LUCIANA SIMÕES RAFAGNIN MARINHO

# PERFIL DA H3K27 NO DESENVOLVIMENTO EMBRIONÁRIO *IN VITRO* NOS MODELOS SUÍNO E BOVINO

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Tese de Doutorado apresentada ao Programa de Pós-graduação em Ciência Animal da Universidade Estadual de Londrina, Área de Concentração Sanidade Animal, como requisito para obtenção do título de Doutor em Ciência Animal.

Orientador: Prof. Marcelo Marcondes Seneda

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## RESUMO

As marcações epigenéticas da lisina 27 da histona H3 (H3K27) parecem exercer importante papel na regulação do desenvolvimento embrionário de mamíferos. O objetivo deste trabalho foi investigar o perfil de alterações da H3K27 e o efeito da inibição da trimetilação da H3K27 em dois modelos animais: embriões suínos e bovinos. Para tanto, foram realizados dois experimentos. O Experimento 1 teve como intuito investigar o perfil de acetilação e metilação (simples, dupla ou tripla) da H3K27 (H3K27ac, me1, me2 e me3, respectivamente) durante a maturação oocitária e desenvolvimento inicial de embriões suínos produzidos in vitro (PIV). O Experimento 2 objetivou investigar o efeito da adição do DZNep, inibidor do Complexo Repressivo Polycomb 2 (PRC2), ao meio de cultivo embrionário em diferentes períodos no desenvolvimento de embriões. Além disso, foi avaliada a expressão de mRNA dos genes das enzimas do PRC2 EZH2, EED e SUZ12; dos genes marcadores de pluripotência OCT4 e NANOG e do gene marcador de diferenciação celular CDX2. No Experimento 1, oócitos e embriões suínos foram fixados em diferentes estágios de desenvolvimento (de vesícula germinativa a blastocistos em D8) e submetidos a um protocolo de imunofluorescência para a identificação das marcas H3K27ac, me1, me2 e me3. No Experimento 2, embriões clivados foram expostos ao DZNep nos dias 3 a 5 (DZNep D3-D5), nos dias 3 a 8 (DZNep D3-D8), nos dias 5 a 8 (DZNep D5-D8) ou cultivados sem a adição do DZNep (Controle). No Experimento 1, o sinal fluorescente da H3K27ac esteve presente em quase todos os estágios, atingindo o mínimo no estágio de 8 células. H3K27me1, me2 e me3 foram detectadas em oócitos e apresentaram sinal fraco ou ausente durante o desenvolvimento embrionário, intensificando-se novamente no estágio de blastocisto. Três padrões distintos da H3K27me3 foram observados em blastocistos suínos em D8. No Experimento 2, o grupo DZNep D3-D8 apresentou menor taxa de blastocisto (6,9%) em comparação ao grupos DZNep D3-D5 (16,7%) e DZNep D5-D8 (14,3%; $p < 0,01$), sendo todas estas menores ao se comparar à taxa do grupo Controle (33,9%). O número médio de células também foi reduzido de 118,2 ± 5,6 (Controle) para70,4 ± 4,3 (DZNep D3-D5), 51,5 ± 2,5 (DZNep D5-D8) e 36,3 ± 1,6 (DZNep D3-D8; $p < 0,01$). Os embriões expostos ao DZNep tiveram também o desenvolvimento atrasado, apresentando menos embriões em estágios avançados de desenvolvimento. O DZNep não influenciou a expressão de mRNA dos genes SUZ12, NANOG, OCT4 e CDX2. Entretanto, o grupo DZNep D3-D8 apresentou maiores níveis de mRNA dos genes EZH2 e EED em comparação ao grupo Controle. Os resultados do Experimento 1 sugerem que as alterações epigenéticas da H3K27 são remodeladas durante a embriogênese suína. Sugerem também que os baixos níveis de H3K27me e altos níveis de H3K27ac indicam um estado transcricionalmente ativo da cromatina durante o desenvolvimento inicial de embriões suínos PIV. Com os resultados obtidos no Experimento 2, é possível concluir que a ação do PRC2 é importante para o desenvolvimento embrionário, uma vez que o DZNep reduziu a taxa de blastocistos, o número de células embrionárias e a velocidade de desenvolvimento. A exposição dos embriões ao DZNep nos dias 3 a 8 aumentou a expressão de mRNA das enzimas Polycomb EZH2 e EED.

**Palavras-chave:** Epigenética. Polycomb. Histona. Embrião. PIVE.

MARINHO, Luciana Simões Rafagnin. **Profile of H3K27 in in vitro embryonic development inporcine and bovinemodels.** 2015. 145 p. Tese (Doutorado em Ciência Animal) – Universidade Estadual de Londrina, Londrina, 2015.

