UNIVERSIDADE ESTADUAL PAULISTA
"JÚLIO DE MESQUITA FILHO"
FACULDADE DE MEDICINA VETERINÁRIA E ZOOTECNIA – BOTUCATU

# CARACTERIZAÇÃO E CONTROLE DA POPULAÇÃO DE OÓCITOS EM BOVINOS NELORE BASEADOS NA CONFIGURAÇÃO DA CROMATINA

JHESSICA NAOMI SAKODA

BOTUCATU – SP
SETEMBRO – 2018

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JHESSICA NAOMI SAKODA

Dissertação apresentada à Faculdade de Medicina Veterinária e Zootecnia da Universidade Estadual Paulista, Campus de Botucatu, para obtenção do título de Mestre na área de Biotecnologia Animal.

Orientador: Prof. Dr. José Buratini Júnior.

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1. Folículo ovariano. 2. Cromatina. 3. Hormônio folículo-estimulante. 4. Nelore (Zebu). 5. Oócitos. 6. Células germinativas.

Palavras-chave: FSH; cromatina; folículo; gado Nelore; vesícula germinativa.

Jhessica Naomi Sakoda

Botucatu, 28 de Setembro de 2018

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## LISTA DE ABREVIATURAS

AFC – do inglês, *antral follicles count*

AMPc – Adenosina monofosfato cíclico

AREG - Ampirregulina

CIV – Cultivo *in vitro*

COC – Complexo cumulus-oócito

$E_2$ – Estradiol

EGF – Fator de crescimento epidermal

EGFR - Receptor do fator de crescimento epidermal

EREG – Epirregulina

FIV – Fertilização *in vitro*

FSH – Hormônio folículo estimulante

GMPc – Guanosina monofosfato cíclico

GnRH – do inglês *Gonadotropin-Releasing Hormone*

GV = vesícula germinativa

GVBD – Vesícula germinativa em quebra (do inglês, *germinal vesicle breaking down*)

LH – Hormônio luteinizante

LHR – Receptor do hormônio luteinizante

MIV – Maturação *in vitro*

MPF – Fatores promotores da maturação

NPPC – do inglês, *natriuretic peptide precursor C*

NPR2 – do inglês, *natriuretic peptide receptor 2*

OPU – do inglês – *ovarium pick up*

$P_4$ – Progesterona

PDE3A – Fosfodiesterase 3A

PIV – Produção *in vitro*

RNAm – RNA mensageiro

TZP – Projeção citoplamática trans-zonal

**SUMÁRIO**

## RESUMO

SAKODA, JN. **Caracterização e controle da população de oócitos em bovinos Nelore baseados na configuração da cromatina.** Botucatu, 2018. p.50. Dissertação (Mestrado) – Faculdade de Medicina Veterinária e Zootecnia – Universidade Estadual Paulista - UNESP.

Na produção *in vitro* (IVP), trabalha-se com uma população de oócitos heterogênea em relação ao estágio da maturação nuclear que estes oócitos se encontram, mais especificamente o estágio de vesícula germinativa (GV), uma vez que estes são obtidos de folículos em diferentes estágios de desenvolvimento. Visto que essa heterogeneidade impacta nos resultados da IVP, torna-se necessário que os processos de seleção de oócitos e de maturação *in vitro* sejam adequados e articulados, para que ocorra o desenvolvimento da competência oocitária para subsequente desenvolvimento. Neste estudo, objetivou-se avaliar a população de ovócitos obtida de folículos antrais grandes, testando a hipótese de que folículos dominantes saudáveis conteriam oócitos com grau intermediário de compactação da cromatina (oócitos em GV2). Em seguida, avaliou-se a população de oócitos obtida em dia aleatório do ciclo estral após OPU e testou-se o efeito de protocolo de sincronização combinando aspiração de folículos e tratamento com FSH para homogeneizar a população e controlar a qualidade dos oócitos. Os resultados sugerem que folículos dominantes saudáveis são predominantemente compostos por oócitos com níveis intermediários de compactação da cromatina e que protocolos de sincronização de aspiração do folículo combinadas ao tratamento com FSH podem ser úteis para controlar a qualidade do oócito para OPU / IVP.



**Palavras Chave**: folículo, vesícula germinativa, cromatina, FSH, gado Nelore

## ABSTRACT

SAKODA, JN. **Characterization and control of the oocyte population in Nellore cattle based on chromatin configuration.** Botucatu, 2018. p.50. Thesis (Master degree) - Faculdade de Medicina Veterinária e Zootecnia – Universidade Estadual Paulista - UNESP.

*In vitro* production (IVP), a heterogeneous oocyte population is employed in relation to the stage of nuclear maturation that these oocytes are found, more specifically the germinal vesicle (GV) stage, once they are obtained from follicles in different stages of development. Since this heterogeneity impacts the results of IVP, it is necessary that the processes of oocyte selection and *in vitro* maturation are adequate and articulated, so that occurs development of oocyte competence for subsequent development. The objective of this study was to evaluate the oocyte population obtained from large antral follicles, testing the hypothesis that healthy dominant follicles would contain oocytes with an intermediate degree of chromatin compaction (GV2 oocytes). Then we evaluated the population of oocytes obtained at random day of the estrous cycle after OPU and tested the synchronization protocol combining follicle aspiration and FSH treatment to homogenize the population and control the quality of oocytes. The results suggest that healthy dominant follicles are predominantly composed of oocytes with intermediate levels of chromatin compaction and that follicle aspiration synchronization protocols combined with FSH treatment may be useful to control oocyte quality for OPU / IVP.



**Keywords:** follicle, germinal vesicle, chromatin, FSH, Nellore cattle.

# CAPÍTULO I

## 1. INTRODUÇÃO

A população de embriões produzidos *in vitro* (IVP) vem aumentando em relação aos embriões derivados da produção *in vivo* nos últimos 20 anos (Perry, 2016), apesar de estar classificado apenas em 11º, com base no índice de intensidade de uso de tecnologias de embriões, o Brasil é líder mundial na produção *in vitro* de embriões bovinos (Viana *et al.,* 2017) pois apresenta o maior rebanho comercial e sua composição por raças zebuínas que apresentam maior contagem de folículos antrais (AFC) e consequentemente maiores taxas de produção embrionária maiores (Santos *et al.,* 2016).

Porém, quando comparados à produção *in vivo* de embriões, os índices produtivos da IVP apresentam-se insatisfatórios, no qual apenas um terço do oócitos aspirados para produção *in vitro* alcançam o estágio de blastocisto (Lonergan e Fair, 2008; Galli, 2017), sendo essa população de oócitos heterogênea em relação ao estágio da maturação nuclear que estes se encontram, uma vez que serão recuperados pela técnica da OPU (*ovum pick up*) de todos os folículos visíveis ao ultrassom. Esses oócitos passarão pelas mesmas condições na maturação in vitro (MIV), fecundação in vitro (FIV) e cultivo in vitro (CIV), quando na verdade, a necessidade do oócito em estágios de desenvolvimento diferente deve ser modulado de acordo com o estágio em que se encontra para que consiga adquirir a competência ao desenvolvimento (Dieci, *et al.,* 2016).

A aquisição de competência pelos oócitos é afetada por fatores endócrinos, parácrinos e autócrinos que alteram a configuração da cromatina na vesícula germinativa, correlacionada à capacidade transcricional oocitária e à comunicação cumulus-oócito (Lodde *et al.,* 2007; Lodde *et al.,* 2008, revisado por Gilchrist, 2011), o que ocorre durante a oogênese e foliculogênese. Quando removido de seu ambiente folicular, a retomada da meiose ocorrerá de forma espontânea, comprometendo a aquisição da competência ao desenvolvimento, uma vez que a sincronização entre as maturações nuclear, citoplasmática e molecular, se faz necessária (Eppig *et al*., 2001). Sendo assim, é importante que os sistemas de cultivo mimetizem as condições fisiológicas (Li *et al*., 2008).

