeas bovinas *indicus-taurus* com alta e baixa contagem de folículos antrais, previamente avaliadas por ultrassonografia, submetidas a protocolos de OPU/ fecundação *in vitro* (FIV) e superovulação/ coleta de embriões;
- Comparar a população de folículos ovarianos pré-antrais, por histologia clássica, entre fêmeas *Bos indicus* e *Bos taurus* com alta e baixa contagem de folículos antrais, previamente avaliadas por ultrassonografia;
- Comparar o teor de lipídios, por dessorção/ ionização de matriz assistida a laser por espectrometria de massa (MALDI-MS), de complexos *cumulus oophorus* (COCs) recuperados por aspiração folicular guiada por ultrassonografia (OPU) entre fêmeas *Bos indicus* e *1/2 indicus-taurus* com alta e baixa contagem de folículos antrais, previamente avaliadas por ultrassonografia.

5 ARTIGOS PARA PUBLICAÇÃO

ARTIGO 1

Population of Antral Follicles and Embryo Production - *in vitro* and *in vivo* - of *Indicus-taurus* Donors from Weaning to Yearling Ages

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Abstract: There is an increasing interest on *indicus-taurus* cattle, since this type of animals seems to present the best characteristics of Zebu and European bovine breeds. The aim of this study was to compare the embryo production amongst *indicus-taurus* donors with high vs. low antral follicle count obtained by *ovum pick-up/in vitro* production (OPU/IVP) and superovulation (SOV)/ embryo collection. Braford females (3/8 Nelore x 5/8 Hereford, n = 137, 9 ± 1-month-old) were submitted to six serial ovarian ultrasonographic scans and were assigned to two groups, according 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 females reached 24 months of age, they (n = 40) were submitted to both OPU/IVP and SOV/ embryo collection. The average number of follicles remained highly stable through all ultrasound evaluations. The mean number of COCs recovered (36.90 ± 13.68 vs. 5.80 ± 3.40) were greater (P < 0.05) among females with high AFC, resulting in a great (P < 0.05) number of total embryos among females with high vs. low AFC (6.10 ± 4.51 vs. 0.55 ± 0.83). The mean number of embryos/collection was also greater (P < 0.05) for G-High vs. G-Low (6.95 ± 5.34 vs. 1.9 ± 2.13). We concluded that a single ultrasound scan to count antral follicles performed at the age of 9 months can be used to predict the embryo production following IVP and SOV/ embryo collection in *indicus-taurus* females.

Keywords: Embryo yield. Antral follicles. OPU. MOET. Cattle.

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Introduction

There is an increasing interest on *indicus-taurus* bovine breeds, considering their adaptability to produce meat and milk under stressful conditions, e.g. high temperature, parasites, and poor pasture (PONTES et al., 2011). These characteristics are maintained in Nelore or Bhraman - Hereford crossbred animals, usually named "Braford", which are popular beef cattle in Central and South America, and potentially other tropical and even non-tropical areas.

Considering the importance of embryo production for genetic improvement, it is important to mention the high variability in embryo production per donor following *ovum pick up* (OPU)/ IVP and superovulation (SOV)/ embryo collection (PONTES et al., 2009). For IVP-derived embryos, it has been already reported that variation in oocyte yield influences the final number of embryos produced (PONTES et al., 2011). Additionally, some females produce the greatest embryo yield following OPU/IVP or SOV/ embryo collection (PONTES et al., 2009).

In recent years, some studies have reported a high variability in the numbers of preantral and antral follicles amongst individual adult cattle (ERICKSON, 1966; BURNS et al., 2005; SILVA-SANTOS et al., 2011). Yet, 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 by ultrasonography females with low, intermediate or high numbers of follicles during waves. However, it is unknown whether the variation in the ovarian follicular reserve and also in the antral follicular count (AFC) could affect the *in vitro* or *in vivo* embryo production in cattle. In this context, the aim of the present study was to compare the efficiency of OPU/ IVP vs. SOV/ embryo collection on embryo production performed in the same donors with consistently high vs. low AFC, after counting antral follicles by ultrasonography in *indicus-taurus* cattle from weaning to yearling ages.

Materials and Methods

Animals

Braford females (3/8 Nelore x 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 old) to yearling ages (20 ± 1 months old). The mean body condition score was 3.0 ± 0.5 (scale, 1 to 5) (LOWMAN et al., 1976) and the average alive body weight was 360 ± 10 kg. Some of these females were selected on the basis of the antral follicular count and were first submitted to OPU and then to SOV/ embryo collection.

Antral follicular counting

Ovaries from each animal were monitored with a 7.5-convex intravaginal array transducer (Áquila PRO, Pie medical, Maastricht, The Netherlands) spaced 60 d 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). Each ovary was scanned from end to end to identify positions of the corpus luteum and antral follicles ≥ 3 mm, which were drawn on an ovarian map. Each follicle was measured and the diameter was averaged and recorded next to the appropriate follicle on each ovarian map. The antral follicle count (AFC - total number of antral follicles ≥ 3 mm in diameter) per pair of ovaries was determined for each animal. After six ultrasound evaluations performed by the same operator, females were assigned to two groups according the number of antral follicles ≥ 3 mm as follows: females with a consistently high (G-High, ≥ 40 follicles; $n = 20$) vs. a 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 mo of age, they were submitted to OPU/IVP procedures and thereafter to SOV/ embryo collection.

Preparation of donor females

Before each procedure, feces 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 anesthesia (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, Maastricht, The Netherlands), 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, aspirated material was washed and filtered through an Emcon embryo filter (Immuno Systems Inc., Spring Valley, WI, USA) with a phosphate buffer solution (PBS-Nutricell, Campinas, SP, Brazil). 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 (6). Both good and regular oocytes were considered viable and were used, whereas atretic follicles 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% fetal calf serum (FCS) and 50 µg gentamycin sulfate, 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 estradiol (estradiol-17β, Sigma E-8875), 2.2 µg pyruvate (Sigma P-4562), and 50 µg gentamycin/mL of medium. The COCs from each category were separately cultured for 24 h in 100 µL drops of maturation medium under mineral oil (D'Altomare, Santo Amaro, SP, Brazil) at 39 °C and 5%

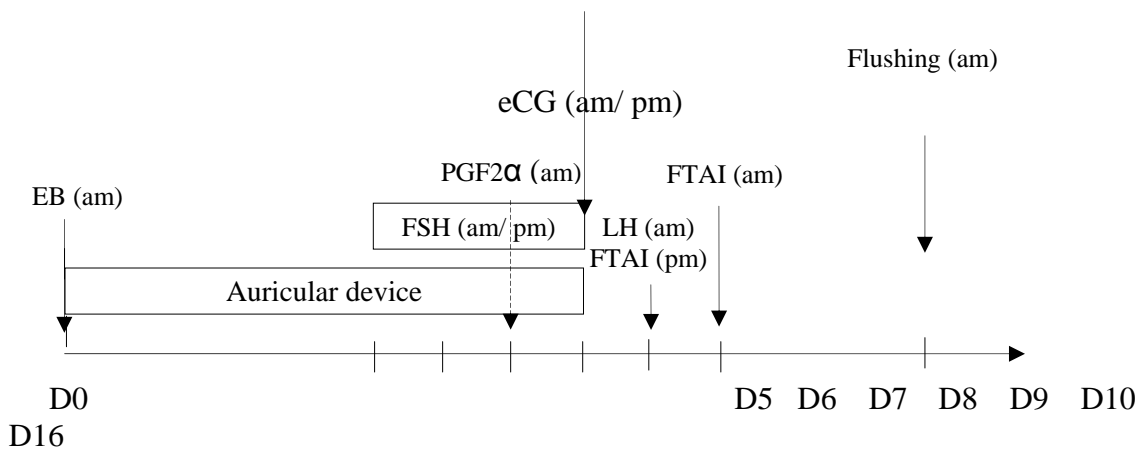
CO₂ in air (GORDON, 1994; SMITH et al., 1996). Frozen-thawed sperm (2×10^7 /dose) from a Braford sire of known fertility, based on previous utilization for *in vitro* fertilization (IVF), was used. For IVF, straws were thawed for 20 s in a 35 °C water bath. Sperm were washed by centrifugation at 200 X g for 30 min through a 90–45% Percoll gradient. 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, 1986). After a visual assessment of motility, the sperm concentration was adjusted to 25×10^6 motile sperm /mL and each fertilization drop received 4 µL of sperm (final concentration 1×10^5 sperm per drop) (SENEDA et al., 2001). After maturation, COCs were washed three times in pre-fertilization medium TCM 199 supplemented with 25 mM HEPES (Gibco Life Technologies, Grand Island, NY, USA) and 0.3% BSA (Sigma A-9647), and they were washed once in TALP fertilization medium supplemented with 10 g/mL heparin and 160 µL PHE solution (PARRISH, 1986; BAVISTER, 1989). Presumptive zygotes had their cumulus cells removed and were transferred to 100 µL drops of culture medium for embryos (SOFaa BSA, containing 8 mg/mL BSA [free of fatty acid] and 1 mM glutamine), under the same conditions of temperature and gaseous atmosphere used for IVF. The osmolarity was maintained at 270 to 280 mOsmol and the pH was 7.4. The embryo rate was obtained from the total of aspirated oocytes. Embryos were evaluated until Day 7 (Day 0 = day of IVF) according to IETS criteria (WRIGHT, 1998). Cleavage and blastocyst rates were recorded at Day 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

After the OPU/IVP procedures, the same females were submitted to a superovulation protocol and embryo recovery 7 d 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 estradiol 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. In the morning of Day 7, donors were given 500 µg cloprostenol

im (PGF2 α , Ciosin, Intervet-Schering Plough, São Paulo, São Paulo Brazil) and, in the morning and in the afternoon of D8, they were given 200 IU eCG im (Novormon, Syntex SA, Argentina). The device was removed at the time of the second dose of eCG (afternoon of Day 8). In the morning of Day 9, females received 12.5 mg LH im (Lutropin-V, Bioniche, Canada) and fixed time artificial insemination (FTAI) was performed 12 to 24 h after the LH injection.

Figure 1 – Treatment schedule for superovulation of donor females with high and low antral follicle numbers.



Embryo recovery

Uterine flushing was performed 7 d after the LH injection and embryos were collected using a two-way Foley catheter passed through the cervix. The catheter's 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). 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 $-196\text{ }^{\circ}\text{C}$.

Semen

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

Statistical analysis

Results are presented as the means \pm SD. All statistical analysis was performed using the software Bioestat 5.0 (AYRES et al., 2007). Means were not normally distributed and were analyzed using the Kruskal-Wallis test, with a Dunn test for comparisons between groups. The embryo production per female with high AFC was normally distributed and was compared with ANOVA. Comparisons amongst the six greatest donors for the *in vitro* or *in vivo* procedures were done with ANOVA, with a t-test for comparisons between procedures. Cleavage, blastocyst rates, proportions of viable oocytes and freezable embryos were evaluated with a Chisquare test. Proportions of vitrifiable embryos were compared using Fisher's Exact test. For all analyses, $P \leq 0.05$ was considered significant.