## ABSTRACT

The epigenetic marks of lysine 27 of histone H3 (H3K27) appear to play an important role in the regulation of embryonic development in mammals. The aim of this study was to investigate the profile of H3K27 modifications and the effect of inhibiting trimethylation of H3K27 in two animal models: porcine and bovine embryos. Therefore, two experiments were conducted. Experiment 1 had the purpose to investigate the profile ofacetylation and methylation (mono-, di- and trimethylation) of H3K27 (H3K27ac, me1, me2 and me3, respectivally) during oocyte maturation and early development of in vitro-produced (IVP) porcine embryos. Experiment 2 aimed to investigate the effect of adding DZNep, an inhibitor of the Polycomb Repressive Complex (PRC2), to the culture media in different periods of culture on embryo development.Furthermore, mRNA expression of genes encoding the PRC2 enzymes EZH2, EED and SUZ12; the markers of pluripotency OCT4 and NANOG and the marker of cell differentiation CDX2 was investigated. In Experiment 1, oocytes and embryos were fixed at different developmental stages (from germinal vesicle to D8 blastocysts) and submitted to an immunocytochemistry protocol to identify the H3K27ac, me1, me2 e me3 marks. In Experiment 2, cleaved embryos were exposed to DZNep from day 3 to 5 (DZNep D3-D5), from day 3 to 8 (DZNep D3-D8), from day 5 to 8 (DZNep D5-D8) or cultured without DZNep (Control). In Experiment 1, the fluorescent signal of H3K27ac was observed in most development stages, reaching a minimum at the 8-cell stage. H3K27me1, me2 and me3 signals were detected in oocytes and showed a weak or absent signal during embryo development, rising again at the blastocyst stage.Three different patterns of the H3K27me3 mark were observed in porcine D8 blastocysts. In Experiment 2, DZNep D3-D8 had lower blastocyst rate (6.9%) than DZNep D3-D5 (16.7%) and DZNep D5-D8 (14.3%; $P< 0.05$), which in turn were lower than the rate of Control group (33.9%). Mean cell number was also reduced from 118.2 ± 5.6 (Control group) to70.4 ± 4.3 (DZNep D3-D5), 51.5 ± 2.5 (DZNep D5-D8) and 36.3 ± 1.6 (DZNep D3-D8; $P< 0.01$). Embryos treated with DZNep also had development delayed, evidenced by fewer embryos at later stages of development. DZNep did not influence mRNA expression of SUZ12, NANOG, OCT4 and CDX2. However, DZNep D3-D8 had higher mRNA levels of EZH2 and EED when compared to the Control group. Results from Experiment 1 suggest that the epigenetic marks of H3K27 are globally remodeled during porcine embryogenesis. Furthermore, the low levels of H3K27me and high levels of H3K27ac indicate a transcriptionally active chromatin state during porcine early embryonic development. With the results obtained from Experiment 2, it is possible to conclude that PRC2 is important for embryo development, considering that DZNep decreased blastocyst rates, blastocyst cell number and speed of development. Exposure of embryos to DZNep from days 3 to 8 increased mRNA expression of the Polycomb enzymes EZH2 and EED.

**Keywords:** Epigenetics. Polycomb. Histone. Embryo. IVEP.

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## LISTA DE ABREVIATURAS E SIGLAS

| | |
|---|---|
| 2C | *twocells* - duas células |
| 4C | *four cells* - quatro células |
| 8C | *eightcells* - oito células |
| ACTB | *beta-actin* - beta-actina |
| AdoHcy | *S-adenosylhomocysteine* - S-adenosil-homocisteína |
| ADP | adenosina difosfato |
| ATH | acetiltransferases de histonas |
| ATP | adenosina trifosfato |
| BSA | *bovineserumalbumin* - albumina sérica bovina |
| CDX2 | *caudal type homeobox 2* - homeobox 2 tipo caudal |
| $CO_2$ | *carbondioxide*– gáscarbônico |
| COCs | *cumulus–oocytecomplexes* - complexos *cumulus*-oócito |
| DACH | deacetilases de histonas |
| DAPI | *4',6-diamidino-2-phenylindole* - 4 ', 6-diamidino-2-fenilindole |
| dbcAMP | *dibutyryl cyclic adenosine monophosphate* - dibutirilmonofosfatocíclico de adenosina |
| DNA | *deoxyribonucleicacid* - ácido desoxirribonucleico |
| DZNep | 3-Deazaneplanocin A |
| EED | *embryonicectodermdevelopment* - desenvolvimento do ectoderma embrionário |
| EGA | *embryonicgenomeactivation* - ativação do genoma embrionário |
| EGF | *epidermalgrowthfactor* - fator de crescimento epidermal |
| EZH2 | *enhancerofzestehomolog 2* - homólogo do intensificador do zeste2 |
| FCS | *fetal calfserum* - soro fetal bovino |
| FSH | *follicle-stimulating hormone* -hormôniofolículoestimulante |
| GV | *germinal vesicle* - vesículagerminativa |
| H3K27 | lisina 27 da histona H3 |
| H3K27ac | acetilação da lisina 27 da histona H3 |
| H3K27me1 | monometilação da lisina 27 da histona H3 |
| H3K27me2 | dimetilação da lisina 27 da histona H3 |
| H3K27me3 | trimetilação da lisina 27 da histona H3 |
| H3K36 | lisina 36 da histona H3 |