Durante a foliculogênese, o aumento dos níveis circulantes de FSH é o principal responsável pelo recrutamento de um novo grupo de folículos antrais

para a próxima onda folicular (Aerts e Bols, 2010), bem como pelo seu crescimento e desenvolvimento (Adams *et al*., 1992) até que atinja a dominância. A atividade esteroidogênica em folículos antrais em crescimento é caracterizada por elevados níveis de estradiol ($E_2$) devido a alto expressão de RNAm aromatase (Bao *et al.,* 1997a) e na fase final de crescimento e maturação do folículo dominante durante a primeira onda folicular de crescimento ocorrerá um aumento de progesterona com o início da regressão folicular (Bao *et al.,* 1997b)

Diante do exposto e da escassa literatura atual sobre a relação entre o microambiente folicular e os estágios de GV de oócitos bovinos. O objetivo do presente estudo foi caracterizar a maturação nuclear oocitária durante o desenvolvimento de folículos antrais em vacas e novilhas da raça Nelore avaliando a relação dos parâmetros do diâmetro folicular, estágio de maturação nuclear e da proporção estradiol:progesterona ($E_2$:$P_4$). Além disso, avaliou-se os efeitos de protocolos de sincronização e estimulação do crescimento folicular utilizando aspiração folicular e o uso de FSH para a homogeinização da população oocitária e obtenção de oócitos e fase de maturação nuclear adequada para a PIV.

## 2. REVISÃO DE LITERATURA

### 2.1. Dinâmica folicular

O desenvolvimento folicular de bovinos ocorre em um padrão de ondas. O número de ondas de crescimento folicular em bovinos varia de duas a três durante o ciclo estral (Savio *et al*., 1988; Ginther *et al.,* 1996), sendo que vacas Nelore apresentam, em sua maioria, 2 ondas enquanto que novilhas apresentam predominantemente 3 ondas de crescimento folicular (Figueiredo *et al.,* 1997). Cada onda folicular compreende a emergência folicular, onde um grupo de pequenos folículos antrais são recrutados e iniciam a fase de crescimento por cerca de 3 dias (Ginther *et al.,* 2003), dos quais apenas o folículo dominante continuará a crescer até o estágio de folículo pré-ovulatório, enquanto os demais iniciam atresia folicular e são classificados como folículos subordinados (Lucy *et al.,* 1992).

Sob estímulos de elevados níveis de progesterona, a onde pré-ovulatória de LH não ocorre (efeito do *feedback* negativo de progestais sob a liberação de GnRH) e mesmo os folículos que atingiram a dominância no meio do ciclo estral, não ovulam e iniciam o processo de atresia, resultando no início de uma nova onda de crescimento folicular (Ginther *et al*., 1989; Webb *et al*., 1999). Quando o folículo dominante se desenvolve durante a última onda de crescimento do ciclo estral, a luteólise e a consequente queda dos níveis de progesterona permitem que ele se diferencie em folículo pré-ovulatório e ovule (Lucy *et al*., 1992; Fortune *et al*., 2004).

O aumento das concentrações plasmáticas de FSH (hormônio folículo estimulante) estimula o crescimento e proliferação celular nos folículos, aumentando gradualmente sua capacidade de síntese de estradiol ($E_2$; Adams *et al*., 1992; Bao *et al.* 1997a). Consequentemente, o FSH é o principal responsável pelo recrutamento de um novo grupo de folículos antrais para a próxima onda folicular (Aerts e Bols, 2010). Através do aumento da concentração de $E_2$ e inibina, com a formação do folículo dominante, os níveis circulantes de FSH diminuem em resposta ao *feedback* negativo da inibina interrompendo o crescimento dos folículos subordinados que involuem (Ginther *et al.,* 1996; Mihm e Bleach, 2003).

A continuidade do desenvolvimento do maior folículo enquanto que os demais iniciam regressão (Ginther *et al*., 1996; 2001) caracteriza o que chamamos de divergência folicular. Em novilhas da raça Nelore (*Bos indicus*), a divergência folicular ocorre no período de 2,5 a 2,7 dias após a emergência folicular (revisado por Baruselli, 2007; Gimenes *et al*., 2005; Sartorelli *et al*., 2005; Castilho *et al*., 2006) e seu folículo dominante pode alcançar o diâmetro ao redor de 12 mm (Figueiredo *et al*, 1997).

### 2.1.1. Folículo Antral

Os folículos antrais são caracterizados pela formação do antro preenchido pelo fluído folicular entre o COC e as células da granulosa murais (Greenwald e Roy, 1994; Callejas, 2001).

A comunicação entre o oócito e as células do cumulus ocorre por projeções citoplasmáticas trans-zonais (TZP), que são extensões das células do cumulus que penetram através da zona pelúcida e atingem o oócito, permitindo o transporte bidirecional de substratos e moléculas através de junções do tipo gap (Albertini *et al.,* 2001). Além das TZPs, a comunicação entre o oócito e células adjacentes ocorre também via sinalização parácrina intrafolicular.

O folículo dominante apresenta maior nível de estradiol e receptores de LH (LHR; Ginther *et al.,* 1996). Os folículos respondem ao estímulo do pico ovulatório de LH mudando a produção de esteroides pelas células da granulosa. O ambiente intrafolicular, anteriormente rico em estradiol, passa a ter predominância da progesterona. Simultaneamente, a produção de ácido hialurônico é ativada, levando à mucificação e expansão das células do cumulus, há retração das TZPs e descontinuidade das junções gap entre estas células do cumulus e o oócito, o que desencadeia a retomada da meiose oocitária (Picton *et al*., 1998, Lucciano *et al.,* 2011; Lodde *et al.,* 2013).

## 2.2. Maturação do complexo cumulus-oócito

A maturação oocitária envolve diversas fase e eventos complexos que permitem a aquisição de competência para sustentar os estágios subsequentes de desenvolvimento embrionário pré-implantação. Para que o oócito adquira

competência para gerar um embrião, é necessário que os eventos nucleares, citoplasmáticos e moleculares ocorram de forma adequada e sincrônica (Eppig *et al*., 2001).

Na maturação nuclear, o oócito que se encontra na prófase I durante a oogênese, apresenta quebra da vesícula germinativa (GVBD – "*germinal vesicle breakdown*") após estímulos gonadotróficos (FSH e LH – hormônio luteinizante). Durante a fase final do crescimento folicular antral, a cromatina se condensa gradativamente e com a GVBD o primeiro corpúsculo polar é extruso, o segundo fuso meiótico se forma (Meinecke *et al*., 2001; Watson, 2007), e o oócito permanece em metáfase II até a fecundação (Van Den Hurk and Zhao, 2005).

A maturação citoplasmática é caracterizada pela redistribuição das organelas intracelulares (Stojkovic *et al*., 2001; Watson, 2007), pela síntese e acúmulo de RNA e de proteínas (Sirard *et al*., 1998; Fair *et al*., 1995; Hamatani, *et al*., 2008) e modificações moleculares (Kubelka *et al.,* 2000). Comparações entre os perfis transcricionais de oócitos bovinos maturados *in vitro* e *in vivo* indicam diferenças que podem afetar o desenvolvimento embrionário (Katz-Jaffe *et al*., 2009).

Em *Bos taurus*, a morfologia da cromatina de oócitos coletados de folículos antrais jovens (diâmetro entre 0,5 e 2 mm) e folículos antrais médios (diâmetro entre 2 e 6 mm) apresenta quatro estágios independentes em função do grau de compactação da cromatina na GV, sendo classificados em GV0, GV1, GV2 e GV3 (Fig.1; Lodde et al., 2007; Lodde *et al.,* 2008).

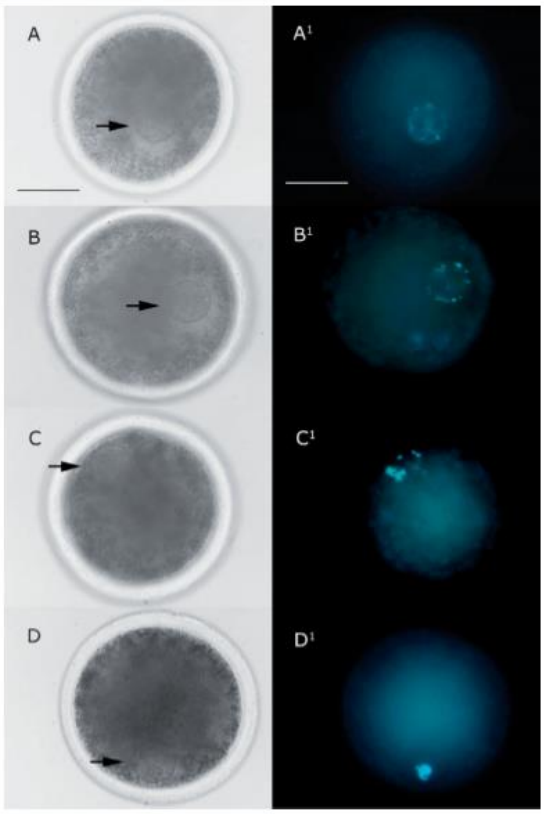


Figura 1. (a) Campo luminoso e imagem fluorescente depois da coloração com Hoechst 33342 de oócitos bovinos com configurações GV0 (A,A1), GV1 (B,B1), GV2 (C,C1), GV3 (D,D1). Setas nos campos luminosos indicam o envelope nuclear. Escala: 50mm (Lodde *et al*., 2007).