Results

Data from embryo production in Braford females with high and low AFC submitted to OPU/IVP and SOV/ embryo collection are presented (Table 1). The average number of follicles remained highly stable through all ultrasound evaluations. After follicular aspiration, both the mean number of COCs recovered (36.90 ± 13.68 vs. 5.80 ± 3.40) and viable oocytes (21.65 ± 10.05 vs. 3.20 ± 2.44) were greater ($P < 0.05$) for females with high AFC, resulting in a greater ($P < 0.05$) number of total embryos for females with high AFC (6.10 ± 4.51 vs. 0.55 ± 0.83). After embryo collection, the number of total recovered structures was greater for G-High (8.80 ± 6.78) compared to 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). There were differences ($P < 0.05$) in the average embryo production following OPU/IVP and SOV/ embryo collection amongst females with low AFC (Table 1).

Table 1 – Mean (\pm SD) data for reproductive performance of Braford females with high (G-High, \geq 40 follicles) and low (G-Low, \leq 10 follicles) antral follicle count (AFC), comparing embryo production following *in vitro* (OPU/IVP) vs. *in vivo* (SOV/ embryo collection) procedures.

	G-High (n = 20)	G-Low (n = 20)
Antral follicles (n)	47 \pm 6	9 \pm 3
Total oocytes recovered	738 ^a	116 ^b
Oocyte recovery rate (%)	78.51 (738/940) ^a	64.44 (116/180) ^b
Oocytes/ OPU (n)	36.90 \pm 13.68 ^a (738/20)	5.80 \pm 3.40 ^b (116/20)
Viable oocytes/ OPU (n)	21.65 \pm 10.05 ^a (435/20)	3.20 \pm 2.44 ^b (64/20)
Proportion 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 \pm 4.51 ^{aA} (122/20)	0.55 \pm 0.83 ^{bB} (11/20)
Vitrifiable embryos/ OPU/IVP (n)	4.10 \pm 3.24 ^a (82/20)	0.20 \pm 0.52 ^b (4/20)
Proportion vitrifiable (%)	67.21 ^a (82/122)	36.36 ^b (4/11)
Total recovered structures/ collection (n)	8.80 \pm 6.78 ^a (176/20)	2.25 \pm 2.63 ^b (45/20)
Total embryos/ collection (n)	6.95 \pm 5.34 ^{aA} (139/20)	1.9 \pm 2.13 ^{bA} (38/20)
Freezable embryos/ collection (n)	5.45 \pm 5.77 ^a (109/20)	1.7 \pm 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 ($P \leq 0.05$).

Females with high and low AFC selected for 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 (Table 2). For both females with high and low AFC, the embryo yield from donors selected for 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 females selected on the basis of greatest embryo production during the *in vivo* procedure, both donors with high and low AFC produced a greater ($P < 0.01$) average number of embryos following SOV/ embryo collection compared to OPU/IVP (13.5 vs.6.2 for G-High; 4.5 vs. 0.3 for G-Low).

Table 2 – Variation in embryo production among Braford females with high (G-High, ≥ 40 follicles) and low (G-Low, ≤ 10 follicles) antral follicle count (AFC), comparing the six greatest donors for *in vitro* (OPU/IVF) or *in vivo* (SOV/ embryo collection) procedures.

	Donors of High AFC							Donors of Low AFC						
	I	II	III	IV	V	VI	Mean	I	II	III	IV	V	VI	Mean
Greatest production following PIV (n = 6)														
PIV	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
PIV	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 ($P \leq 0.01$).

Discussion

Comparisons between the *in vitro* and *in vivo* methods for bovine embryo production have been reported (FARIN; FARIN, 1995; HANSEN; BLOCK, 2004; GJORRET; KNIJN, 2005; PONTES et al., 2009), though this is the first comparative study showing that ultrasound scan to count antral follicles performed at

weaning ages can predict the number of embryos yielded from *indicus-taurus* donors at yearling ages following IVP and SOV/ embryo collection procedures.

Selection of cattle based on the count of antral follicles during the follicular waves by ultrasound examination is possible because there is a highly repeatability within individuals regardless of breed, age, season, stage of lactation or management conditions (BURNS et al., 2005; IRELAND et al., 2007; 2008). In agreement, we also observed a repeatability in 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 number of antral follicles present in ovaries, because a single ultrasound scan was useful to predict the superovulatory response in cattle (SINGH et al., 2004). Beyond supporting the use of antral follicles count to predict the superovulatory response, the present study shows that this count can be performed in cattle at weaning ages to predict the success of IVP programs.

Extreme variation in numbers of oocytes recovered and the superovulatory response remain one of the problems in bovine embryo production (PONTES et al., 2009; 2011). It has been reported that success of IVP and responsiveness to SOV have a inverse association with reduction in numbers of follicles and oocytes in the ovaries of cattle (KAWAMATA, 1994; CUSHMAN et al., 1999; TANEJA et al., 2000; SINGH et al., 2004; PONTES et al., 2011), but there are no studies comparing the efficiency between the *in vitro* and *in vivo* procedures performed in the same cattle. In the present study, females with high AFC submitted to first OPU/IVP and thereafter to SOV/ embryo collection showed best results compared to the low counterparts. The embryo yield obtained by OPU/IVP from females of G-High vs. G-Low (6.10 ± 4.51 vs. 0.55 ± 0.83) was lower compared to SOV/ embryo collection (6.95 ± 5.34 vs. 1.9 ± 2.13 ; Table 1). There were differences in embryo production between procedures within females of G-Low. Similarly, other authors reported a reduced number of IVP-derived embryos (1.3 vs. 4.9) obtained from abattoir ovaries of females with low numbers of antral follicles (< 15 follicles) and a lower production of embryos 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 there were differences in absolute numbers, we did not observe differences in the proportions of freezable embryos

among females of 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 also associated with embryo production and pregnancy rates (PONTES et al., 2009; 2011). Donor cows producing a remarkably large number of oocytes (59 oocytes/OPU) produced significantly more embryos and pregnancies (~ 6-fold 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), albeit there were no differences in the proportions of viable oocytes (58.94 vs. 55.17%) among donors with high and low AFC. 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, or 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 within 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). In the current study, six greatest embryo productions per donor following OPU/IVP and SOV/ embryo collection (Table 2) also support this high variation amongst females and suggest that some donors present greater efficiency in embryo production following OPU/IVP whereas others have great efficiency following SOV/ embryo collection. For both high- and low-AFC groups, although six females of each group have been previously selected for high embryo yield following OPU/IVP, there were no differences amongst them comparing the *in vitro* vs. *in vivo* procedure (11.5 vs. 7.2 for G-High; 1.7 vs. 1.5 for G-Low). However, both females with high and low AFC selected on the basis of a greater number of embryos following the *in vivo* procedure produced higher numbers of embryos following SOV/ embryo collection compared to OPU/IVP (13.5 vs. 6.2 for G-High; 4.5 vs. 0.3 for G-Low).

In conclusion, this study presented the embryo yield from donors with high and low antral follicle numbers after almost one year of follicular growth monitoring by ultrasound, comparing both the *in vitro* and *in vivo* procedures in the same donor cows. Although consistent individual variation in embryo yield exists,

OPU/ IVP and SOV/ embryo collection procedures performed in the same cattle resulted in greatest number of embryos from females with higher population 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 and can be used as an easy and cheap tool to identify cattle potentially good embryo producers.

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ARTIGO 2

Comparison Among Antral and Preantral Follicle Population in *Bos indicus* and *Bos taurus* Purebred Cows

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Abstract: The number of oocytes recovered by OPU from *Bos indicus* females are much greater than that recovered from *Bos taurus*, although there are no differences in the numbers of preantral follicles. Some females have higher numbers of antral follicles during follicular waves and this characteristic is repeatable within individuals. The aim of the present study was to compare the population of preantral ovarian follicles among *Bos indicus* and *Bos taurus* purebred females with high and low numbers of antral follicles during follicular waves. Ovaries from Nelore (*Bos indicus*, n = 100) and Angus (*Bos taurus*, n = 100) cows (72-96 months-old) were collected at abattoirs and examined by ultrasonography. Antral follicles ≥ 3 mm were counted and cows were assigned into groups with high (G-High) or low (G-Low) antral follicle count (AFC) as follows: *Bos indicus* cows with high-AFC (≥ 57 follicles, n = 8) or low-AFC (≤ 21 follicles, n = 8) and *Bos taurus* females with high- (≥ 45 follicles, n = 10) or low-AFC (≤ 13 follicles, n = 10). Ovaries were processed for histological evaluation and the number of preantral follicles was estimated using a correction factor. Comparisons between groups were made with ANOVA ($P \leq 0.05$). The mean numbers of antral follicles (mean \pm SD) in *Bos indicus* cows was 63 ± 8 (G-High) and 15 ± 5 follicles (G-Low), and 59 ± 23 (G-High) and 11 ± 3 follicles (G-Low) for *Bos taurus* females. A large variation in the number of preantral follicles was observed among animals. There was no difference between the average number of preantral follicles of *Bos indicus* of G-High ($48,349 \pm 30,149$ follicles) or G-Low ($33,037 \pm 31,710$ follicles), or between *Bos taurus* of G-High ($35,050 \pm 36,060$ follicles) or G-Low ($30,481 \pm 43,360$ follicles). There was also no difference between the population of preantral follicles in *Bos indicus* and *Bos taurus* cows with high- and low-AFC. Therefore, the numbers of preantral follicles are not influenced by the population of antral follicles in ovaries of *Bos indicus* and *Bos taurus* purebred cattle with high and low AFC.

Keywords: Preantral follicles. Antral follicular count. *Bos indicus*. *Bos taurus*. Cattle.

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Introduction

Since greater amounts of oocytes can be obtained by *ovum pick up* (OPU) performed in *Bos indicus* compared to *Bos taurus* cattle (PONTES et al., 2009; 2010), researchers have been stimulated to perceive the mechanism that could account for this greater oocyte production. There are some differences regarding number of follicular waves (FIGUEIREDO et al., 1997; VIANA et al., 2000), follicles per wave (CARVALHO et al., 2008), antral follicles < 5 mm in diameter (SEGERSON et al., 1984) and size of corporea lutea (CL) (RHODES et al., 1995; SARTORELLI et al., 2005) among *Bos indicus* and *Bos taurus* females. But no studies justify differences in the oocyte production between these breeds.