| | |
|---|---|
| H3K4 | lisina 4 da histona H3 |
| H3K4me3 | trimetilação da lisina 4 da histona H3 |
| H3K79 | lisina 79 da histona H3 |
| H3K9 | lisina 9 da histona H3 |
| H4K20 | lisina 20 da histona H4 |
| HMTase | *histone methyltransferase* - metiltransferase de histonas |
| hpf | *hours post fertilization* - horas pós-fecundação |
| ICM | *inner cell mass* - massacelularinterna |
| IVEP/PIVE | *in vitro embryoproduction* - produção *in vitro* de embriões |
| IVC/CIV | *in vitro culture* - cultivo *in vitro* |
| IVF/FIV | *In vitro fertilization* - fecundação*in vitro* |
| IVP/PIV | *in vitro-produced* - produzido*in vitro* |
| LH | *luteinizing hormone* - hormônioluteinizante |
| LSD1 | *lysinespecificdemethylase 1* - demetilase específica de lisinas |
| MII | *metaphase II* - metáfase II |
| mRNA | *messenger RNA* - RNA mensageiro |
| NANOG | *NANOG gene* - gene NANOG |
| OCT4 | *octamer-bindingtranscriptionfactor 4* - fator de transcrição 4 vinculativo a octâmeros |
| PBS | *phosphatebufferedsolution* - solução tamponada com fosfato |
| PC | Polycomb |
| PcG | *Polycombgroup* - grupo Polycomb |
| PCR | *polymerasechainreaction* - reação em cadeia da polimerase |
| PH | *polyhomeotic* - poli-homeótico |
| PN | *pronuclus/pronuclei/pronuclear* - pronúcleo/pronúcleos/pronuclear |
| PRC1 | *Polycombrepressivecomplex 1* - complexo repressivo Polycomb1 |
| PRC2 | *Polycombrepressivecomplex 2* - complexo repressivo Polycomb2 |
| PTM/MPT | *post-translationalmodifications* - modificações pós-traducionais |
| PZM | *porcinezygotemedium* - meio para zigotos suínos |
| RING1 | *ring finger protein 1* - proteína*ring finger* 1 |
| RNA | *ribonucleicacid* - ácido ribonucleico |
| RPM | *rotations per minute/* rotações por minuto |
| rRNA | *ribosomal RNA* - RNA ribossômico |
| SUZ12 | *suppressorofzeste 12 homolog* - homólogo do supressor do zeste 12 |

| | |
|---|---|
| TBM | *Tris-bufferedmedium/* meio tamponado com Tris |
| TCM | *tissueculturemedium* - meio para cultivo de tecidos |
| TE | *trophectoderm* - trofectoderma |

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# 1 INTRODUÇÃO

Todas as células de um animal, com poucas exceções, carregam a mesma sequência de DNA, originada do zigoto formado após a fecundação. Entretanto, complexos mecanismos epigenéticos garantem o desenvolvimento normal do indivíduo por meio da regulação da expressão dos genes apropriados a cada tipo celular. Cada tecido possui uma espécie de assinatura epigenética que reflete um padrão de expressão gênica, resultando em diferentes estruturas e funções entre células geneticamente idênticas (Boland *et al.*, 2014).

As alterações de histonas desempenham papel fundamental no desenvolvimento embrionário e falhas no estabelecimento e remoção destas marcações podem ser letais para embriões mamíferos no estágio de pré-implantação (Beaujean, 2014a). Em especial, as marcações epigenéticas da lisina 27 da histona H3 (H3K27) têm sido apontadas como reguladoras do desenvolvimento embrionário inicial, participando ativamente da determinação da pluripotência e das linhagens celulares (Hammoud *et al.*, 2009; Zhou *et al.*, 2014). O silenciamento dos genes relacionados à diferenciação nas células pluripotentes, estabelecido pela ação das enzimas do grupo Polycomb através da trimetilação da H3K27, é posteriormente revertido e permite a especificação dos tipos celulares no momento oportuno(Jorgensen *et al.*, 2006). Entretanto, este processo não é inteiramente conhecido.

Recentemente, as alterações relacionadas à H3K27, bem como as enzimas Polycomb, têm atraído a atenção científica como moduladoras da diferenciação de células embrionárias e de células-tronco em mamíferos (Creppe *et al.*, 2014; Thornton *et al.*, 2014). O conhecimento mais aprofundado do funcionamento destas proteínas e da sua importância no desenvolvimento embrionário pode representar uma estratégia para aumentar a eficiência de biotecnologias como a produção *in vitro* (PIV) de embriões, a transferência nuclear de células somáticas e os métodos de diferenciação de células-tronco para terapias paciente-específicas.

Desta forma, este trabalho teve como objetivo investigar o papel das alterações epigenéticas da H3K27 no desenvolvimento embrionário de mamíferos, utilizando embriões suínos e bovinos como modelos experimentais. Para tanto, foram realizados dois experimentos. O Experimento 1 objetivou avaliar o perfil de alterações da H3K27 (acetilação e metilação simples, dupla e tripla) em oócitos e no desenvolvimento inicial de embriões suínos PIV. O Experimento 2 teve como objetivo investigar o efeito da adição do 3-Deazaneplanocin A (DZNep), inibidor do complexo repressivo Polycomb 2, ao meio de cultivo embrionário em diferentes períodos do cultivo no desenvolvimento de embriões e expressão de mRNA de genes codificadores das enzimas Polycomb, bem como de genes relacionados à pluripotência e diferenciação celular em bovinos.

# 2 REVISÃO DE LITERATURA

Os mecanismos reguladores da expressão gênica de organismos eucariotos permanecem pouco compreendidos. Embora as diferenças genéticas possam elucidar divergências entre espécies, não explicam como mais de 200 tipos celulares de um mesmo organismo possuem perfis variados de expressão, específicos a cada tipo celular (Inbar-Feigenberg *et al.*, 2013). Visando a compreensão dos fatores não genéticos moduladores do fenótipo, a epigenética tem sido definida como o estudo das alterações nucleares herdáveis por meio de divisões celulares não relacionadas a alterações na sequência de DNA (Holliday, 1994).