A fase GV0 é caracterizada por um padrão de cromatina difusa e filamentosa em toda a área do núcleo, que representa a grande maioria dos oócitos em folículos antrais iniciais, mas está ausente em folículos antrais médios. Os oócitos em estágio GV0 são transcricionalmente ativos e incapazes de progredir até o estágio de metáfase II da divisão meiótica. De acordo com Lodde e colaboradores (2007; 2008), no estágio GV1 a cromatina apresenta alguns focos de condensação, o que representa o estabelecimento de um estado transcricional moderado (Fig. 2) e, apesar da competência meiótica completa, o oócito apresenta uma capacidade limitada para completar o desenvolvimento pré-implantação após fecundação *in vitro* (FIV).

Em contrapartida, os estágios GV2 e GV3 apresentam maior competência para o desenvolvimento, devido à maior carga de transcritos adquiridos durante a maturação nuclear. Em GV2, observa-se aglomerados distintos de cromatina condensada, enquanto que em GV3 o nível máximo de condensação é

alcançado com a cromatina organizada em um único aglomerado (Lodde *et al*., 2007).

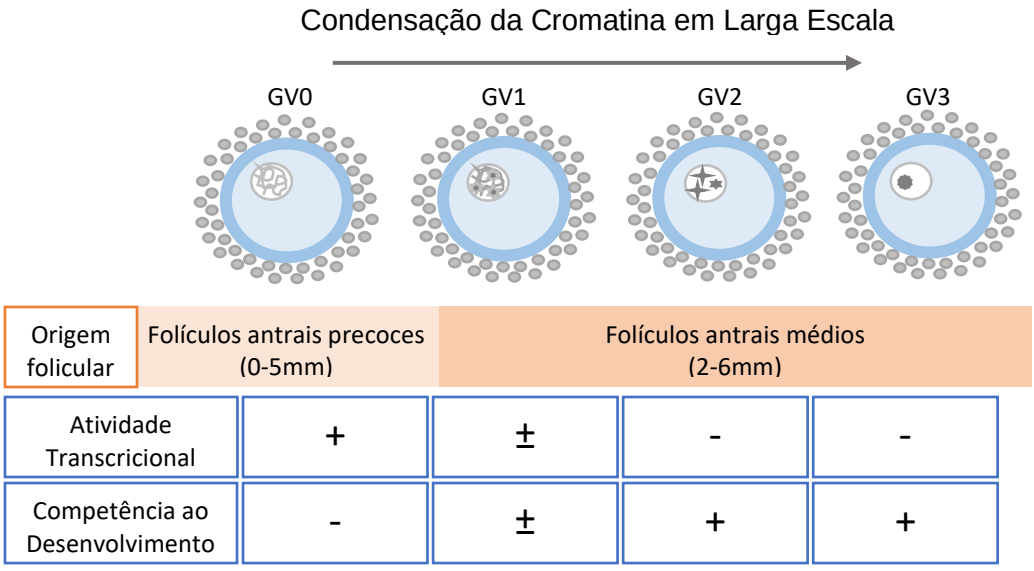


Figura 2. Atividade transcricional e competência de desenvolvimento do oócito em relação a configuração da cromatina (+ e – correspondem a presença ou ausência enquanto ± corresponde a uma condição intermediária). Adaptado de Luciano *et al.*, 2014.

Com o início da regressão folicular parece haver transição de GV2 para GV3, sendo que COCs em GV3 apresentam sinais de apoptose (Dieci, *et al.,* 2016).

### 2.2.1. Retomada da meiose

Quando os oócitos são retirados do folículo, ou seja, do seu ambiente fisiológico e cultivados *in vitro*, ocorre a retomada da meiose de forma espontânea e precoce, mesmo sem os estímulos dos hormônios gonadotróficos (Pincus e Enzmann, 1935; Zhang *et al.,* 2009). E quando comparada à maturação *in vivo*, o oócito apresenta baixa capacidade de desenvolvimento, o que se acredita ser majoritariamente decorrente da assincronia entre a maturação nuclear e a citoplasmática (Eppig *et al*., 2001).

A retomada da meiose *in vivo* ocorre com estímulos gonadotróficos que induzem a transcrição dos fatores de crescimento epidermal (EGF), como

ampirregulina (AREG) e epirregulina (EREG) (revisado por Gilchrist, 2011; Park *et al*., 2004). Esses fatores se ligam aos seus receptores (EGFR) presentes nas células da granulosa e do cumulus e ativam a via ERK1/2, que faz com que as conexinas 34 e 47 se fosforilem (Sakaguchi *et al*., 2000; Su *et al.,* 2003; Conti *et al*., 2006). Essas conexinas são canais hexaméricos que formam as junções do tipo gap permitindo a comunicação bidirecional entre o oócito e suas células adjacentes (Albertini *et al.,* 2001). Quando esses canais se fecham, impedem a passagem de moléculas fundamentais para o crescimento, metabolismo e maturação oocitária, como a adenosina monofosfato cíclico (AMPc) e o monofosfato cíclico de guanosina (GMPc; Lodde *et al*., 2007; Gilchrist *et al*., 2008).

Elevados níveis de AMPc e GMPc no oócito são responsáveis por manter o oócito em parada meiótica. Quando os níveis de AMPc diminuem o fator promotor da maturação (MPF) leva à quebra da vesícula germinativa (Dekel, 1988). O GMPc é responsável pela inibição da fosfodiesterase 3 (PDE3A), enzima que hidrolisa o AMPc (Tsafriri *et al*., 1996; Webb *et al*., 2002; Zhang *et al*., 2009). Assim, quando ocorre a queda dos níveis de GMPc intra-oocitário, a enzima PDE3A (fosfodiesterase 3A) degrada o AMPc levando à retomada da meiose (Norris *et al*., 2009; Zhang *et al*., 2010; Zhang *et al*., 2011).

### 2.2.2. Peptídeo natriurético do tipo C (NPPC)

O NPPC (pepitídeo natriurético do tipo C) e seu receptor (NPR2 – receptor do peptídeo natriurético 2) são essenciais na manutenção da parada meiótica em oócitos de camundongos, suínos, cabras e bovinos (Zhang *et al.,* 2010; Zhang *et al*., 2011; Zhang *et al.,* 2014; Hiradate *et al.*, 2014; Franciosi *et al.,* 2014; Soares *et al.,* 2017). O GMPc é produzido nas células do cumulus após ativação da NPR2 pelo seu ligante NPPC, sugerindo uma relação funcional na manutenção da parada meiótica (Zhang *et al*., 2010), no qual o $E_2$ apresenta papel importante na manutenção dos receptores de NPPC (NPR2) funcionais nas células do cumulus (Zhang *et al*., 2011). Em bovinos, a interação entre esteroides (estradiol, progesterona e androstenediona) com NPPC na MIV mostrou-se importante para o atraso da retomada da meiose, aumento da expressão de NPR2 e prolongamento da comunicação cumulus-oócito pelas junções do tipo gap (Soares *et al*., 2017). Contudo, o pico ovulatório de LH reduz a expressão do NPPC e do NPR2 nas células da granulosa (Kawamura *et al*., 2011), diminuindo os níveis de GMPc do oócito o que leva a retomada da meiose.