Variation in the numbers of antral follicles in the ovaries of bovine females has been associated with anti-Müllerian hormone (AMH) concentrations (IRELAND et al., 2008; 2009; RICO et al., 2009; 2011; ERENO et al., 2012). Anti-Müllerian hormone has been used to predict the ovarian response to a stimulatory treatment in women (VAN ROOIJ et al., 2002; GRUIJTERS et al., 2003; BROER et al., 2009). Also, antral follicle count (AFC) has been considered as a test to predict poor ovarian response after *in vitro* fertilization (IVF) in women, with the same level of accuracy and clinical value as AMH (HENDRIKS et al., 2005; 2007; BROER et al., 2009). In cattle, both AMH and AFC can be used as determinants of embryo production capacity (MONNIAUX et al., 2010; RICO et al., 2009; 2011; 2012). There is repeatability in the numbers of antral follicles during follicular waves (BURNS et al., 2005; IRELAND et al., 2007; 2008; 2009; 2011; SILVA-SANTOS et al., 2012), which allows to apart females using an easy tool as intravaginal ultrasound. Besides, cows with a low AFC present lower fertility (MOSSA et al., 2012).

Preantral follicle population also presents high variation among females (ERICKSON, 1966; SILVA-SANTOS et al., 2011; 2012). It was observed correlation between the antral follicle population and the number of healthy follicles and oocytes in *Bos taurus* females with high- and low- AFC (IRELAND et al., 2008). However, comparison between different purebreds of cattle with high vs. low AFC has not been made. Therefore, the aim of the present study was to compare the population of preantral follicles in the ovaries of *Bos indicus* (Nelore) and *Bos taurus* (Aberdeen Angus) purebreds females with consistently high vs. low AFC.

Material and Methods

Ovary collection and antral follicular count

Ovaries (n = 200) were collected at abattoirs from 72- to 96-months-old purebred cows (Nelore, *Bos indicus*, n = 100, and Aberdeen Angus, *Bos taurus*, n = 100). Females were kept on cultured pasture and fed mineral salt *ad libitum*. At slaughter, mean body condition was 4 ± 0.5 (scale, 1–5) (LOWMAN et al., 1976). All cattle were carefully evaluated according to body condition and health parameters before the slaughter. Ovaries were transported to the laboratory in saline at 32-35 °C within three hours of collection. Immediately after arrive in the laboratory, ovaries were evaluated by ultrasonography to count follicles ≥ 3 mm (SILVA-SANTOS et al., 2012). Each ovary was scanned with a 7.5-convex intravaginal array transducer (Áquila PRO, Pie medical, Maastricht, The Netherlands) from end to end to count antral follicles ≥ 3 mm. Antral follicle count (AFC - total number of antral follicles ≥ 3 mm in diameter) per pair of ovaries was determined for each animal. Following ultrasound evaluation, females were assigned to two groups according the number of antral follicles ≥ 3 mm: females with a consistently high (G-High) vs. a low AFC (G-Low). The number of follicles per group (G-High vs. G-Low) was defined using the mean number of antral follicles per animal \pm the standard deviation (SD) as follows: Nelore cows of G-High (≥ 57 follicles; n = 8) and G-Low AFC (≤ 21 follicles; n = 8), and Aberdeen Angus cows of G-High (≥ 45 follicles; n = 10) and G-Low AFC (≤ 13 follicles; n = 10). Animals with intermediate AFC (Nelore, 22-56 follicles; Angus, 14-44 follicles) were eliminated from any further studies.

Following antral follicle count, ovaries were cut longitudinally into halves and fixed in Bouin's fixative for 24 h. Ovaries were then placed in 70% alcohol. For estimate of preantral follicles, only ovaries without CL were used, to ensure good histological processing, and only one ovary per female was analyzed (SILVA-SANTOS et al., 2011).

Histological evaluation and preantral follicle classification

Ovarian halves were dehydrated in alcohol, cleared with xylene, embedded in paraffin, and all the tissue was serially sectioned at 7 μ m with a rotating

microtome (Leica[®], Wetzlar, Germany). In all ovaries, every 120° histological sections (CAHIL et al., 1979) was mounted and stained with periodic acid Schiff (PAS) and hematoxylin. All sections were used to evaluate the number of healthy follicles. Preantral follicles were classified according to the developmental stage as primordial (one layer of flattened or flattened-cuboidal granulosa cells surrounding the oocyte), primary (a single layer of cuboidal granulosa cells around the oocyte), or secondary (oocyte surrounded by more than one complete layer cuboidal granulosa cells) (HULSHOF et al., 1994; CARÁMBULA et al., 1999), and as normal or degenerated according to their morphological appearance. Follicles were considered degenerated if they had one or more of the following aspects: condensed oocyte nucleus, shrunken oocyte, pycnotic bodies in the granulosa cells, low cellular density, or basement membrane breakdown. Based on these parameters, only morphologically healthy follicles were evaluated (LUCCHI et al., 2002). Sections were examined and photographed using a light microscope (Nikon[®], Tokyo, Japan). Using an ocular micrometer, average diameters of oocytes were determined by measuring two follicles of each category (primordial, primary, and secondary) per section in which the nucleolus of the oocyte was observed (equatorial section). Each follicle and its associated oocyte were measured in two dimensions, and the arithmetic mean of the two measures was determined. The strategy for considering oocyte nuclei was important to avoid counting the same follicle in two sections. All procedures were performed by the same operator.

Estimate of preantral follicles

The number of preantral follicles was estimated by counting all follicles in each histological section; counting was done by only one operator in a blinded trial. To avoid counting the same follicle twice in the same section, a pen mark was made at the border of the histological section. Evaluation started from this point and followed a clockwise direction until the cortical portion had all been evaluated. Only follicles in which the oocyte nuclei was visible in each histological section were counted. The nucleus of the oocyte was used as a marker, according to the correction factor described (GOUGEON; CHAINY, 1987) and the following formula:

$$Nt = \frac{No \times St \times ts}{So \times do}$$

Nt = Estimated total number of follicle of each category; No = number of follicles observed in the ovary; St = total number of cuts done in the ovary; ts = cutting thickness; So = total number of sections evaluated; and do = mean diameter of the follicle nucleus of each category.

Statistical analysis

Results are presented as means \pm SD. Bioestat 5.0 software (AYRES et al., 2007) was used to test the normality of the samples. Comparison between groups was made with ANOVA. The number of antral follicles was compared using the Kruskal-Wallis test and comparisons between groups using the Dunn test. For all analyses, a 5% level of probability at least was required.

Results

After ultrasound evaluation of 200 ovaries from *Bos indicus* (n = 100) and *Bos taurus* females (n = 100), the average number of antral follicles in *Bos indicus* cows was 63 ± 8 (G-High AFC) and 15 ± 5 (G-Low AFC), and 59 ± 23 (G-High AFC) and 11 ± 3 (G-Low AFC) in *Bos taurus* females (Table 1).

The average number of preantral follicles per ovary was similar ($P > 0.05$) among *Bos indicus* and *Bos taurus* females. There were (mean \pm SD) $48,349 \pm 30,149$ preantral follicles in the ovaries of *Bos indicus* cows with high AFC and $33,037 \pm 31,710$ follicles in *Bos indicus* cows with low AFC, and $35,050 \pm 36,060$ and $30,481 \pm 43,360$ in *Bos taurus* females with high- and low-AFC (Table 1).

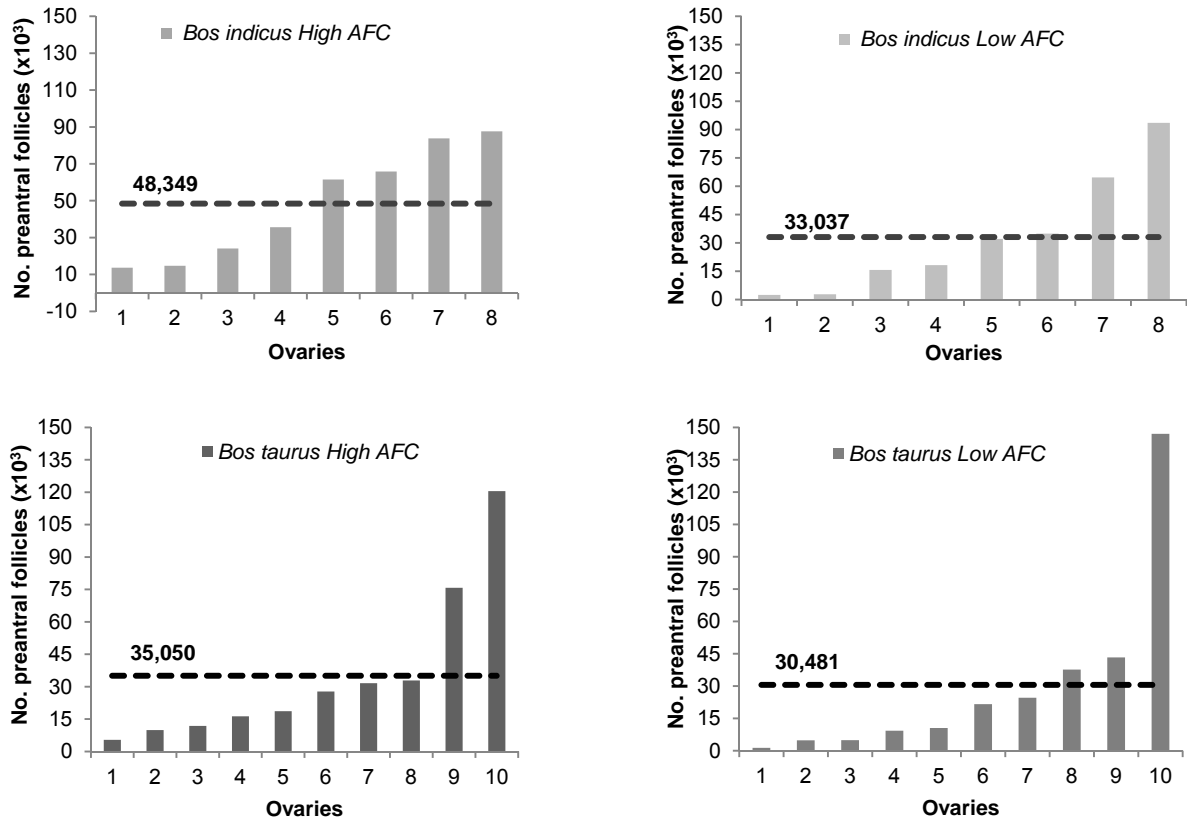
Table 1 – Mean (\pm SD) number of preantral follicles per ovary and antral follicles per female of *Bos indicus* (Nelore) and *Bos taurus* (Aberdeen Angus) cows (72–96 mo) with high (G-High) vs. low (G-Low) AFC.