As alterações epigenéticas incluem metilação de DNA, modificações de histonas, RNAs não codificantes e remodeladores da cromatina, cuja transmissão se dá por mitose e em alguns casos meiose (Wu e Morris, 2001; Bonasio *et al.*, 2010). Estes mecanismos definem o local e o momento no qual os fatores de transcrição têm acesso ao DNA, conduzindo o embrião em desenvolvimento ao crescimento e diferenciação apropriados (Reik, 2007).

## 2.1 MODIFICAÇÕES PÓS-TRADUCIONAIS DE HISTONAS

No núcleo das células eucarióticas, o DNA é altamente comprimido e compactado por proteínas histonas e não histonas, formando a cromatina (Li e Reinberg, 2011). Os diferentes níveis de organização da cromatina dependem da dinâmica estruturação dos nucleossomos, a unidade básica da cromatina (Inbar-Feigenberg *et al.*, 2013). Cada nucleossomo consiste em um octâmero com duas unidades de cada uma das histonas H2A, H2B, H3 e H4; mais a histona de ligação H1. O octâmero de histonas é circundado pelo DNA, garantindo estabilidade à estrutura e capacidade de regular a expressão gênica (Inbar-Feigenberg *et al.*,

2013). As histonas são pequenas proteínas básicas formadas por um domínio globular e uma cauda amino-terminal, capaz de projetar-se do seu próprio nucleossomo e interagir com os nucleossomos adjacentes (Beaujean, 2014b).

As histonas podem sofrer modificações pós-traducionais (MPT) nas caudas amino-terminais, como metilação, acetilação, fosforilação, sumoilação, ubiquitinação e ADP-ribosilação. Cada MPT das histonas é catalisada por enzimas específicas, as quais podem inserir ou remover cada modificação (Kouzarides, 2007). As MPT de histonas são encontradas em posições estratégicas ao longo do genoma, como regiões codificadoras, promotoras e potencializadoras. Nestas posições, além de modularem a cromatina através da ativação/inativação dos genes, também exercem papéis fundamentais na replicação e reparo do DNA e na condensação dos cromossomos (Kouzarides, 2007; Bannister e Kouzarides, 2011).

As MPT de histonas controlam o contato entre o nucleossomo e o DNA, levando à compactação ou ao relaxamento da cromatina. Em regiões menos compactas, o DNA está mais acessível à transcrição e à replicação, formando a eucromatina. Em regiões mais compactas,os genes estão transcricionalmente silenciados, caracterizando a heterocromatina (Tamaru, 2010). Além de exercerem influência direta sobre o nível de compactação da cromatina, as MPT de histonas recrutam enzimas remodeladoras que utilizam energia derivada da hidrólise de ATP para reposicionar os nucleossomos(Bannister e Kouzarides, 2011).

A acetilação é a alteração com o maior potencial para descompactar a cromatina, uma vez que neutraliza a carga básica das lisinas presentes nas caudas das histonas, atenuando a interação eletrostática entre histonas e DNA(Hasan e Hottiger, 2002). Desta forma, a cromatina assume uma estrutura menos resistente, facilitando o acesso ao DNA das proteínas envolvidas na transcrição. A acetilação de lisinas é uma alteração altamente dinâmica,

tipicamente encontrada na eucromatina e regulada por duas classes de enzimas: acetiltransferases de histonas (ATH) e deacetilases de histonas (DACH) (Wang *et al.*, 2014).

Diferentemente da acetilação, a metilação é uma pequena modificação neutra (não altera a carga das histonas) e pode ocorrer em todos os resíduos básicos: argininas, lisinas e histidinas. Lisinas podem ser mono-, di- ou trimetiladas e as posições mais estudadas de metilação em lisinas são histona H3 lisina 4 (H3K4), H3K9, H3K27, H3K36, H3K79 e H4K20 (Greer e Shi, 2012). Acetilação e metilação de histonas participam intensamente da formação de estados de ativação e repressão dos genes durante a embriogênese e foliculogênese, contribuindo para o estabelecimento dos padrões adequados de expressão gênica necessários para o desenvolvimento embrionário (Seneda *et al.*, 2008; Rodriguez-Sanz *et al.*, 2014).

Pelo fato de o grupo metil apresentar rotatividade mais lenta do que as outras MPT, a metilação de histonas foi por muitos anos considerada uma modificação estável e estática, sendo considerada até mesmo irreversível(Byvoet *et al.*, 1972). Entretanto, a identificação da H3K4 demetilase LSD1 *(lysinespecificdemethylase1)*em 2004 revelou quea metilação de histonas é, de fato, reversível (Shi *et al.*, 2004). Atualmente, um grande número de metiltransferases e demetilases têm sido identificadas, cuja função é mediar a adição e a remoção de grupos metil dos diferentes resíduos de lisinas em histonas. Dependendo do contexto no qual a célula se encontra, algumas metilações de lisinas necessitam ser mantidas de maneira estável, como em casos de metilações envolvidas na herança mitótica de um estado silenciado de heterocromatina. Em outro contexto, as metilações são passíveis de alterações, como na diferenciação celular ou na resposta celular a alterações do ambiente(Greer e Shi, 2012). Interessantemente, existem mais domínios reconhecedores de metilações de lisinas do que de qualquer outra MPT, refletindo a importância desta alteração (Bannister e Kouzarides, 2011).