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# CAPÍTULO II

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# CHARACTERIZATION AND CONTROL OF THE OOCYTE POPULATION IN NELLORE CATTLE BASED ON CHROMATIN CONFIGURATION

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**Abstract**

*In vitro* embryo production in cattle is still inefficient. The identification of oocyte competence markers and development of strategies to optimize oocyte quality for OPU may strongly impact on IVP efficiency. In this study, we first assessed the oocyte population obtained from large antral follicles testing the hypothesis that healthy dominant follicles would contain oocytes with an intermediate degree of chromatin compaction. We then assessed oocyte population obtained by OPU at a radom day of estous cycle with regard to chromatin configuration, while testing a synchronizing protocol combining follicle aspiration and FSH treatment to control oocyte quality. Oocytes with an intermediate degree of chromating compaction (GV2 oocytes) were predominat in healthy large antral follicles, whereas oocytes with high degree of chromatin compaction (GV3 oocytes) were predominant in transitional and atretic large follicles. In Nellore cows, a protocol combining aspiration of all follicles >2mm at a random day (day 0) with a 40mg FSH treatment divided in 2 injections on day 2 and followed by OPU on day 5 decreased the percentage of small follicles at OPU, but did not alter oocyte germinal vesicle (GV) patterns. Conversely, the same protocol utilizing half of FSH dose did not alter follicle size patterns but decreased the percentage of oocytes with high chromatin compaction (GV3 oocytes) at OPU. In Nellore peripuberal heifers, the same protocol with 20mg FSH and OPU on day 4 did not alter oocyte GV pattern but doubled the proportion of medium follicles while halfing the proportion of small follicles at OPU. Moreover, Nellore heifers presented a higher percentage of GV2 oocytes and lower percentage of GV3 oocytes at OPU in relation to cows. These data suggest that healthy dominant follicles contain oocytes with intermediate levels of chromatin compaction and that synchroning protocols combining follicle aspiration and FSH treatment may be useful to control oocyte quality for OPU/IVP.

**Keywords:** follicle, germinal vesicle, chromatin, FSH, Nellore cattle.

## 1. Introduction

Embryo *in vitro* prodution (IVP) in cattle is still inefficient, and in addition to the cuture systems utilized for oocyte *in vitro* maturation (IVM), *in vitro* fertilization (IVF) and *in vitro* embryo culture (IVC), oocyte quality is a major determinant of its success (Smitz *et al.*, 2011). Previous studies have indicated that the oocyte population obtained by ovum pick up (OPU) at a random day of the estrous cycle is heterogeneous with regard to chromatin configuration, which is directly associated with oocyte developemental competence (Lodde *et al*., 2007; Lodde *et al*., 2008; Luciano *et al.*, 2011). This study aimed to characterize the oocyte population obtained at a random day of the bovine estrous cycle, as well as to assess the effect of the follicular status on germinal vesicle stage. Moreover, we tested protocols utilizing follicular aspiration combined with FSH treatment to promote a more homogeneous oocyte population for IVP.

*In vivo*, resumption of meiosis is triggered by the preovulatory LH surge only in oocytes derived from dominant follicles. The activation of LH receptors on mural granulosa cells leads to the secrection of EGF-like peptides (ampiregulin and epiregulin most importantly in cattle; Caixeta *et al.*, 2013), which reach cumulus cells to stimulate there their own expression and secretion. EGF-like peptides induce oocyte meiosis resumption mainly through the ERK1/2 pathway (reviewed by Gilchrist, 2011; Gilchrist *et al.*, 2008). On the other hand, before ovulation, meiosis progression is actively blocked by NPPC, which is produced by mural and cumulus granulosa cells in cattle, and activates the receptor NPR2 in cumulus cells to induce cGMP production. Cyclic GMP then flows through traszonal projections and gap junctions to reach the oocyte, where it will reduce the activity of phosphodiesterase 3A (PDE3A) on cAMP, that inhibits activation of the maturation promoting factor, thus preventing meiosis resumption (Mayes and Sirard, 2002; Thomas *et al*., 2002; Conti *et al.*, 2012). Estradiol has been shown to cooperate

with NPPC to block meiosis resumption in rodents, porcine, goat and cattle by stimulating the expression of NPR2 in cumulus cells (Zhang *et al.,* 2010; Zhang *et al.,* 2014; Zhang *et al.,* 2015; Hiradate *et al.,* 2014; Franciosi *et al.,* 2014; Soares *et al.,* 2017).

During antral follicular development, oocyte chromatin configuration changes in association with transcriptional activity. Oocyte from early antral follicles present uncondensed chromatin and high transcriptional activity (Lodde *et al.,* 2008). A classification system has been proposed for the germinal vesicle of bovine oocytes, in which stages GV0 to GV3 represent increasing levels of chromatin compaction, starting from no compaction (GV0) to highly condensed (GV3) (Lodde *et al.,* 2007). There is evidence that GV2 oocytes present higher developmental competence for IVP and that GV3 oocytes are associated with atresia and apoptosis (Dieci *et al.,* 2016; Lodde *et al.,* 2007).

Previous studies report that the distribution of the different GV stages described above does not change with follicular size (Dieci *et al.,* 2016). However, it is well known that follicle size per se is not a reliable parameter to determine follicular status (Sirard, 2011; Sánchez et al., 2015). Nevertheless, based on data on oocyte developmental competence and on the identification of markers of apoptosis in cumulus cells, it has been speculated that growing and preovulatory dominant follicles would contain GV2 oocytes, and that the transition to GV3 would coincide with the beginning of follicle atresia (Dieci *et al.*, 2016). Therefore, the first aim of this study was to test the hypothesis that healthy dominant follicles, as determined by the ratio between intrafollicular concentrations of estradiol and progesterone, would contain predominantly GV2 oocytes, whereas transitional and atretic follicles would present higher percentages of GV3 oocytes. We then hypothesized that protocols combining follicle aspiration and FSH treatment would

induce a more homogeneous population of oocytes for OPU/IVP in Nellore cows and heifers, with greater representation of GV2 oocytes in relation to random OPU.

## 2. Material and Methods

The experimental design was approved by the animal experiment ethics committee of the Faculty of Veterinary Medicine and Animal Science of Universidade Estadual Paulista (UNESP) - Campus Botucatu number 0144/2017 - CEUA.

### Post-mortem samples

To assess the effect of follicular status on chromatin configuration, cumulus oocyte complexes were obtained from abattoir ovaries, which were transported to the laboratory in thermal bottles containing sterile saline solution (0.9% NaCl) at 35-37° C. In laboratory, ovaries were washed in saline solution, sterilized with 70% alcohol, and follicles larger than 10 mm in diameter were dissected and individually recovery oocyte. For each individual follicle, the follicular fluid containig the COC was stored in 1.5ml tubes at -80ºC until steroid measurements and the follicle was classified as heathy, transitional or atretic in accordance with the estradiol/progesterone ratio (ratios >1, 1–0.01 and <0.01, respectively) as previously suggested (Ireland *et al.,* 1994; Grimes and Ireland, 1986). The COC was then recovered under a stereomicroscope, the oocyte was mechanically denuded, fixed and stained for chromatin evaluation.

### Evaluation of chromatin configuration

After denudation, oocytes were fixed in 60% methanol solution for 30 minutes, and washed again in DPBS solution. The oocytes were stained in 1 μg/mL of Hoechst 33342 and chromatin configuration was evaluated under a fluorescence microscope (Nikon Eclipse 80i). Oocytes were classified in accordance with the degree of chromatin

condensation, presence of the germinal vesicle and presence of genetic material as GV0, GV1, GV2, GV3, GVBD (germinal vesicle breakdown) and DEG (degenerated; absence of genetic material) (Lodde *et. al*, 2007).

**Steroid Assays**

Estradiol and progesterone concentrations were measured in follicular fluid samples using Ultra-Sensitive Estradiol RIA (Beckman-Coulter Inc.) and Progesterone RIA kit (ImmuChem$^{TM}$ Coated Tube Inc.), respectively, following kit instructions. The sensitivity of the assays were 0.002ng/mL for estradiol and 0.15ng/mL for progesterone.

For progesterone, the standard curve and samples were diluted in PBS gelatin in 1:5 ratio, and the intraassay coefficient of variation was 7.6%. For estradiol, dilutions of 1:100 and 1:20 were used, and intraassay and interassay coefficients variations was 2.9% and 15.7% respectively.

***In vivo* studies**

Three experiments, two in Nellore cows and one in Nellore peripuberal heifers, were conducted to characterize the oocyte population obtained by OPU at a random day of the estrous cycle, while testing protocols combining aspiration of all follicles larger than 2mm (aiming for wave synchronization) with FSH treatment (aiming to enhance follicular recruitment) to homogenize the oocyte population increasing the percentage of GV2 stage. Experimental designs are described and ilustrated in Fig 1.