Groups	No. preantral follicles per ovary				No. antral follicles per female
	Primordial	Primary	Secondary	Total	
<i>Bos indicus</i> G-High AFC (≥ 57 follicles, n = 8)	331,639 ($\pm 22,398$)	12,714 ($\pm 14,341$)	3,996 ($\pm 3,288$)	48,349 ($\pm 30,149$)	63 \pm 8 ^a
<i>Bos indicus</i> G-Low AFC (≤ 21 follicles, n = 8)	18,766 ($\pm 18,493$)	9,116 ($\pm 9,359$)	5,155 ($\pm 6,794$)	33,037 ($\pm 31,710$)	15 \pm 5 ^b
<i>Bos taurus</i> G-High AFC (≥ 45 follicles, n = 10)	18,424 ($\pm 24,338$)	12,383 ($\pm 11,397$)	4,243 ($\pm 2,737$)	35,050 ($\pm 36,060$)	59 \pm 23 ^a
<i>Bos taurus</i> G-Low AFC (≤ 13 follicles, n = 10)	11,273 ($\pm 20,713$)	15,617 ($\pm 21,383$)	3,591 ($\pm 3,200$)	30,481 ($\pm 43,360$)	11 \pm 3 ^b

Within a column, means without a common superscript differ ($P \leq 0.05$).

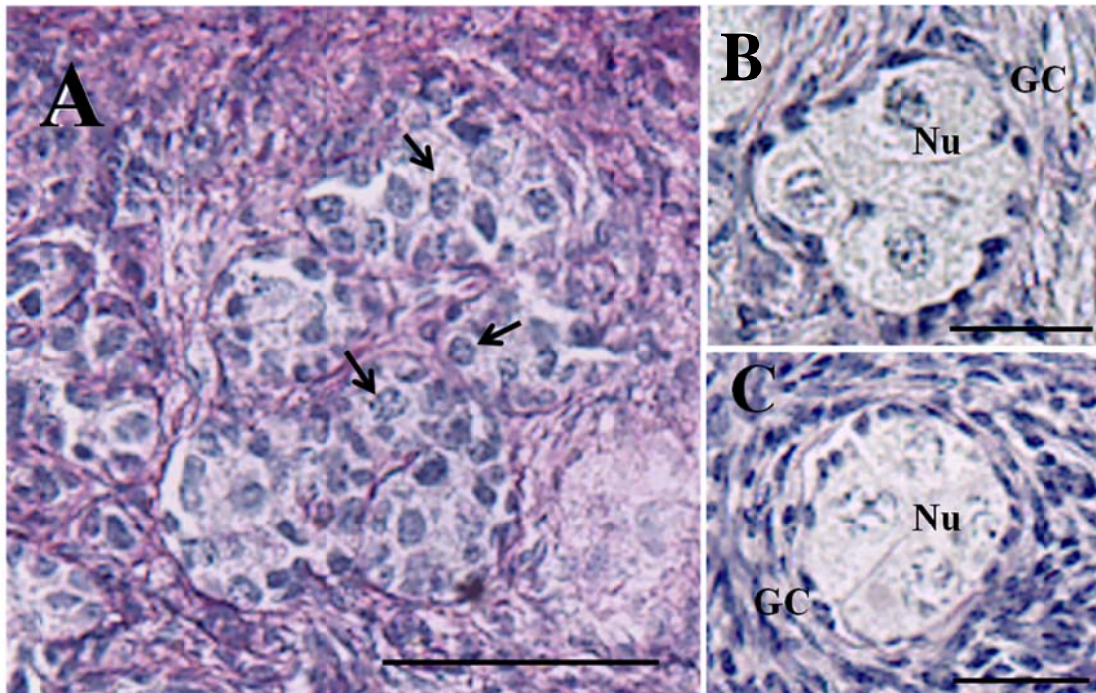
The number of preantral follicles varied among individuals within the same group and between breeds (Figure 1). Variation within Nelore females ranged from 13,660 to 87,638 (G-High) and 2,462 to 93,560 follicles (G-Low). For Angus females, the number of preantral follicles ranged from 5,388 to 120,493 (G-High) and 1,348 to 147,006 follicles (G-Low; Figure 1). For both groups, amongst females that presented the greatest preantral follicle population, a greater number of preantral follicles was observed in females from the low AFC (93,560 in Nelore and 147,006 follicles in Angus) compared to the high AFC groups (87,638 in Nelore and 120,493 follicles in Angus). Besides, in 37.5% of Nelore of G-High AFC the average numbers of preantral follicles was smaller compared to the average population of Nelore of G-Low and 25% of Nelore of G-Low AFC had greatest average numbers of preantral follicles compared to the average population of Nelore of G-High. For Angus cows, 60% of cows of G-High AFC had smallest population of preantral follicles compared to the average numbers of Angus of G-Low and in 20% of Angus of G-Low AFC the population of preantral follicles was greater compared to the average of Angus of G-High (Figure 1).

Figure 1 –Variation in the preantral follicle population among individuals of the same group (G-High vs. G-Low AFC) and between breeds (Nelore – *Bos indicus* vs. Aberdeen Angus – *Bos taurus*).



Among the ovaries assessed (n = 26), 14 (54%) had multiocyte follicles (primordial, primary and secondary). Multiocyte follicles were observed in 25% of *Bos indicus* with high AFC and 50% of *Bos indicus* with low AFC, and in 40% of *Bos taurus* cows with high AFC and 40% of *Bos taurus* with low AFC. The number of oocyte nuclei varied (2-5) within the multiocyte follicles. Ovigerous cords were observed in two *Bos taurus* cows of the high and low AFC groups (Figure 2).

Figure 2 –Histological classification of polyovular follicles and ovigerous cords. Ovigerous cords (A) and multiocyte follicle (B) in the ovary of a *Bos taurus* cow with low AFC and multiocyte follicle in the ovary of a *Bos indicus* cow with low AFC (C). Presumptive nucleus of oogonias (arrows) within the ovigerous-like cords and presumptive nucleus of oocytes (Nu) enclosed within a follicle-like cell with a single layer of granulosa cells (GC). Sections were stained with periodic acid Schiff (PAS) and hematoxylin. Bars = 50 μ m. Original magnification X400.



Discussion

To our knowledge, this is the first comparative study of the population of preantral ovarian follicles between two purebreds (Nelore – *Bos indicus* and Aberdeen Angus – *Bos taurus*), previously selected from a large number of cows based on the count of antral follicles by ultrasonography. Although there is a remarkable variation in the numbers of antral follicles during follicular waves among cattle, repeatability within individuals allows identifying cattle with high or low numbers of antral follicles based on the AFC (BURNS et al., 2005; IRELAND et al., 2007; 2008; 2011; SILVA-SANTOS et al., 2012). Observations that AFC have the same level of accuracy and clinical value as AMH to predict the response in assisted reproduction therapy (ART) in women (HENDRIKS et al., 2005; 2006; BROER et al.,

2009) stimulates its use in bovine females, since it is an easy and cheap tool that could improve field results.

In the present study, the average number of preantral follicles was similar among *Bos indicus* and *Bos taurus* females with high vs. low AFC. As observed in previous studies from our group (SILVA-SANTOS et al., 2011; 2012) preantral follicle population had a great variation between groups and among females within the same group. Conversely, it was observed differences in the numbers of preantral follicles in *Bos taurus* females with high- compared to the low-AFC group (IRELAND et al., 2008). The influence of breed may have accounted for this difference. In the present study, it were evaluated ovaries of purebred cattle genetically selected for several generations, whereas in the previous study it was compared the population of preantral follicles between crossbred beef cattle. *Bos taurus* females within the high-AFC group here presented had ≥ 45 antral follicles versus ≥ 25 antral follicles reported (IRELAND et al., 2008).

There are some studies on the total number of preantral follicles in the ovaries of cattle (ERICKSON, 1966; IRELAND et al., 2008; SILVA-SANTOS et al., 2011), but no one compared the ovarian reserve among two purebred beef cows with remarkably high vs. low AFC selected from a large number of animals. The average number of preantral follicles in the ovaries of *Bos taurus* females with high AFC (35,050 follicles) in the present study was slightly more than that reported for *Bos taurus* cows with high AFC (29,056 follicles) (IRELAND et al., 2008).

With this study we confirmed that population of preantral follicles is similar among *Bos indicus* and *Bos taurus*, and among *Bos indicus* and 1/2 *indicus-taurus* cows, regardless of the numbers of antral follicles in the ovaries (SILVA-SANTOS et al., 2011; 2012). However, *Bos indicus* have more follicular waves (VIANA et al., 2000), more follicles per wave (CARVALHO et al., 2008) and more oocytes recovered by OPU (PONTES et al., 2009; 2010) than *Bos taurus* females. Furthermore, lower numbers of antral follicles are associated with lower reproductive performance and is suggestive of suboptimal fertility (BURNS et al., 2005; IRELAND et al., 2007; 2008; 2009; 2011; JIMENEZ-KRASSEL et al., 2009; MOSSA et al., 2012), but whether or not high numbers of antral follicles per follicular wave are positively associated with fertility remains to be determined. It seems that follicular atresia can account for the differences mentioned above among *Bos indicus* and *Bos taurus* females. Indeed, it had already been suggested the oocyte generation in

postnatal mammalian adult ovaries (JOHNSON et al., 2004; 2005; ZOU et al., 2009) and the epigenetic regulation of histone 3 at lysine 4 (H3K4) in folliculogenesis (SENEDA et al., 2008), as occurs in mammalian spermatogenesis (GODMANN et al., 2007). Finally, lineages of *Bos indicus* and *Bos taurus* females may have influenced our results, although purebred animals, since there was observed great variation within both groups (high and low) and some cows with high AFC had lowest population of preantral follicles compared to the average population of the low AFC group and, some females with low AFC, greatest numbers compared to the average of the low AFC group (Figure 1).

There were observed ovigerous cords and multiocyte follicles in *Bos indicus* and *Bos taurus* cows with high and low AFC (Figure 2). The highest frequency of multiocyte follicles was observed in the ovaries of *Bos indicus* cows with low AFC (50%). Conversely, higher frequencies of multiocyte follicles were observed in *Bos taurus* fetuses, heifers and cows compared to *Bos indicus* cattle at the same age (SILVA-SANTOS et al., 2011) These structures are typically described in fetuses participating in the primordial follicles formation (DINIZ et al., 2005; YANG; FORTUNE et al., 2008; SILVA-SANTOS et al., 2011b). At the moment, it is not well understood the role of the ovigerous cords and multiocyte follicles in the ovaries of adult cows, although they have been reported in cattle (IRELAND et al., 2008; SILVA-SANTOS et al., 2011).

In conclusion, *Bos indicus* and *Bos taurus* females with high and low numbers of antral follicles during follicular waves had no difference regarding the population of preantral follicles. There was variation in the numbers of preantral follicles between groups and among individuals within the same group. Therefore, mechanisms that account for the greater numbers of oocytes recovered by OPU in *Bos indicus* females are yet to be established.