A localização do radical metil na cauda de uma histona, bem como o grau de metilação, simples (me1), dupla (me2) ou tripla (me3), tem sido associada a diferentes estados de expressão dos genes. Por exemplo, H3K4me3 está geralmente associada à ativação da transcrição enquanto H3K27me3 está associada à repressão (Santos-Rosa *et al.*, 2002; Erhardt *et al.*, 2003).

Modificações epigenéticas na lisina 27 da histona H3 parecem exercer grande influência sobre o desenvolvimento embrionário (Goke *et al.*, 2011; Cotney *et al.*, 2012; Bogdanovic *et al.*, 2013). A H3K27me3 tem sido alvo de diversos estudos; porém, as funções da H3K27me1 e da H3K27me2 permanecem menos conhecidas. A H3K27me2 parece estar conectada ao silenciamento de *enhancers* não específicos, enquanto a H3K27me1 está associada à ativação gênica (Barski *et al.*, 2007; Ferrari *et al.*, 2014). A acetilação da H3K27 (H3K27ac), por sua vez, está presente em promotores de genes ativos e é bioquimicamente incompatível com a metilação (O'meara e Simon, 2012).

## 2.2 Proteínas do Grupo Polycomb

A H3K27me3 é estabelecida por enzimas do grupo Polycomb e está associada ao silenciamento estável e herdável dos genes (Ross *et al.*, 2008). Nas células pluripotentes, os genes relacionados ao desenvolvimento e à diferenciação celular têm a sua transcrição inibida pela H3K27me3 (Rada-Iglesias *et al.*, 2011). As proteínas Polycomb também são conhecidas pelo seu papel no estabelecimento das linhagens celulares, *imprinting*genômico, inativação do cromossomo X e tumorigênese(Rougeulle *et al.*, 2004; Bracken e Helin, 2009; Gieni e Hendzel, 2009).

Diferentemente da maior parte das metilações de histonas, catalisadas por várias enzimas diferentes, a H3K27me3 possui uma única metiltransferase conhecida: a enzima

*enhancerofzestehomolog2*(EHZ2) (Kuzmichev *et al.*, 2002).A EZH2 faz parte do complexo repressivo Polycomb2 (PRC2), juntamente com as enzimas *embryonicectodermdevelopment* (EED)e *suppressorofzeste 12 homolog* (SUZ12), todas indispensáveis para o desenvolvimento embrionário (Faust *et al.*, 1995; O'carroll *et al.*, 2001; Pasini *et al.*, 2004). O complexo repressivo Polycomb1 (PRC1) é composto principalmente pelas proteínas PC *(polycomb)*, PH *(polyhomeotic)* e RING1 *(ringfingerprotein 1)*(Ross et al., 2008).

Ambos os complexos são recrutados em locais específicos da cromatina para induzir e manter a repressão transcricional. A função do PRC2 é catalisar a metilação da H3K27, a qual recruta o PRC1, atingindo e propagando de forma estável o estado de silenciamento gênico (Cao *et al.*, 2002; Schuettengruber *et al.*, 2007).

A ação das proteínas do PRC2 pode ser interrompida pelo 3-Deazaneplanocin A (DZNep), causando a subsequente inibição da H3K27me3 (Tan *et al.*, 2007). Em muitos tipos de câncer há uma superexpressão da metiltransferase EZH2, levando ao silenciamento de genes supressores de tumores. O tratamento com DZNep resultou na redução da expressão da proteína EZH2 e consequentemente da H3K27me3, levando à morte das células cancerígenas (Tan *et al.*, 2007; Benoit *et al.*, 2013). O cultivo de embriões de *zebrafish* com DZNep também resultou em redução dos níveis de H3K27me3 (Ostrup et al., 2014).

## 2.3 Genes Reguladores do Desenvolvimento Embrionário

### 2.3.1 OCT4

O fator de transcrição OCT4, codificado pelo gene OCT4 ou Pou5f1, pertence à família Pou de fatores de transcrição e é considerado um importante regulador da pluripotência celular (Osorno *et al.*, 2012). A expressão de OCT4 é ativada previamente ao estágio de 8 células em embriões murinos, e é abundante e uniforme em todas as células no

estágio de mórula(Rosner *et al.*, 1990; Palmieri *et al.*, 1994). Entretanto, no momento em que as células externas diferenciam-se em trofectoderma (TE), os níveis de expressão de OCT4 são reduzidos e passam a ser restritos às células da massa celular interna (MCI) no blastocisto(Rosner *et al.*, 1990).

O gene OCT4 é considerado de grande importância no estabelecimento de totipotência e pluripotência celular, e a redução da sua expressão tem sido relacionada à perda da pluripotênciacelular (Jaenisch e Young, 2008; Osorno *et al.*, 2012).

### 2.3.2 NANOG

O gene NANOGcodifica uma proteína pertencente a um grupo de fatores de transcrição envolvidos na manutenção da pluripotência em células indiferenciadas, juntamente com OCT4 e SOX2(Wang *et al.*, 2013).

A expressão do gene NANOG está presente em células pluripotentes e ausente em células diferenciadas (Chambers *et al.*, 2003). A diminuição dos níveis de expressão do NANOG induz a diferenciação celular, sugerindo um papel fundamental na pluripotência embrionária desempenhado por este gene(Hyslop *et al.*, 2005). Existem evidências de que NANOG e OCT4 exercem funções complementares na manutenção da pluripotência celular (Wang *et al.*, 2013).