Empty Nellore cows (n=26) aged 3 to 8 years were used from the Teaching, Research and Extension Farms of the Department of Animal Production FMVZ-UNESP-Botucatu, kept on pasture with access to mineral salt and water *ad libitum*, with a body score of 2.8 to 3.5. Nellore heifers (n=40) from Santo Antônio Farm, located in the county

of São Manuel were kept on pasture with corn silage supplementation, access to mineral salt and water *ad libitum*, with a body score of 3.5.

Before OPU, cows received low epidural anesthesia and then follicles larger than 2 mm were aspirated. During OPU, numbers of small, medium and large follicles (3-5mm, 5-8mm and> 8mm, respectively) were registered. The recovered fluid was deposited in a conical tube with solution of DPBS and heparin, washed and filtered for later recovery and evaluation of the COCs in a Petri dish (90mm) under a stereomicroscope. COCs were morphologically classified (Grades I, II and III) and oocytes were denuded and fixed in 60% methanol solution for 30 minutes for subsequent assessment of the germinal vesicle as described above.

**Statistical analysis**

The effects of the synchronization treatments on oocyte recovery, follicle size and GV statuses were assessed in cross-over designs and therefore tested by the paired Student T-Test after percentage data arcsine transformation. Statistical analysis was performed using the JMP software (SAS Institute, Cary, NC, USA) and the results are presented as means ± standard error of the mean (EPM). Differences were considered significant when $P < 0.05$.

## 3. Results

Different patterns of distribution of GV stages were observed in large antral follicles with different health statuses (Fig. 2). While in transitional and atretic follicles the presence of GV3 oocytes was predominant, in healthy large follicles GV2 oocytes were more frequent. Ooocytes at GV1 were retrieved from a much smaller percentage of transitional and atretic large antral follicles, but not from healthy large follicles. In

addition, GV0 oocytes were not observed in large antral follicles and degenerated oocytes were found only in atretic large follicles as expected.

The protocol combining follicular aspiration and FSH treatment altered the follicular size pattern at OPU when a higher total dose of FSH was used (40mg; Fig. 3A), but not when half of this dose was tested (20mg; Fig. 3B). In both cases however, most follicles available for OPU at a random day or after synchronization with follicle aspiration and FSH were small from 3 to 5mm in diameter (Table 1 and 3). The protocol combining follicle aspiration and 40mg of FSH significantly reduced the percentage of small follicles at the OPU day (Fig. 3A). Regardless the dose of FSH utilized, the combination of follicle aspiration with FSH treatment did not alter oocyte recovery rate in relation to all follicles aspirated, which was on average around 50-60% (Fig. 4).

In Nellore cows, the protocol combining follicular aspiration and FSH treatment only significantly altered oocyte GV patterns at OPU at the lower total dose of FSH (20mg; Table 2), when a reduction in the percentage of GV3 oocytes was observed (Fig. 3B). Differently, in Nellore peripuberal heifers, the protocol combining follicle aspiration and FSH (20mg) did not alter oocyte GV patterns at OPU (Table 5, Fig. 6A), but increased the percentage of medium follicles available to OPU from around 10 to 45%, while reducing the percentage of small follicles from around 80 to 45% (Table 6, Fig.6B). Furthermore, oocyte GV patterns at OPU appeared to vary between Nellore cows and peripuberal heifers; a higher proportion of GV2 oocytes and a lower proportion of GV3 oocytes were observed in heifers in relation to cows (Figs 5 and 6A).

## 4. Discussion

This is the first report to our knowledge investigating the patterns of oocyte chromatin configuration in relation to the physiological status of the ovarian follicle and at random versus synchronized OPU. Data presented herein confirm previous suggestions that healthy large follicles contain oocytes with intermediate degree of chromatin compaction (GV2) and that protocols utilizing follicle aspiration and FSH treatment may be applied to control follicular and oocyte patterns at OPU to improve oocyte developmental competence.

Previous data demonstrated that oocyte chromatin configuration does not change with follicular size in bovine follicles. Small, medium and large follicles presented equivalent percentages of GV1, GV2 and GV3 oocytes, which exhibit incrasing levels of chromatin compaction (Dieci *et al*., 2016). Because follicle size does not reflect the follicular health status, being the steroidogenic activity a more reliable indicator (Sirard, 2011; Sánchez *et al.,* 2015), we investigated the GV pattern in different follicular categories (healthy, transitional and atretic) as determined by the intrafollicular estradiol/progesterone ratio. As hypothesized a higher percentage of GV2 oocytes presenting an intermediate degree of chromatin compaction was observed in large healthy follicles. This observation combined with previous data suggesting that GV2 oocytes are more competent (Lodde *et al*., 2007), reinforce the concept that GV2 oocytes would be the target in strategies aiming to control the oocyte population for OPU/IVP.

Higher percentages of GV3 oocytes bearing a highly condensed chromatin were observed in transitional and atretic follicles. It appears therefore that once follicle regression starts chromatin compaction increases. The present data also suggest that the intrafollicular mechanisms maintaining oocytes in GV2 in healthy follicles may involve higher availability of estradiol. Estradiol has been shown to enhance NPPC activity in

cumulus cells by increasing the expression of the receptor NPR2 in different species including cattle (Zhang *et al.*, 2011). This appears to cause an increase in cGMP production which would maintain cAMP levels high enough in the oocyte to block meiosis progression. Moreover more recently NPPC was demonstrated to maintain transzonal projections between the cumulus cells and the oocyte contributing to cGMP deliver (Clarke, 2018).

The strategie combining follicle aspiration and FSH treatment only affected the follicular size pattern at OPU when the higher dose (40 mg) was used in Nellore cows, when a reduction in the percentage of small follicles at OPU was observed, which was accompanied by numerical increases in the percentages of medium and large follicles. Therefore, it appears that 20 mg of FSH was not sufficient to stimulate follicular growth as observed with 40 mg.

On the other hand, the synchronization protocol only affected the oocyte GV pattern when 20 mg of FSH was used. This may suggest that FSH at 40 mg might stimulate the transition from GV2 to GV3 in Nelore cows, which would not occur at the same degree with 20 mg.

The synchronizing protocol with 20 mg of FSH did not change the GV pattern in Nellore peripuberal heifers. Interestingly, although we cannot directly compare data obtained from different experiments, a higher percentage of GV2 oocytes and a lower percentage of GV3 oocyte were bserved in peripuberal heifers as compared with cows. The reasons behind this finding are intriguing, difficult to explain, and likely related to endocrinological and intrafollicular differences between cows and peripuberal heifers (Figueiredo *et al.*, 1997). Although speculative, these differences may include lower LH activity on granulosa cells of heifers. Once LH has been shown to suppress NPPC activity

(Kawamura *et al.*, 2011), lower LH activity would favor NPPC action and thus meiotic arrest.

Although the synchrozing protocol did not change the GV pattern in peripuberal heifers, it altered the follicular size pattern at OPU by halfing the percentage of small follicles and doubling the percentage of medium follicles. The same was not observed in Nellore cows with either 20 mg or twice the dose, suggesting that peripuberal Nellore heifers are more sensitive to FSH than Nellore cows.

In conclusion, this study provides for the first time experimental evidence that healthy dominant follicles contain predominantly GV2 oocytes, and that synchronization protocols combining follicular aspiration and FSH treatment may be useful to control the oocyte population for OPU aiming to improve IVP outcomes.