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ARTIGO 3

MALDI-MS Lipid Profiles of Oocytes Recovered by Ovum Pick-up from *Bos indicus* and 1/2 *Indicus-taurus* with High versus Low Oocyte Yield

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Abstract: A greater number of oocytes can be obtained from Nelore (*Bos indicus*) compared to *Bos taurus* donors, but *indicus-taurus* crosses also have good oocyte yield and there is little knowledge on the molecular composition and mechanisms related to these oocyte yield differences. Lipids are known to effect oocyte developmental competence during oocyte maturation. The aim of this study was therefore to compare the lipid profile of Nelore (*Bos indicus*) and 1/2 Nelore x Angus (1/2 *indicus-taurus*) females with high versus low oocyte yield. Females (Nelore, n = 20; 1/2 Nelore X Angus, n = 20) were randomly subjected to follicular aspiration during their estrous cycle (D0) to withdraw all follicles ≥ 3 mm and to induce the growth of a new follicular wave. Ovaries were examined by ultrasonography during days 4, 19, 34, 49 and 64, and antral follicles ≥ 3 mm were counted. After these evaluations, cows were assigned to one of two groups: a) high antral follicle count (AFC) / oocyte yield (mean ≥ 30 follicles; Nelore NH-group, n = 3; 1/2 Nelore x Angus, AH-group, n = 5) and b) low AFC (≤ 15 antral follicles; Nelore, NL-group, n = 4; 1/2 Nelore x Angus, AL-group, n = 4). Following OPU recovery, the lipid profiles of the oocytes were obtained via matrix-assisted desorption/ionization – mass spectrometry (MALDI-MS). Differences on sphingomyelin (SM), phosphatidylcholine (PC) and triacylglycerol (TAG) species in the profiles were revealed by principal component analysis (PCA). COCs from preantral follicles and expanded oocytes presented membrane lipids structural differences compared to G1, GII and GIII quality oocytes. Considering just G1, GII and GIII oocytes, Nelore samples tend to cluster separately from 1/2 Nelore x Angus samples, specially the NH-group, whereas 1/2 Nelore x Angus oocytes (AH- and AL-group) were mixed. The lipid species [PC (P-38:5) + H]⁺ and/or [PC (P-36:2) + Na, [PC (38:2) + H]⁺, [PC (38:5) + Na]⁺ and [TAG (60:8) + NH₄]⁺ were more abundant in Nelore (NH- and NL-group) compared to 1/2 Nelore x Angus (AH- and AL-groups) oocytes. Membrane and lipid droplet structural differences occur therefore between Nelore and 1/2 Nelore x Angus oocytes recovered from females with high versus low oocyte yield and that the greatest amount of lipid content in Nelore oocytes seems to be related to membrane metabolic rate differences and contribute to oocyte competence and embryo development.

Keywords: Lipid content. Oocyte yield. Antral follicle count. Mass spectrometry. Bovine.

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Introduction

Bos indicus animals usually produce greater numbers of oocytes compared to *Bos taurus* donors (MACHADO et al., 2006; PONTES et al., 2009; 2010). Good oocyte yield can be obtained from *Bos indicus* and *Bos taurus* crosses (PONTES et al., 2010). The use of *indicus-taurus* crossbred females in tropical and subtropical areas has been improved stimulated by the better adaptability of these animals to the tropical climate. There is however great variation in the oocyte production within individuals (PONTES et al., 2009; 2011). Although in general it is possible to produce more embryos and pregnancies following OPU/ *in vitro* embryo production (IVP) compared to the superovulation/ embryo transfer (ET) in Nelore cows (Pontes et al., 2009), individual variation is observed in the oocyte production of cows with Nelore background.

Triacylglycerides (TAG) predominate in the lipid content of the cytoplasm of mammalian cells forming lipid droplets (FERGUSON et al., 1999; HULBERT, 2003; AARDEMA et al., 2011; MCKEEGAN; STURMEY, 2011). These lipids are crucial for oocytes and embryos since they serve as a mean to store energy (STURMEY et al., 2009). For eukaryotic cell membranes, phospholipids (PL) are the most abundant lipid and particularly phosphatidylcholines (PC) and sphingomyelins (SM) influence fluidity, permeability, and thermal phase behavior (EDIDIN, 2003).

Immature oocytes show very low mitochondrial activity compared to *in vitro*-matured ones (VAN BLERKOM et al., 2002; TARAZONA et al., 2006) and developmental competence seems not to be influenced by the number of active mitochondria in immature porcine oocytes (CASTANEDA et al., 2013). In oocytes with low developmental competence, early activation of mitochondria are therefore possibly triggered by lipids (CASTANEDA et al., 2013).

Oocyte developmental capacity to undergo maturation, fertilization and early cleavage is influenced by both lipid content and mitochondrial activity (MCEVOY et al., 2000; CUMMINS, 2004). During maturation, lipase activity remains constant (CETICA et al., 2002) whereas triacylglyceride and cholesterol stores are reduced (FERGUSON; LEESE, 1999; KIM et al., 2001) and mitochondrial activity increases (TARAZONA et al., 2006). These trends suggest that lipids are used as a cellular energy source. Also, lipid-organelle interactions probably occur, because

mitochondria, lipid, and smooth endoplasmic reticulum remain in clusters during oocyte maturation (FLEMING; SAACKE, 1972; HYTTEL et al., 1997).

Among the analytical techniques for lipid analysis of mammalian oocytes and preimplantation embryos, mass spectrometry (MS) is emerging as a powerful approach. Using MS, lipid structural information from individual or small pools (2-5) of oocytes from human, bovine, sheep, mouse, canine and feline species as well as individual preimplantation embryos have been obtained from cattle and mouse (FERREIRA et al., 2010; FERREIRA et al., 2012a; 2012b; SUDANO et al., 2012; APPARICIO et al., 2012). The major MS technique used for lipid profiling in oocytes and embryos has been matrix-assisted desorption/ionization (MALDI) (FERREIRA et al., 2010) whereas a more direct approach using an ambient desorption/ionization technique (DESI) has also been recently demonstrated (FERREIRA et al., 2012b). MALDI-MS has been used under conditions which favor the detection of phosphatidylcholine (PC), sphingomyelin (SM) and triacylglycerols (TAG).

In the present study, MALDI-MS lipid profiles of oocytes from *Bos indicus* and 1/2 *indicus-taurus* donors with high versus low oocyte yield were compared in order to establish whether substantial lipid structural differences would occur. We envisage that lipids can become biomarkers for early diagnosis of high oocyte yield animals and can contribute to the understanding of the molecular mechanisms involved in the higher oocyte yield condition.

Materials and Methods

Animals

Nelore females (*Bos indicus*, n = 20) and 1/2 Nelore x Angus (*Bos taurus*, n = 20), 6-8 years-old, body weight 450 ± 10 kg and body condition score, 3.5 ± 0.5 (scale, 1 to 5) (LOWMAN et al. 1976) were subjected to follicular aspiration on random days during the estrous cycle (D0) to promote the ablation of all follicles ≥ 3 mm and the beginning of a new follicular wave. Animals were maintained in *Brachiaria brizantha* pasture supplemented with mineral salt *ad libitum*. Some of them were selected on the basis of the antral follicle count.

Antral follicular counting

Ovaries from each animal were monitored with a 7.5-convex intravaginal array transducer (Áquila PRO, Pie medical, Maastricht, The Netherlands) spaced 15 d apart after OPU (Days 4, 19, 34, 49, and 64) and antral follicles were counted as described previously (BURNS et al., 2005; IRELAND et al., 2008). Each ovary was scanned from end to end to identify positions of the CL and antral follicles ≥ 3 mm, which were drawn on an ovarian map. Each follicle was measured and the diameter was averaged and recorded next to the appropriate follicle on each ovarian map. The antral follicle count (AFC - total number of antral follicles ≥ 3 mm in diameter) per pair of ovaries was determined for each animal. After five ultrasound evaluations performed by the same operator, females were assigned to two groups according the number of antral follicles ≥ 3 mm in diameter: females with a consistently high- (≥ 30 follicles; Nelore, n = 3; 1/2 Nelore x Angus, n = 5) or low-AFC (≤ 15 follicles; Nelore, n = 4; 1/2 Nelore x Angus, n = 4) in all ultrasound scans. Animals with intermediate AFC (>15 and < 45 follicles) were not studied further.

Follicular aspiration and oocyte recovery

Oocytes were obtained by follicular aspiration from Nelore and 1/2 Nelore x Angus females with high- and low-AFC. Briefly, each visible follicle was aspirated using a real-time B-mode ultrasound scanner (Áquila PRO, Pie medical, Maastricht, The Netherlands), 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). Immediately after recovery, aspirated material was washed and filtered through an Emcon embryo filter (Immuno Systems Inc., Spring Valley, WI, USA) with a phosphate buffer solution (PBS-Nutricell, Campinas, SP, Brazil). The cumulus oocyte complexes (COCs) were classified according to the presence of cumulus cells and the oocyte quality using the following

criteria: (i) COCs from preantral follicles, (ii) grade I (GI), (iii) grade II (GII), (iv) grade III (GIII), (v) denuded, (vi) expanded, and 4) atretic oocytes.

Experimental design

From 83 oocytes recovered by OPU, only oocytes classified as GI, GII and GIII (total n = 53; 23 mass spectra) have been considered for the lipid profile and statistical analysis considering breed and oocyte yield comparison. The four experimental groups were defined as follows: oocytes from Nelore cows with high AFC (NH-group; n = 31; 10 mass spectra), Nelore cows with low AFC (NL-group; n = 5, 3 mass spectra), 1/2 Nelore x Angus cows with high AFC (AH-group; n = 12, 6 mass spectra) and 1/2 Nelore x Angus with low AFC (AL-group, n = 5, 4 mass spectra).

Lipid analysis by MALDI-MS

Sample preparation. Each Nelore and 1/2 Nelore x Angus *cumulus* oocyte complexes with *cumulus* cells and homogeneous cytoplasm were denuded of cells by gentle pipetting in 0.5% hyaluronidase. All oocytes were washed three times in drops of a PBS solution with 0.1% of polyvinyl alcohol and stored in microtubes containing 100 μ l of a 50% aqueous methanol solution (ACS/HPLC grade; Burdick and Jackson)/ultrapure water (Millipore) at – 80 °C for transportation and immediate analysis. Sample preparation involved placing each oocyte in a given spot of the target plate under the stereomicroscope. Samples were allowed to dry at room temperature, and their location was recorded in order to place the laser at the correct location during analysis. Just before analysis, 1 μ l of 1.0 mol/l 2,5-dihydroxybenzoic acid (DHB) in methanol was placed in each target spot and allowed to dry at room temperature.

MALDI-MS data acquisition. – Oocytes were washed in ultrapure H₂O/methanol 1:1 (v/v) and placed (1 to 4 oocytes of the same quality/spot) in the MALDI target plate. After drying at room temperature, samples were then covered with 1 μ L of 2,5-DHB 0,5 M dissolved in pure methanol as the MALDI matrix. The MALDI target plate was then immediately placed in a Q-ToF Premier (Synapt HDMS) mass spectrometer (Waters, Manchester, UK) equipped with a 200 Hz solid-state

laser in the m/z 700-1200 range, operated in the reflectron and QTOF modes. Instrument operating conditions were: laser energy 250 a.u., sample plate 20 V. Therefore, unmodified (i.e. no extraction procedure) oocytes have been directly analyzed. All mass spectra were manually collected for approximately 1 min in the positive ion mode. Mass spectra were processed using the software MassLynx 4.1 (Waters Corp. Milford, MA, USA).