A atividade do gene NANOG parece sofrer influência dos genes OCT4 e SOX2 e de fatores epigenéticos, como a H4 histona acetiltransferase e H3K4 metiltransferase(Ang *et al.*, 2011; Li *et al.*, 2012). A enzima Polycomb EZH2 também participa da regulação deste gene, e atrimetilação da H3K27 na região promotora do gene NANOG, estabelecida pela EZH2, está inversamente correlacionada à sua expressão em células-tronco (Villasante *et al.*, 2011).

### 2.3.3 CDX2

CDX2 (*caudal typehomeobox2*) é um fator de transcrição pertencente à família de genes *caudal typehomeobox*. A proteína CDX2 é expressa em células do epitélio intestinal e está envolvida no crescimento e diferenciação celular, regulando o desenvolvimento do trato intestinal em embriões em estágio inicial de desenvolvimento(Yan *et al.*, 2014).

A expressão do CDX2 é indispensável para a diferenciação e funcionamento adequado do trofectoderma e falhas na regulação deste gene estão relacionadas à incapacidade de implantação embrionária(Meissner e Jaenisch, 2006; Wu *et al.*, 2010).

Embriões murinos portadores de uma mutação no gene CDX2 são capazes de originar células-tronco embrionárias, mas falham em formar o trofectoderma(Chawengsaksophak *et al.*, 2004). Em humanos, foi sugerido que provocar uma mutação no gene Cdx2 em células somáticas previamente à transferência nuclear poderia formar blastocistos capazes de produzir células–tronco, porém incapazes de se desenvolverem em seres humanoscompletos(Hurlbut, 2005).

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# 3 HIPÓTESES

As marcações epigenéticas da H3K27 (acetilação, mono-, di- e trimetilação) são dinâmicas e apresentam variação durante a maturação oocitária e o desenvolvimento inicial de embriões suínos.

A inibição do complexo repressivo Polycomb2 (PRC2) pelo DZNep prejudica o desenvolvimento embrionário bovino e altera a expressão de mRNA dos genes EZH2, EED, SUZ12, OCT4, NANOG e CDX2.

# 4 OBJETIVOS

## 4.1 OBJETIVOS GERAIS

- Avaliar o perfil de alterações da H3K27 em oócitos e embriões suínos produzidos *in vitro;*
- Avaliar o efeito da adição do DZNep, inibidor das enzimas do PRC2, no desenvolvimento de embriões bovinos produzidos *in vitro*.

## 4.2 OBJETIVOS ESPECÍFICOS

- Investigar o perfil de acetilação da H3K27 em oócitos e embriões suínos produzidos *in vitro;*
- Investigar o perfil de mono-, di- e trimetilação da H3K27 em oócitos e embriões suínos produzidos *in vitro;*
- Verificar o efeito da adição do DZNep ao meio de cultivo embrionário em diferentes períodos na taxa de blastocisto, número de células e estágio de desenvolvimento de embriões bovinos produzidos *in vitro;*
- Verificar o efeito da adição do DZNep ao meio de cultivo embrionário em diferentes períodos na expressão de mRNA dos genes EZH2, EED, SUZ12, OCT4, NANOG e CDX2 em embriões bovinos produzidos *in vitro*.

# 5 ARTIGOS PARA PUBLICAÇÃO

ARTIGO 1

ACETYLATION AND METHYLATION PROFILES OF H3K27 DURING IVP PORCINE EMBRYO DEVELOPMENT

# Acetylation and methylation profiles of H3K27 during IVP porcine embryo development

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**Abstract**

Methylation and acetylation of histone H3 lysine 27 (H3K27) play an important role in regulating chromatin structure and gene expression in early embryo development. Acetylation of H3K27 (H3K27ac) is associated with active gene expression, whereas H3K27 methylation (H3K27me) is closely linked with transcriptional repression. The aim of this study was to investigate the profile of acetylation and methylation (mono-, di- and trimethyl) of H3K27 during oocyte maturation and early development of IVP porcine embryos. Oocytes/embryos were fixed at different developmental stages from germinal vesicle to D8 blastocysts and submitted to an immunocytochemistry protocol to identify the epigenetic marks. H3K27ac was strongly observed in most of the development stages, reaching a minimum at the 8-cell stage. H3K27me1 and me2 signals were detected in oocytes, decreased through cleavage stages and rose again at the blastocyst stage. Similarly, H3K27me3 was detected in oocytes and showed very weak signal in early stages of embryo development. However, different patterns of the H3K27me3 mark were observed at the blastocyst stage. Our results suggest that H3K27 epigenetic marks are globally remodeled during porcine embryogenesis. Furthermore, the low levels of H3K27me and high levels of H3K27ac indicate a transcriptionally active chromatin state during preimplantation embryonic development.

Keywords: epigenetics; histone; pig; IVEP

## Introduction

Epigenetic phenomena are highly responsible for programming early post-fertilization embryos, essentially by determining and maintaining cell lineage. Events that are decisive for the process of embryogenesis in mammals, like genetic imprinting, inactivation of the X chromosome and reprogramming of the two parental haploid genomes into one diploid genome are mainly regulated by epigenetic mechanisms (Morris, 2009; Hales *et al.*, 2011).