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Table 1. Effects of treatments combining follicular aspiration and FSH administration of 40mg on the number of small (3-5mm); medium (5-8mm) and large (>8mm) follicles aspirated during OPU in Nellore cows (n=14 cows in crossover).

| | ***3-5mm*** *(n±EPM)* | ***5-8mm*** *(n±EPM)* | ***>8mm*** *(n±EPM)* | ***Total*** |
|---|---|---|---|---|
| *Control* | 207 ± 6,2 | 61 ± 5,3 | 17 ± 1,9 | 285 |
| *Synchronized* | 169 ± 8,4 | 103 ± 7,3 | 34 ± 3,5 | 306 |

n = number of aspirated follicles
EPM = Standard error of the mean

Table 2. Effects of treatments combining follicular aspiration and FSH administration of 40mg on the number of degenerated oocytes, oocytes exhibiting germinal vesicle breakdown and oocytes with different degrees of chromatin compaction (GV0 to GV3) after OPU in Nellore cows (n=14 cows in crossover).

| | ***GV0*** *(n±EPM)* | ***GV1*** *(n±EPM)* | ***GV2*** *(n±EPM)* | ***GV3*** *(n±EPM)* | ***GVBD*** *(n±EPM)* | ***DEG*** *(n±EPM)* | ***Total*** |
|---|---|---|---|---|---|---|---|
| *Control* | 2 ± 1,1 | 2 ± 0,3 | 90 ± 9,9 | 62 ± 9,6 | 1 ± 0,1 | 7 ± 9,0 | 164 |
| *Synchronized* | 0 ± 0 | 9 ± 1,5 | 72 ± 9,3 | 57 ± 7,4 | 0 ± 0 | 22 ± 8,8 | 160 |

n = number of classified oocytes
EPM = Standard error of the mean

Table 3. Effects of treatments combining follicular aspiration and FSH administration of 20mg on the number of small (3-5mm); medium (5-8mm) and large (>8mm) follicles aspirated during OPU in Nellore cows (n=12 cows in crossover).

| | ***3-5mm*** *(n±EPM)* | ***5-8mm*** *(n±EPM)* | ***>8mm*** *(n±EPM)* | ***Total*** |
|---|---|---|---|---|
| *Control* | 417 ± 4,5 | 39 ± 3,8 | 12 ± 1,5 | 468 |
| *Synchronized* | 397 ± 3,9 | 50 ± 3,1 | 16 ± 1,2 | 463 |

n = number of aspirated follicles
EPM = Standard error of the mean

Table 4. Effects of treatments combining follicular aspiration and FSH administration of 20mg on the number of degenerated oocytes, oocytes exhibiting germinal vesicle breakdown and oocytes with different degrees of chromatin compaction (GV0 to GV3) after OPU in Nellore cows (n=12 cows in crossover).

| | ***GV0*** *(n±EPM)* | ***GV1*** *(n±EPM)* | ***GV2*** *(n±EPM)* | ***GV3*** *(n±EPM)* | ***GVBD*** *(n±EPM)* | ***DEG*** *(n±EPM)* | ***Total*** |
|---|---|---|---|---|---|---|---|
| *Control* | 0 ± 0 | 5 ± 1,4 | 92 ± 9,5 | 58 ± 11,3 | 0 ± 0 | 18 ± 3,4 | 173 |
| *Synchronized* | 0 ± 0 | 8 ± 2,1 | 93 ± 10,0 | 67 ± 9,1 | 0 ± 0 | 31 ± 4,1 | 199 |

n = number of classified oocytes
EPM = Standard error of the mean

Table 5. Effects of a treatment combining follicular aspiration and FSH administration (total dose of 20mg) on the number of degenerated oocytes and oocytes with different degrees of chromatin compaction (GV0 to GV3) in Nellore heifers subjected to OPU (n=40 cows in crossover).

| | ***GV0*** *(n±EPM)* | ***GV1*** *(n±EPM)* | ***GV2*** *(n±EPM)* | ***GV3*** *(n±EPM)* | ***DEG*** *(n±EPM)* | ***Total*** |
|---|---|---|---|---|---|---|
| *Control* | 6 ± 0,9 | 51 ± 1,7 | 620 ± 8,4 | 223 ± 6,1 | 144 ± 6,6 | 1044 |
| *Synchronized* | 6 ± 0,9 | 46 ± 2,3 | 580 ± 8,8 | 174 ± 6,5 | 69 ± 6,2 | 875 |

n = number of aspirated follicles
EPM = Standard error of the mean

Table 6. Effects of a treatment combining follicular aspiration and FSH administration (total dose of 20mg) on the number of follicles according to follicle size patterns in Nellore heifers subjected to OPU (n=40 cows in crossover).

| | ***3-5mm*** *(n±EPM)* | ***5-8mm*** *(n±EPM)* | ***> 8mm*** *(n±EPM)* | ***Total*** |
|---|---|---|---|---|
| *Control* | 1085 ± 4,8 | 141 ± 4,0 | 41 ± 2,1 | 1267 |
| *Synchronized* | 599 ± 6,5 | 531 ± 6,4 | 76 ± 3,1 | 1206 |

n = number of classified oocytes
EPM = Standard error of the mean

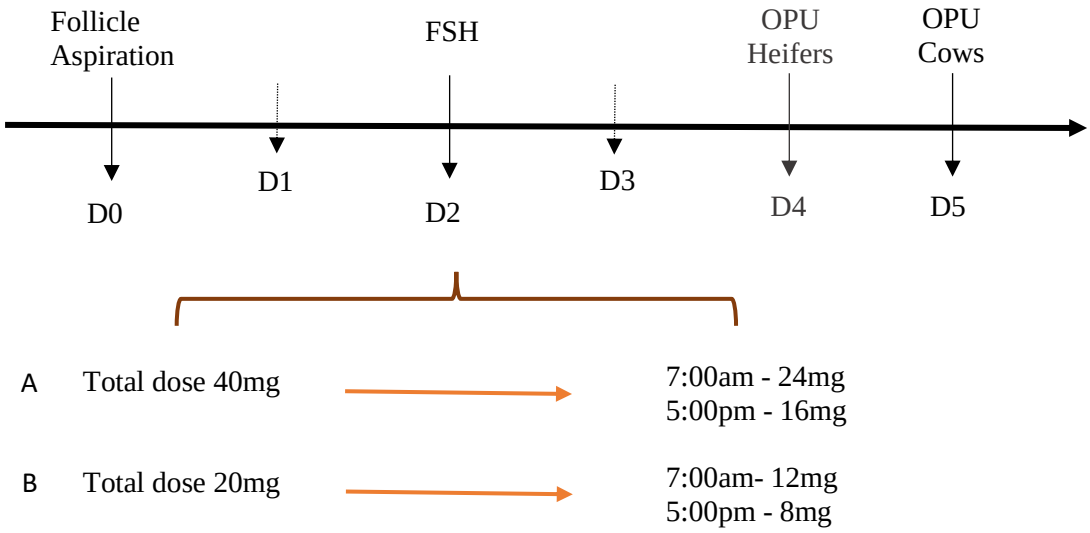


Fig. 1: Experimental design of *in vivo* studies aiming to characterize the oocyte population obtained by OPU at a random day and to test treatments combining follicle aspiration and FSH administration to homogenize the oocyte population for OPU. All treatments included aspiration of all follicles >2 mm on day 0 (D0), two injections of FSH (Folltropin) 10 hours apart on day 2 (D2), and OPU on day 4 for heifers and 5 (D5) for cows. The experiment was performed twice in Nellore cows using different two different FSH regimens [A (n=14 cows); B (n=12 cows)] and once in Nellore heifers (n=40 cows) utilizing the lower FSH total dose (B) and aspirated on day 4, always in cross-over designs. During the control treatment, animals were subjected to OPU at a random day of the estrous cycle.

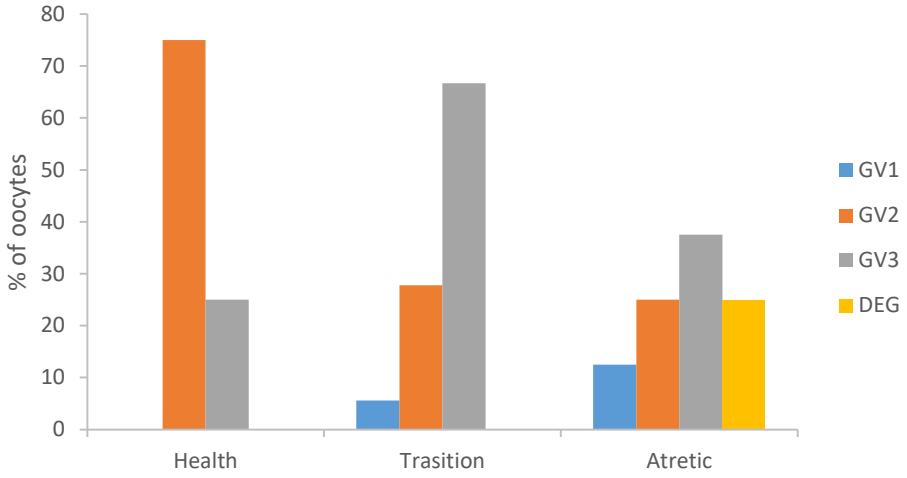


Fig. 2: Effect of follicular health status on oocyte chromatin configuration in oocytes from large bovine antral follicles (≥10mm; n=34 follicles) as assessed by the percentage of oocytes showing increasing degrees of chromatin compaction (GV1 to GV3; Lodde *et al*. 2007) and with degenerated chromatin (DEG). Large antral follicles were classified as healthy (n=8), transitional (n=18) and atretic (n=8) according with the estradiol:progesterone ratio (>1, 1–0.01 and <0.01, respectively).