Statistical analysis

A list of relative ion abundances of the samples organized in columns was organized using the Excel software. Data was uploaded as a cvs file in the online software metaboAnalyst (XIA et al., 2009; 2011; 2012) as peak intensity table in unpaired columns. Since relative ion abundances (and not absolute ion counts) have been used, no further normalization has been performed. Data has been submitted to partial least squares-discriminant analysis (PLS-DA). This is a supervised clustering method where previous knowledge of the classes is used during the classification process. PLS-DA projects the data into a low-dimensional space that maximizes the separation between different groups of data in few dimensions (called latent variables), which are ranked by how well they explain the variance of the data. PLS-DA in MetaboAnalyst produces the variable importance measures by weighting a sum of squares of the PLS loadings that takes into account the amount of explained variance of each component (XIA; WISHART, 2011). Calculation of average values of lipid ions abundance and standard deviation has been performed using Excel software.

Results

Bovine oocytes recovered by OPU were classified according to their quality in (i) COCs from preantral follicles, (ii) GI, (iii) GII, (iv) GIII, and (v) expanded oocytes. When only information on the oocyte quality regardless of breed and oocyte yield/ antral follicle count were plotted by PLS-DA analysis, preantral and expanded samples were separated from the other groups (Figure 1). COCs from preantral follicles presented the lowest abundance of sphingomyelin [SM (16:0) + Na]⁺ of m/z 725.4; phosphocholine [PC (38:2) + H]⁺ of m/z 814.8; plasmalogen [PC (P-38:5)+ H]⁺

and/or [PC (P-36:2)+ Na]⁺ of *m/z* 792.8; and [PC (38:7)+ H]⁺ and/or [PC (36:4)+ Na]⁺ of *m/z* 804.4; and higher abundances of [PC (32:1)+ Na]⁺ of *m/z* 754.4 (Table 1), whereas the expanded oocytes presented higher abundances of *m/z* 725.4, 814.8, 792.8 and 804.4 and lower abundances of *m/z* 754.5. Oocytes GI, GII and GIII were not distinguishable by their lipid profiles. Figure 2 shows representative MALDI mass spectra. To eliminate interference from oocyte quality for further PLS-DA analysis considering the four experimental groups, only GI, GII and GIII oocytes have been considered.

Figure 1 –Tridimensional PLS-DA score plot of the oocytes collected by OPU to be used in this work (n = 83, 31 mass spectra). The analysis considered the lipid profile according to the oocyte quality, independently of breed or oocyte yield. Expanded oocytes (n = 8, 3 spectra) are represented by red triangles; GI oocytes (n = 10, 4 spectra) by green triangles; GII oocytes (n = 12; 5 spectra) by dark blue triangles; GIII oocytes (n = 31, 14 spectra) by light blue triangles; and COCs from preantral follicles (n = 23; 5 spectra) by pink triangles. Each component indicates the percentage of variability of the data explained.

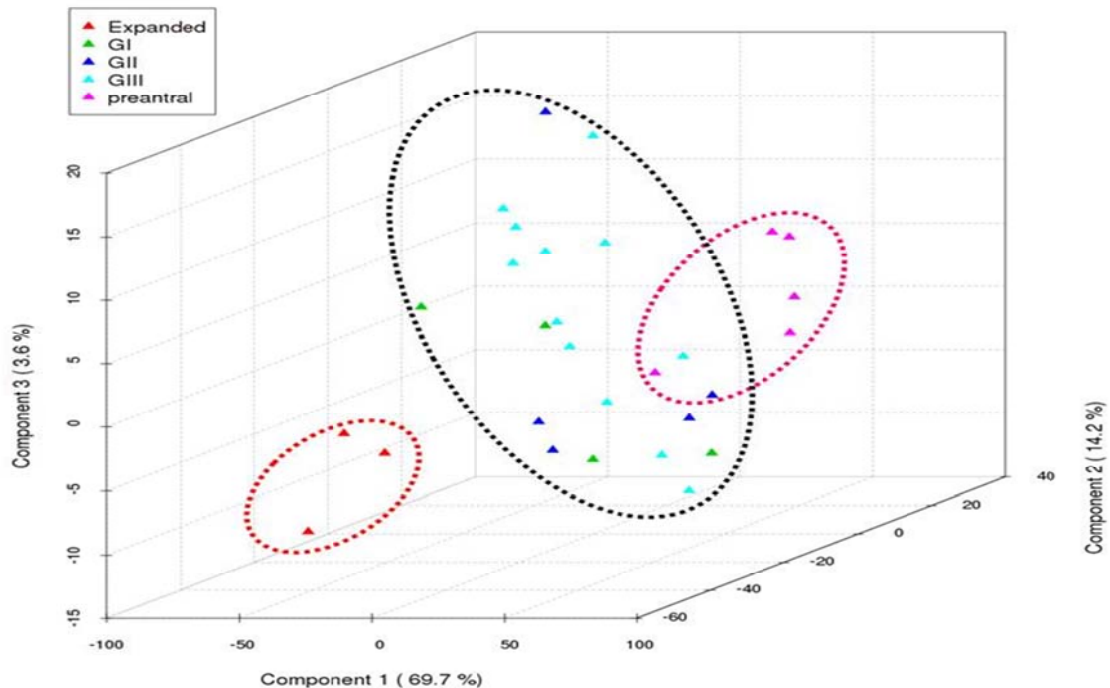


Table 1 – Lipid species presenting the highest PLS-DA scores in the MALDI-MS lipid profiles of bovine oocytes used in this work.

<i>m/z</i>	Attribution [class (number or carbons:unsaturation) + proton/adduct] ⁺
725.4	[SM (16:0) + Na] ^{+*}
754.4	[PC (32:1)+ Na] ^{+*}
792.4	[PC (P-38:5)+ H] ⁺ , [PC (P-36:2)+ Na] ^{+**}
804.4	[PC (38:7)+ H] ⁺ and/or [PC (36:4)+ Na] ^{+*}
814.4	[PC (38:2) + H] ^{+*}
830.8	[PC (38:5) + Na] ^{+**}
990.8	[TAG (60:8) + NH ₄] ^{+**}

*Attribution has been performed based on our previous work using the same methodology of lipid analysis in mammalian oocytes^{5,6}

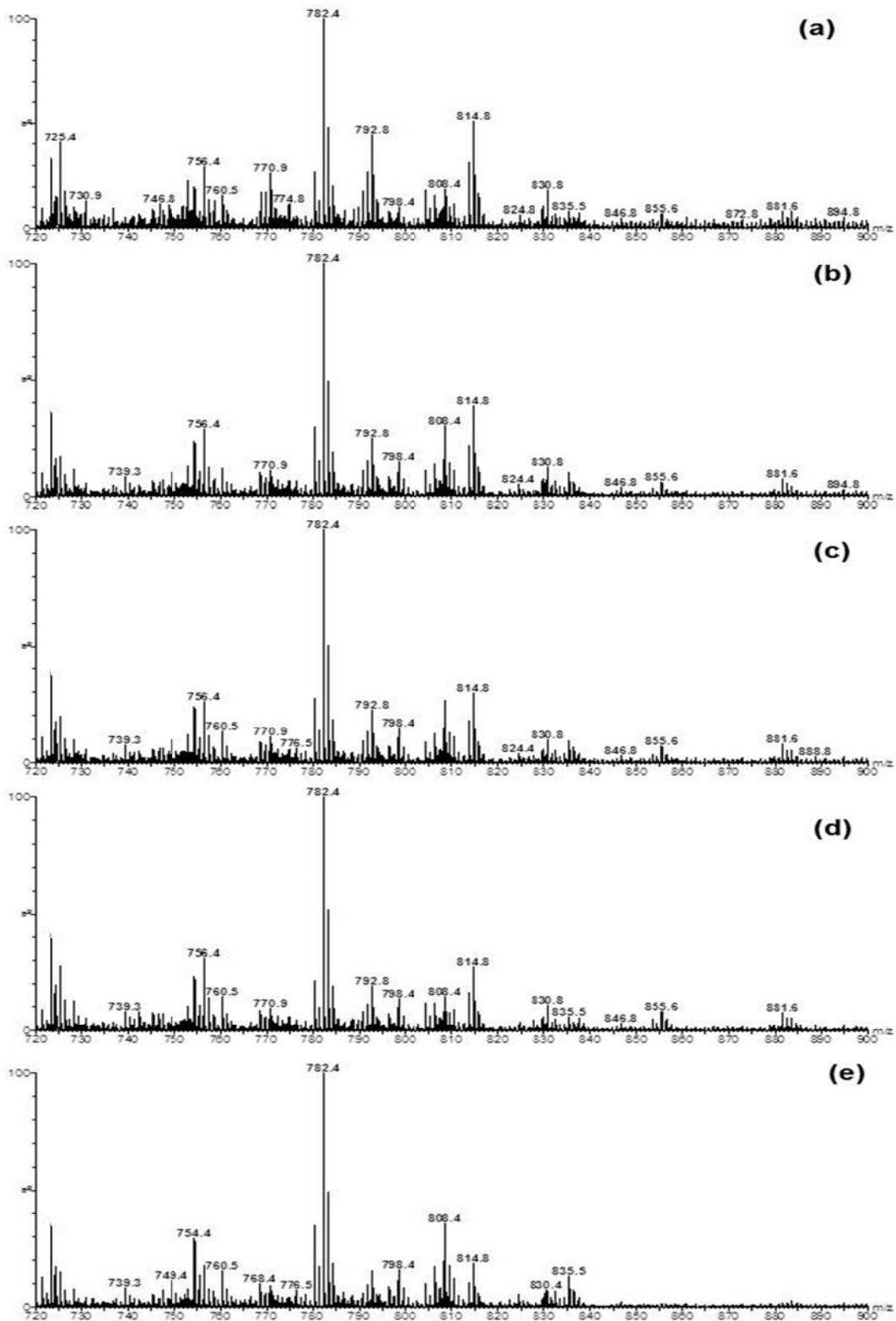
**Attribution has been performed based on LipidMaps (The LIPID MAPS Lipidomics Gateway, <http://www.lipidmaps.org/>) considering the MALDI in the positive ion mode with DHB as organic matrix is highly selective for SM, PC and TAG detection.

An error of up to 0.3 Da was considered acceptable due to the TOF resolution.

Abbreviations used: SM – sphingomyelin; PC – phosphatidylcholine, TAG - triacylglycerol

'P-' prefix is used for the 1Z-alkenyl ether (Plasmalogen) substituent.