Histone post-translational modifications are found throughout the genome and are known to modulate chromatin structure and gene expression (Beaujean, 2014). Histone acetylation usually occurs on the lysine residues of core histones and neutralizes the basic charge of lysine residues, therefore decreasing their affinity for DNA (Hasan e Hottiger, 2002). This mark is almost invariably associated with activation of gene transcription in cells and is involved in regulation of totipotency and proliferation events during cell reprogramming and embryo development (Wang *et al.*, 2007; Rodriguez-Sanz *et al.*, 2014). H3K27 acetylation (H3K27 ac), particularly, plays an important role in stimulation of pluripotency and regulation of key developmental genes in stem cells (Creyghton *et al.*, 2010; Pasini *et al.*, 2010).

The exact role of mono- and dimetylated H3K27 (H3K27me1 and me2, respectively) remains to be investigated. However, H3K27me1 appears to be associated to gene activation, while H3K27me2 has been connected to silencing of nonspecific enhancers (Barski *et al.*, 2007; Ferrari *et al.*, 2014). On the other hand, trimethylation of H3K27 (H3K27me3)is known to be strongly associated with transcriptional repression, promoting stable and heritable gene silencing (Schwartz e Pirrotta, 2007). This epigenetic mark regulates lineage specification by temporarily repressing genes involved in development and cell differentiation of pluripotent

cells (Surface *et al.*, 2010; Shpargel *et al.*, 2014). Genes associated to organogenesis, morphogenesis and embryonic development are temporarily suppressed by H3K27me3 until the moment when their transcription is required (Boyer *et al.*, 2006).

It is well established that mammalian embryos undergo several epigenetic modifications during early development(Boland *et al.*, 2014). Nonetheless, the regulatory mechanism of lineage commitment is still not fully known. Understanding how individual species organize their chromatin following fertilization is fundamental for understanding the epigenetic regulation of early embryonic development. Furthermore, knowing the molecular mechanisms of pluripotency that rule stem cell self-renewal is greatly promising for the development of stem-cell based therapies.

Therefore, the objective of this study was to evaluate the profile of acetylation and methylation (mono-, di- and trimethyl) of histone H3 at lysine 27 during oocyte maturation and early development of in vitro-produced porcine embryos.

## Materials and Methods

Unless otherwise indicated, chemicals were purchased from Sigma Chemical Company (Sigma-Aldrich, Oakville, ON, Canada).

### *Oocyte collection and in vitro maturation*

Ovaries from prepubertal gilts were collected from a local abattoir (Olymel S.E.C./L.P.) and transported to the laboratory for approximately one hour in 0.9% NaCl at 30 to 35 °C. Cumulus–oocyte complexes (COCs) were aspirated from 3 to 6-mm diameter

follicles using an 18-gauge needle. Only COCs surrounded by a minimum of three cumulus-cell layers, with an evenly granulated cytoplasm were selected for in vitro maturation (IVM). Groups of 20 to 25 COCs were cultured in 100 µL of maturation medium under mineral oil, in a humidified atmosphere of 5% $CO_2$ and 95% air at 38.5 °C. Maturation medium consisted of TCM 199 (Life Technologies, Burlington, ON, Canada), supplemented with 0.1 mg/mL cysteine, 0.91 mM sodium pyruvate, 3.05 mM D-glucose, 0.5 µg/mL follicle-stimulating hormone (FSH; SIOUX Biochemical Inc.), 0.5 µg/mL luteinizing hormone (LH, SIOUX Biochemical Inc.), 10 ng/mL epidermal growth factor (EGF; Life Technologies), 20 µg/mL gentamicin (Life Technologies), 1 mMdibutyryl cyclic adenosine monophosphate (dbcAMP), and 20% (v/v) porcine follicular fluid. After 22 h, the oocytes were transferred to the same maturation medium, but without FSH, LH, and dbcAMP for additional 22 to 24 h, under the same conditions.

*In vitro fertilization (IVF) and in vitro culture (IVC)*

Matured oocytes were freed from cumulus cells by vortexing in Hepes-buffered (Life Technologies) medium supplemented with 0.1% hyaluronidase for 7 min. The denuded oocytes were washed in Hepes-buffered medium before being transferred to fertilization media.

For fertilization, the oocytes were washed twice in fertilization medium, which consisted of Tris-buffered medium (TBM) supplemented with 2.5 mg/mL fatty acid-free bovine serum albumin (BSA), and placed in 90 µL drops of the same medium. A pool of fresh semen collected from multiple males was diluted in the extender gDILand left at 18 °C for 24 h and tubes with 1 mL were submitted to centrifugation at 4,000 RPM for 3 min. Supernatant

was discarded and the sperm pellet was re-suspended in 1 mL of fertilization medium and homogenized. The semen was centrifuged again (4,000 RPM for 2 min), the supernatant was discarded and the sperm pellet was re-suspended in 500 μL of fertilization medium. Sperm concentration was adjusted to obtain a final concentration of 2,000 live sperm/oocyte. Sperm and oocytes were co-incubated for 4 h under mineral oil at 38.5 °C, 5% $CO_2$ in air and 100% humidity.

After IVF, the oocytes were washed three times in PZM-3 supplemented with 3 mg/mL BSA and then cultured in the same medium under mineral oil. Culture conditions were humidified atmosphere of 5% $CO_2$ and 95% air at 38.5 °C.