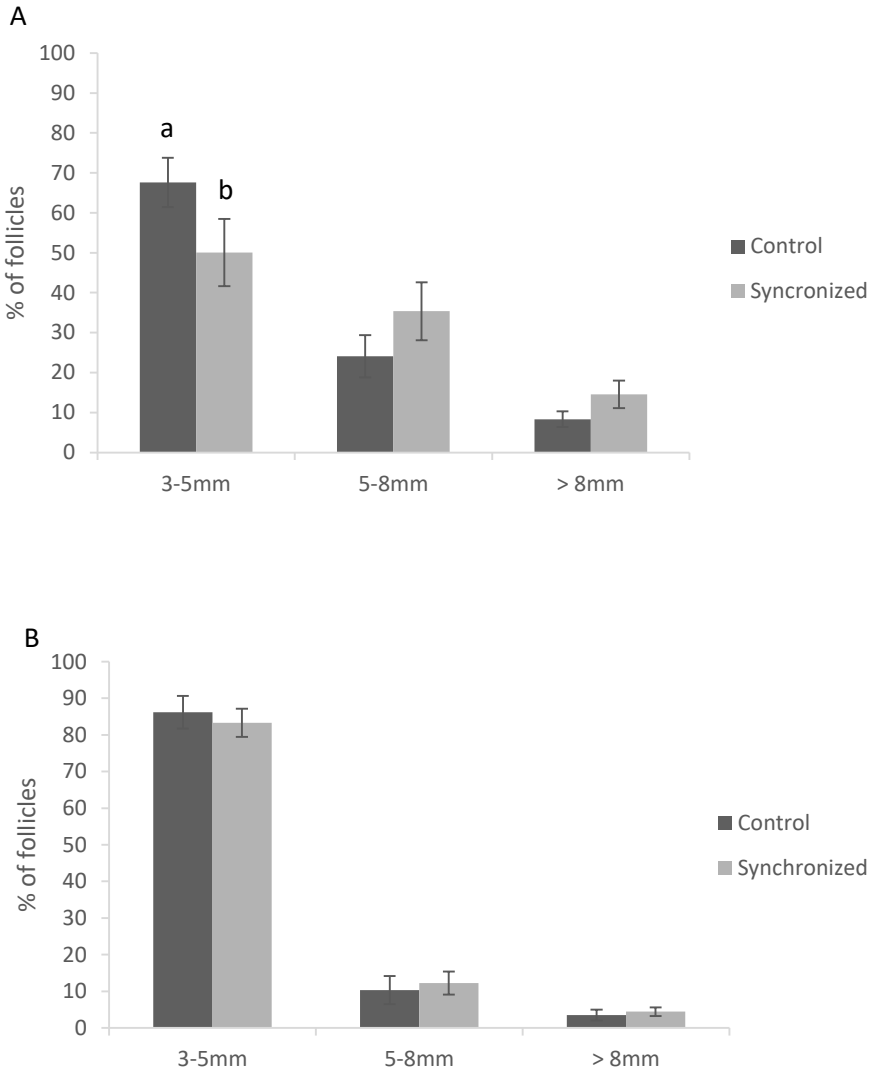


Fig. 3: Effects of treatments combining follicular aspiration and FSH administration [total doses of 40mg (A, n=14 cows in crossover) and 20mg (B; n=12 cows in crossover)] on the percentage of small (3-5mm); medium (5-8mm) and large (>8mm) follicles aspirated during OPU in Nellore cows. Different letters represent significant statistical differences ($P<0.05$).

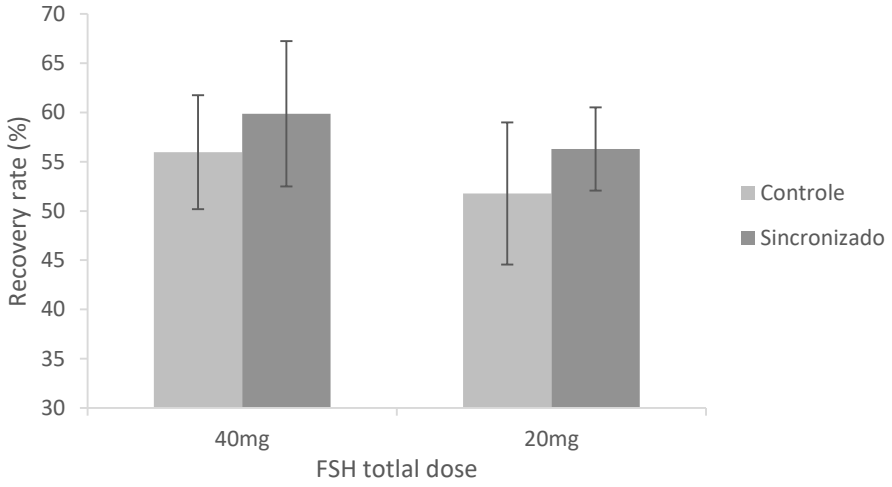


Fig. 4: Effects of treatments combining follicular aspiration and FSH administration [total doses of 40mg (A; n=14 cows in crossover) and 20mg (B; n=12 cows in crossover)] on oocyte recovery rate (% oocytes obtained in relation to the number of follicles aspirated) after OPU in Nellore cows. Different letters represent significant statistical differences ($P<0.05$).

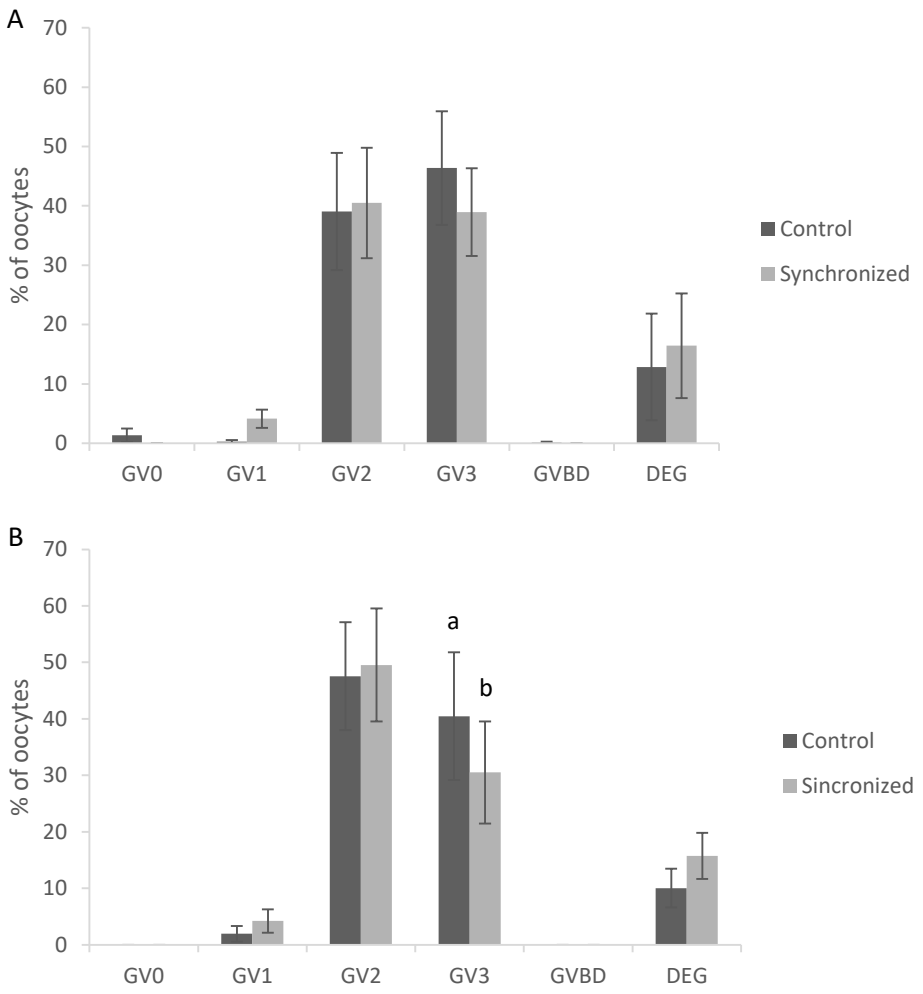


Fig. 5: Effects of treatments combining follicular aspiration and FSH administration [total doses of 40mg (A; n=14 cows in crossover) and 20mg (B; n=12 cows in crossover)] on the percentage of degenerated oocytes, oocytes exhibiting germinal vesicle breakdown and oocytes with different degrees of chromatin compaction (GV0 to GV3) after OPU in Nellore cows. Different letters represent significant statistical differences ($P<0.05$).