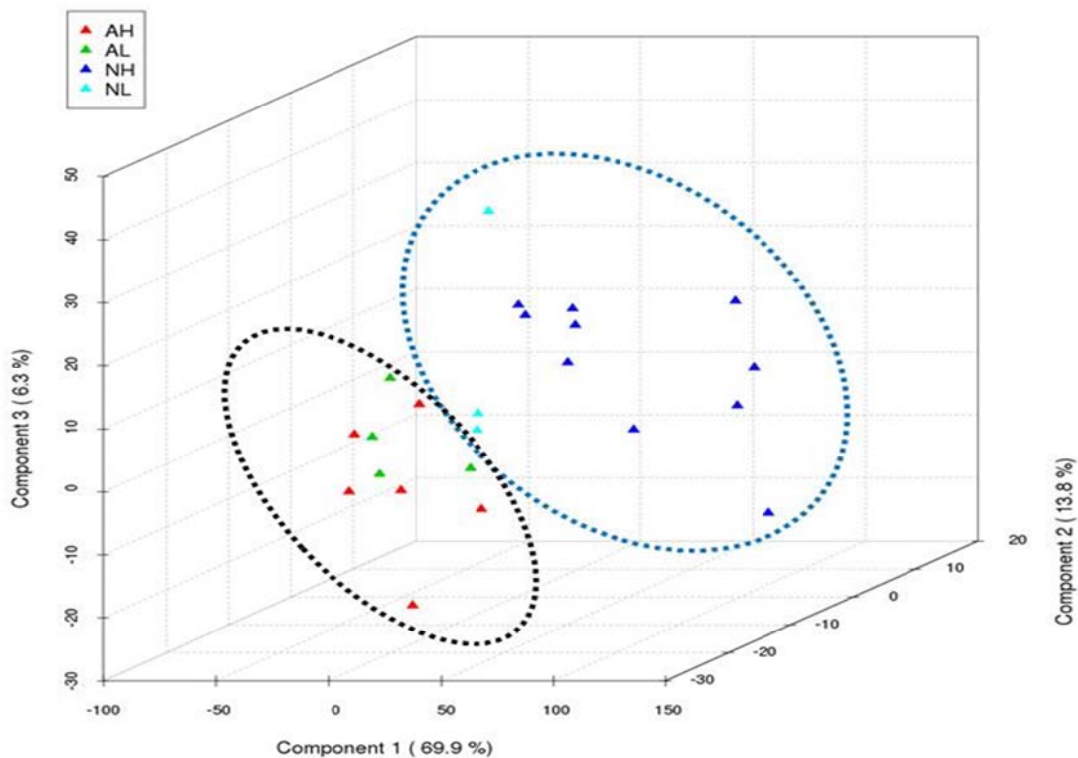
Figure 2 –Representative MALDI-MS of (a) expanded oocytes; (b) GI; (c) GII; (d) GIII oocytes; (e) COCs from preantral follicles.



When considering just GI, GII and GIII oocytes, it is possible to observe in the PLS-DA plot that Nelore samples tend to cluster separately from 1/2

Nelore x Angus samples, specially the NH-group (Figure 3), whereas 1/2 Nelore x Angus oocytes (AH- and AL-group) were mixed. The most important ions that explain this variability are those of m/z 792.8, 814.8, $[\text{PC (38:5)} + \text{Na}]^+$, of m/z 830.8 and $[\text{TAG (60:8)} + \text{NH}_4]^+$ of m/z 990.8 which were more abundant in the NH- and NL-group compared to AH- and AL-groups.

Figure 3 –Tridimensional PLS-DA score plot of the representative oocytes for each of the four experimental groups. 1/2 Nelore x Angus cows with high AFC (AH-group, $n = 12$, 6 mass spectra) are represented by red triangles; 1/2 Nelore x Angus with low AFC (AL-group, $n = 5$, 4 mass spectra) by green triangles; Nelore cows with high AFC (NH-group; $n = 31$; 10 mass spectra) by dark blue triangles; and Nelore cows with low AFC (NL-group; $n = 5$, 3 mass spectra) by light blue triangles. Each component indicates the percentage of variability of the data explained.



The relevance of the four most abundant ions present in Nelore oocytes was further confirmed by plotting the respective relative ion abundances in individual samples (Figure 4). Figure 5 shows representative spectra of the four experimental groups.

Figure 4 –Line graphic showing the relative ion abundances (y-axis) in the lipid mass spectra in each sample of 1/2 Nelore x Angus (AH-group samples: A1 to A6; AL-group samples: A7 to A10), and Nelore (NH-group: N1 to N10; NL-group: N11 to N13).

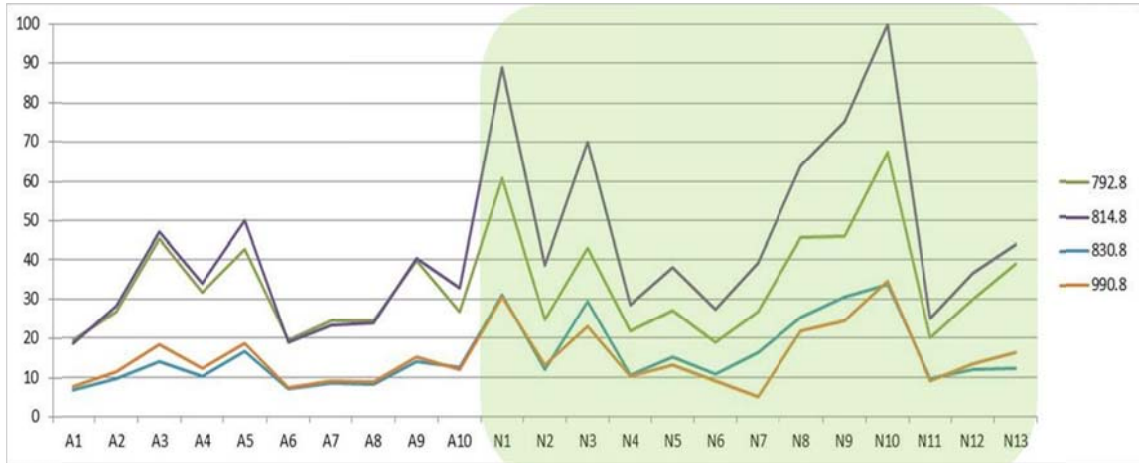
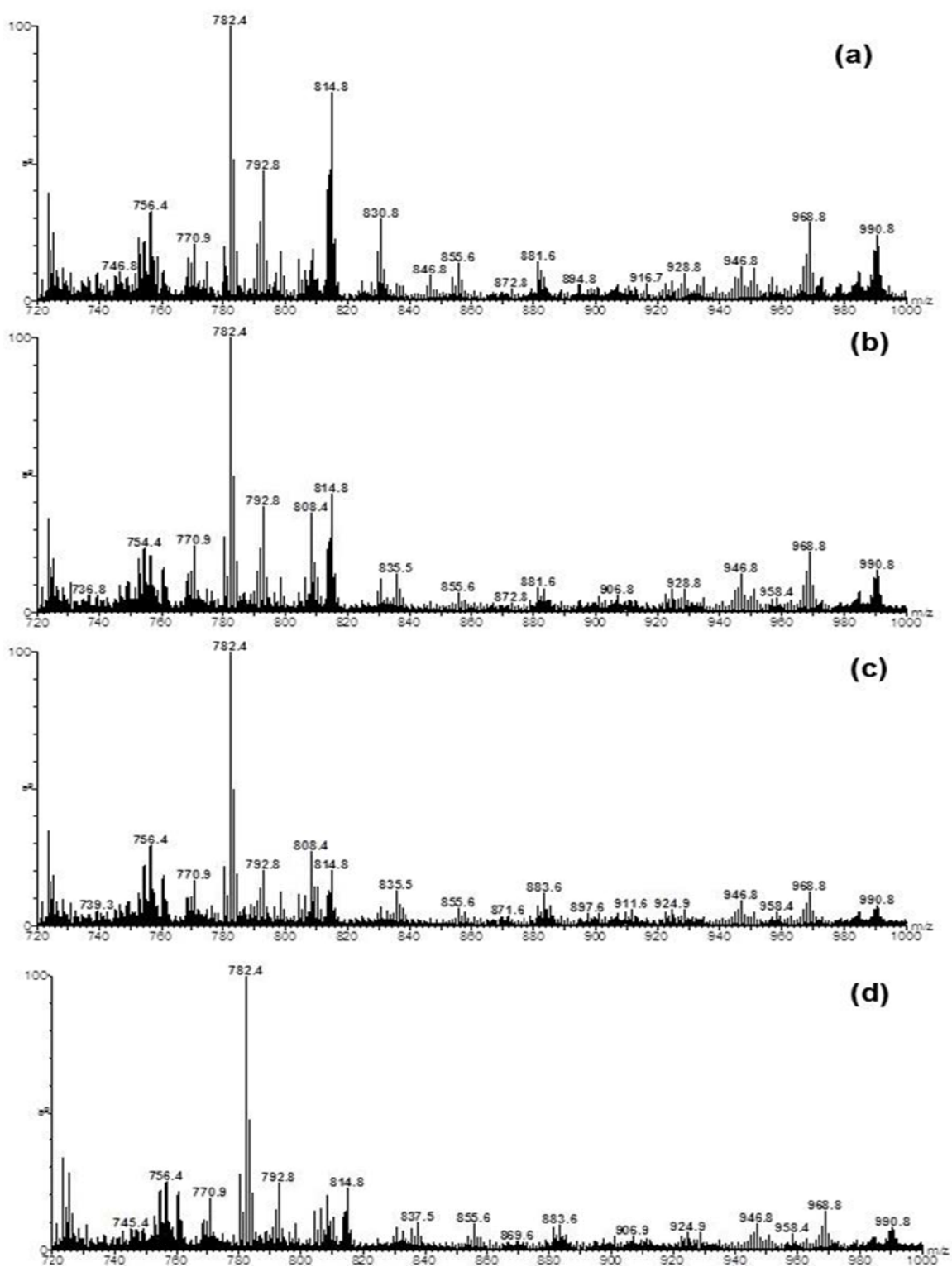


Figure 5 –Representative MALDI-MS of (a) NH-group; (b) NL-group; (c) AH-group; and (d) AL-group.



Discussion

Herein, we report the first comparative study of lipid profile obtained via MALDI-MS of immature oocytes recovered by OPU from *Bos indicus* and 1/2 *indicus-taurus* females with remarkably high versus low antral follicle count/ oocyte yield.

Lipids have been targeted and may represent reliable biomarkers for oocyte development since they play many significant roles such as in energy storage and cell structure by tuning its physical properties (KIM et al., 2001). In the present study, independent of the breed and oocyte yield/ antral follicle count, COCs from preantral follicles and expanded oocytes tended to be separated from the other groups (Figure 1) due to substantial differences in their lipid profiles. COCs from preantral follicles presented lower abundances of sphingomyelin [SM (16:0) + Na]⁺, of *m/z* 725.4; phosphocholine [PC (38:2) + H]⁺, of *m/z* 814.8; plasmalogen [PC (P-38:5)+ H]⁺ and/or [PC (P-36:2)+ Na]⁺ of *m/z* 792.8; and [PC (38:7)+ H]⁺ and/or [PC (36:4)+ Na]⁺ of *m/z* 804.4; and higher abundances of [PC (32:1)+ Na]⁺ of *m/z* 754.4. Conversely, expanded oocytes presented the highest abundance of *m/z* 725.4, 814.8, 792.8 and 804.4 and the lowest abundance of *m/z* 754.5. These changes in lipid molecular design observed in the COCs from preantral follicles could be related to the metabolic status, the presence of less cytoplasmic membrane content or the membrane specialization in oocytes at growing stages, compared to oocytes present in the antral follicles.

Large amounts of intracellular lipids, represented by lipid droplets, are present in the oocyte cytoplasm (KIM et al., 2001). Lipid droplets store neutral lipids such as TAG and are surrounded by a monolayer of phospholipids and associated proteins (BARTZ et al., 2007). Indeed, TAG are the major components of intracellular lipid in the oocytes (HOMA et al., 1986; MCEVOY et al., 1997; 2000). A metabolic role for TAG in the *in vitro* maturation of pig and bovine oocytes have been demonstrated (MCEVOY et al., 2000; FERGUSON; LEESE, 1999), which indicates that oocytes may use lipid as an energy source, providing ATP for the protein synthesis that is necessary for continuation of cytoplasmic maturation and meiosis (KRUIP et al, 1983). During *in vitro* maturation, the TAG content of pig oocytes were found to decrease (STURMEY; LEESE, 2003). Similarly, higher amounts of triglyceride have been measured in immature oocytes compared to *in vitro* matured

ones (FERGUSON; LEESE, 1999; KIM et al., 2001). Conversely, a study has found no lipid variation of bovine oocytes after *in vitro* maturation (AARDEMA et al., 2008).

TAG can also be used as a source of energy during early embryo development (HILLMAN; FLYNN, 1980; WATERMAN; WALL, 1988; KHANDOKER; TSUJII, 1998; SPINDLER et al., 1999; GOMEZ et al., 2001; 2002). It was reported that *in vitro*-produced bovine zygotes were able to cleave in complete absence of exogenous nutrients (FERGUSON; LEESE, 1999). Indeed, substrate oxidation by five- to eight-cell embryos was continuous (FERGUSON; LEESE, 2006), which suggests the use of lipid internal stores. In the present study, the oocytes of Nelore females clustered separately from the oocytes of 1/2 Nelore x Angus, specially the NH-group, when only the oocytes GI, II and III were considered, whereas the 1/2 Nelore x Angus oocytes (AH- and AL-group) were mixed (Figure 3). This difference was due mainly by the greater abundance of four ions, that is, those of m/z 792.8, 814.8, $[\text{PC (38:5) + Na}]^+$, of m/z 830.8 and $[\text{TAG (60:8) + NH}_4]^+$, of m/z 990.8 in the NH- and NL-group compared to AH- and AL-groups. These findings suggest different metabolic rates among Nelore and 1/2 Nelore x Angus oocytes and that Nelore females with high AFC/ oocyte yield have an energy store advantage over 1/2 Nelore x Angus donors with high and low oocyte yield. These PC lipid species may be associated with the lipid droplets and other cellular membranes and have an active role in the metabolism of stored lipids during oocyte maturation.

A recent study (PONTES et al., 2011) showed that oocytes recovered by OPU from Nelore donors with high oocyte production produced significantly more embryos and more pregnancies compared to females with low oocyte production. The greater abundance of the four lipid ions observed in the present study could therefore be related to these differences among Nelore donors with high vs. low oocyte yield reported by Pontes et al. (2011). However, a great variation in lipid droplet number among oocytes from the same ovary has been observed (AARDEMA et al., 2008).

Since PC are lipids involved in the bilayer membrane structure, the present results indicate membrane structural differences among Nelore and 1/2 Nelore x Angus oocytes from females with high versus low AFC/ oocyte yield. The physiological significance of the greatest abundance of the four lipid species in Nelore oocytes compared to 1/2 Nelore x Angus could be related to membrane metabolic rate differences and contribute to oocyte competence and embryo

development. Results also suggest that expanded oocytes may have lipid metabolic changes related to pre-ovulation, and this intriguing possibility will be further investigated by MALDI-MS using a larger set of samples.

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6 DISCUSSÃO GERAL

Nos últimos anos, muitos avanços ocorreram na área da foliculogênese em mamíferos. Em especial, destaca-se a obtenção de embrião a partir do cultivo *in vitro* de folículos pré-antrais, o que representa grande avanço, com possibilidade de maior aproveitamento de oócitos inclusos em folículos pré-antrais através da criopreservação e também, futuramente, na produção de embriões em larga escala.

O Brasil apresenta-se em destaque no cenário mundial de produção de embriões, principalmente, produzidos *in vitro*. A liderança na produção *in vitro* nos últimos anos certamente está relacionada à quantidade de laboratórios de PIV no Brasil, bem como à capacidade técnica dos profissionais envolvidos no processo; à quantidade de fêmeas bovinas no rebanho brasileiro, composto em sua maioria por animais *Bos indicus*; e também pela elevada quantidade de oócitos obtidos naturalmente de fêmeas *Bos indicus* em procedimentos de OPU comparado a fêmeas *Bos taurus*. O potencial produtivo de fêmeas *Bos indicus* certamente seria ampliado com a compreensão dos fatores que contribuem para esta diferença na produção de oócitos entre fêmeas *Bos indicus* e *Bos taurus*. Considerando que a resposta para esta questão seria a quantidade de folículos ovarianos pré-antrais, nosso grupo desenvolveu trabalho comparando a população de folículos pré-antrais entre fêmeas *Bos indicus* e *Bos taurus*. Entretanto, constatamos grande variabilidade individual e nenhuma diferença entre os grupos.

Alguns trabalhos com fêmeas *Bos taurus* mostraram haver herdabilidade e repetibilidade no número de folículos antrais por onda de crescimento folicular. Ainda, fêmeas *Bos taurus* cruzadas com alta CFA apresentaram maior quantidade de folículos pré-antrais comparado a fêmeas com baixa CFA. A baixa quantidade de folículos antrais está relacionada com subfertilidade e parâmetros de baixa produtividade em fêmeas bovinas. O hormônio anti-Mülleriano (AMH) possui correlação com a quantidade de folículos antrais e poderia ser utilizado como critério de separação entre fêmeas com alta e baixa CFA. Em mulheres, mostrou-se haver alta acurácia e valor clínico entre o AMH e a CFA na resposta a tratamentos superovulatórios, o que apresenta grande importância clínica na reprodução assistida.

No Artigo 1, objetivamos comparar a produção de embriões *in vivo* e *in vitro* entre fêmeas *indicus-taurus* com alta e baixa CFA, após avaliação dos ovários por ultrassonografia do desmame ao sobreano. Com os resultados obtidos, podemos afirmar que fêmeas de alta CFA apresentaram melhor desempenho reprodutivo, independente do método utilizado (OPU/ PIV vs. SOV/ coleta de embrião). Ainda, com este trabalho, confirmamos o padrão de repetibilidade da população de folículos antrais presentes no ovário. Além disso, confirmamos a possibilidade de estimativa prévia da resposta da doadora através da avaliação da população de folículos antrais (CFA), questão de extremo interesse já que permitiria ampliar o potencial produtivo utilizando uma ferramenta extremamente simples, rápida e de baixo custo.

No Artigo 2, objetivamos comparar a quantidade de folículos ovarianos pré-antrais entre fêmeas *Bos indicus* e *Bos taurus* puras com alta e baixa contagem de folículos antrais por onda de crescimento folicular. Neste trabalho, não houve diferença entre fêmeas *Bos indicus* e *Bos taurus* dos grupos de alta e baixa CFA, nem entre fêmeas do mesmo tipo racial. Em conjunto com estudo prévio conduzido pelo nosso grupo, os resultados mostram que a população de folículos pré-antrais entre fêmeas *Bos indicus* e *Bos taurus* não difere, independente da raça e da população de folículos antrais.

Por fim, no Artigo 3, avaliamos se o teor de lipídios de oócitos de fêmeas *Bos indicus* e *1/2 indicus-taurus* com alta e baixa CFA poderia contribuir para a quantidade de oócitos recuperada por OPU. Verificamos que fêmeas *Bos indicus* dos grupos de alta e baixa CFA apresentaram maior quantidade de quatro lipídios estruturais em relação a fêmeas *1/2 indicus-taurus* dos grupos de alta e baixa CFA, o que sugere diferentes taxa de metabolismo de membrana entre os grupos e, conseqüentemente, possível influência na competência oocitária e desenvolvimento embrionário.

Finalmente, apresentamos comparações envolvendo população de folículos pré-antrais, teor de lipídios de oócitos e produção de embriões entre fêmeas bovinas com alta e baixa quantidade de folículos antrais, cujos resultados contribuem para a utilização de fêmeas de alta CFA como doadoras de oócitos e embriões. Entretanto, a diferença em relação à quantidade de oócitos ainda deve ser esclarecida e, provavelmente, está relacionada com mecanismos moleculares. Ainda, algumas questões devem ser consideradas antes da utilização da CFA como

seleção de doadoras bovinas: (i) fertilidade de fêmeas de alta CFA, (ii) acurácia da CFA em relação ao AMH, e (iii) impacto da seleção de fêmeas de alta CFA.

7 CONCLUSÕES

Os resultados obtidos nos permitem concluir que:

- Fêmeas *indicus-taurus* (3/8 Nelore x 5/8 Hereford) avaliadas do desmame ao sobreano apresentaram repetibilidade no número de folículos antrais presentes nos ovários. Fêmeas com alta CFA apresentaram maior produção de embrião nos métodos *in vivo* e *in vitro* comparado a fêmeas com baixa CFA, o que sugere que a avaliação da população de folículos antrais poderia ser um bom indicador para prever a resposta da doadora em programas de produção de embrião em fêmeas bovinas;
- Fêmeas *Bos indicus* (Nelore) e *Bos taurus* (Angus) com alta e baixa CFA durante as ondas de crescimento folicular não apresentaram diferença entre a população de folículos pré-antrais ovarianos e o número de folículos pré-antrais variou entre os grupos e entre fêmeas do mesmo grupo;
- Oócitos recuperados por OPU de fêmeas *Bos indicus* (Nelore) com alta e baixa CFA apresentaram maior quantidade de quatro íons lipídicos estruturais comparado a oócitos provenientes de fêmeas 1/2 *indicus-taurus* (Nelore x Angus) com alta e baixa CFA, sugerindo haver diferentes taxas de metabolismo de membrana entre estes tipos raciais, o que poderia contribuir para a competência oocitária e o desenvolvimento embrionário em fêmeas *Bos indicus*.