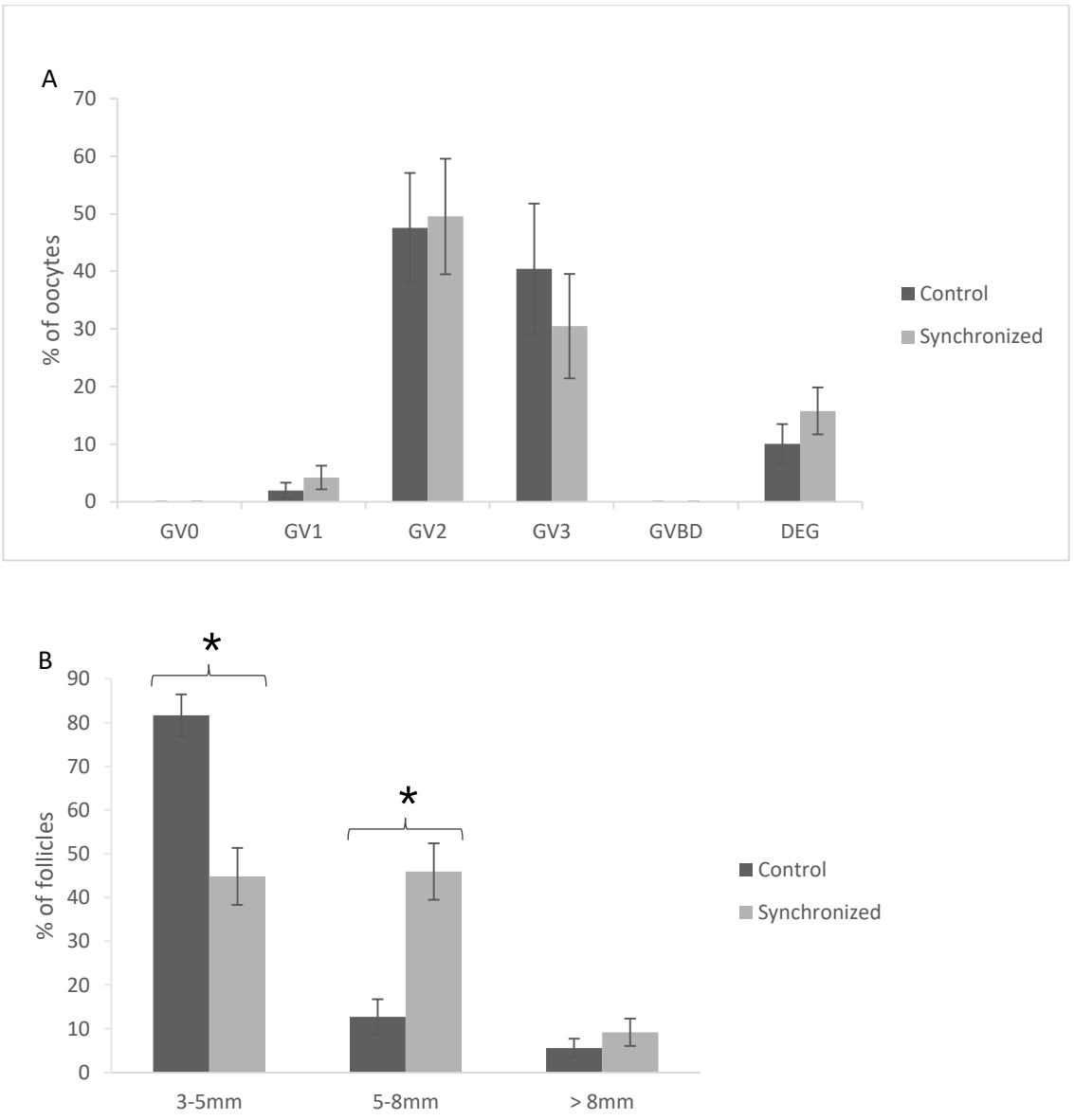


Fig. 6: Effects of a treatment combining follicular aspiration and FSH administration (total dose of 20mg) on the percentage of degenerated oocytes, oocytes with different degrees of chromatin compaction (GV0 to GV3; A), and on follicular size patterns in Nellore heifers subjected to OPU (n=40 cows in crossover). Asterisks represent significant statistical differences ($P<0.05$).

# CAPÍTULO III

**Final Considerations**

Synchronization protocols as a reproductive biotechnological tool maximizes IVP indexes, aiming to obtain a maximum number of viable embryos after the stimulation of gonadotrophic responsible for the recruitment of a new follicular wave and follicular growth, in order to meet the growing comercial demand of food in the world.

In OPU-IVF practice, oocytes are classified according to the morphological analysis of ooplasm and cumulus cells investment ((Blondin and Sirard, 1995; Hazeleger *et al.*, 1995; Dieci *et al*., 2016), but this analysis can not predict the stage of maturation and therefore if acquisition of development competence was acquired. Thus, in addition to increasing the oocyte recovery rate, making the population more homogeneous for IVP, is attractive in terms of the quality of oocytes.

Ultrasound-guided transvaginal follicle aspiration, as a method of follicle ablation, induce and synchronize wave emergence and ovulation in heifers selected at unknown stages of the estrous cycle (Bergfelt *et al.*, 1994).

FSH is the main responsible for the recruitment of a new group of antral follicles, growth and development (Adams *et al.*, 1992; Aerts and Bols, 2010). Carvalho and collaborators (2007) report that *Bos indicus* females recruit more follicles per follicular growth wave than *Bos taurus* females (33.4 ± 3.2 and 25.4 ± 2.5, respectively). This increase in the number of follicles present in the ovaries may be related to the IGF system since they are synergistic to FSH in the promotion of follicular growth and estradiol production (Bó *et al*., 2003; Fortune *et al.*, 2004). This would explain the higher sensitivity to FSH in superovulation treatments, and possible reduction in doses compared to apply *Bos taurus* (Baruselli *et al*, 2003).

Previous parallel studies have shown that Holstein cattle present a pattern of distribution of oocyte population with different chromatin configuration when compared

to Nellore cows, presented a higher proportion of immature oocytes (oocytes in GV0 and GV1) on random days of the estrous cycle, which may be responsible for the lower efficiency for IVP in relation to Nellore (Soares *et al.,* 2017). The data also showed that the synchronization protocol combined with the FSH treatment (110mg, Folltropin) to synchronize the emergency and increase the follicular recruitment, was efficient increasing the percentage of oocytes in GV2 in Holstein cows.

Nellore cows present the majority of their oocyte population at advanced stages of chromatin condensation (oocytes in GV2 and GV3) when compared to holstein, but when evaluated the population in Nellore heifers, GV2 oocytes represent approximately 60% of their composition, which may be due that the ovulatory wave is longer for females with 2 waves than for those with 3 waves (Figueiredo *et al.,* 1997) allowing greater advancement in oocyte maturation.

Further improvement of ASP-FSH protocols and fine-tuning with IVM culture system are needed before their real impact on IVP outcomes can be determined. However, these data demonstrate a potential for advances in embryo production in Brazil, mainly associated to the increase in demand for the commercial herd of dairy and beef farms in the production optimization, in addition the use of embryo technologies for large scale production of cross-breeding approaching the world market.

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