*Immunocytochemistry and experimental design*

Oocytes and embryos were fixed at the following developmental stages: germinal vesicle (GV) and metaphase II (MII) oocytes, pronuclear (PN; 18 hours post fertilization - hpf), two-cell (2C), four-cell (4C), eight-cell (8C), D6 blastocysts (D6) and D8 blastocysts (D8). Samples were rinsed in PBS, fixed in 4% paraformaldehyde for 15 to 20 min and stored at 4°C in PBS with 0.2% TritonX-100 and 0.3% BSA. Cell permeabilization was performed with 0.5% TritonX-100 in PBS with 0.3% BSA for 1 h at 37°C. Oocytes/embryos were then washed twice (10 min each) in blocking solution (3% BSA and 0.2% Tween-20 in PBS) and exposed overnight at 4°C to the primary antibodies diluted in blocking solution (1:1000). Polyclonal rabbit anti-H3-K27 acetyl (Abcam; ab4729), anti-monomethyl H3-K27 (Upstate; 07-448), anti-dimethyl H3-K27 (Upstate; 07-452), and anti-trimethyl H3-K27 (Upstate; 07-449) were used as primary antibodies. Samples were then washed three times for 20 min each in blocking solution and incubated for 2 h at room temperature in the presence of 1:1000

diluted Alexa Flour 488 goat antirabbit (Molecular Probes, Eugene, OR, USA) secondary antibodies. Finally, the samples were washed three times (20 min each) in blocking solution, the second one with 10 µg/mL DAPI (4',6-diamidino-2-phenylindole) in PBS for DNA staining. Oocytes/embryos were mounted on microscope slides using a drop of Mowiol and examined by epifluorescence using a Nikon eclipse 80i microscope (Nikon, Tokyo, Japan) with 200x magnification, and images were individually recorded using a Retiga 2000R monochromo digital camera (Qimaging, BC, Canada). All images were captured using the same settings and saved in TIFF format. The fluorescent signal was corrected to the background signal of negative controls embryos.

A total of 48 oocytes/embryos were evaluated for H3K27ac, 38 for H3K27me1, 32 for H3K27me2 and 65 for H3K27me3. Control groups were included in each immunostaining replicate, in which samples were processed as described above, except the primary antibody was omitted (samples were stained with only the secondary antibody).

**Results**

H3K27ac was detected in the nuclei of GVand in the DNA of MII oocytes. Strong fluorescent signals were also observed in both PN and in 2C and 4C stages. At the 8C stage, the signal reached a minimum. Intense signals were observed again in D6 and D8 blastocysts (Fig. 1).

The signal of H3K27me1 was intensely detected in GV and MII oocytes, and in one pronucleus. Fluorescent signal strongly decreased after PN stage, and was weak in 2C, 4C and 8C embryos. In D6 and D8 blastocysts, the signal became strong again (Fig. 1).

H3K27me2 presented with strong signal in GV and MII stage oocytes, but was absent in PN stage zygotes. Embryos at 2C, 4C and 8C stages showed very weak or absent signal. However, in D6 and D8 blastocysts, H3K27me2 mark was again observed (Fig. 1).

H3K27me3 was strongly detected in GV and MII oocytes. In PN-stage embryos, only one pronucleuswas tagged (Fig. 2). Two-cell, 4C, 8C embryos and D6 blastocysts showed very weak or absent fluorescent signal (Fig. 1). Of the 29 D8 blastocyst submitted to immunocytochemistry, 7 (24.1%) showed no fluorescent signal; 19 (65.5%) had some or all cells tagged, displaying no evident pattern; and 3 (10.3%) showed H3K27me3 only in the inner cell mass(ICM; Fig. 3).

**Discussion**

Epigenetic changes are known to regulate reprogramming of the information carried over by the oocyte and the sperm to the new developing embryo and to control totipotency and cell lineage commitment. Here we report the global profile ofH3K27 acetylation, mono-

di- and trimethylation during oocyte maturation and early development of porcine embryos. To our knowledge, this is the first report of H3K27me2profile during early development of porcine embryos.

Trimethylated H3K27 was detected in immature and matured oocytes, in accordance with the findings reported in porcine (Park *et al.*, 2009) and bovine oocytes (Ross *et al.*, 2008). In pronuclear (PN) stage zygotes, only one of the two PN was positive for this mark. It was most likely the feminine PN because polyspermic embryos displayed only one PN positive for H3K27me3. Ross et al. (Ross *et al.*, 2008) also observed an asymmetric staining pattern in bovine embryos at PN stage and affirmed that the feminine PN was the one that was positivefor H3K27me3. In the referred study, in addition to polyspermic embryos (where one PN is derived from the oocyte and the remaining is derived from the sperm) having only one PN positive for this mark, parthenogenetic embryos (in which both PN are derived from the oocyte) had all PN positively stained. The same patternof H3K27me3 staining was described for pronuclei of both in vivo- and IVP porcineembryos (Jeong *et al.*, 2007; Park *et al.*, 2009), as well as bovine (Breton *et al.*, 2010) and murine PN-stage embryos (Erhardt *et al.*, 2003; Santos *et al.*, 2005).

Trimethylated H3K27 fluorescence intensity was severely reduced in 2C, 4C and 8C embryos. This agrees with observations in pig (Park *et al.*, 2009) and bovine embryos (Ross *et al.*, 2008; Breton *et al.*, 2010). Here we report three different patterns of the H3K27me3 mark in D8 blastocysts: 1) embryos with no stained cells, 2) embryos with some or all of the cells stained and 3) embryos with only the ICM stained. Park et al. (Park *et al.*, 2009) did not detect H3K27me3 in porcine blastocysts. However, the referred study investigated the presence of this epigenetic markin blastocysts at D6 of development (144 hpf).On the other hand, Gao et al. (Gao *et al.*, 2010) reported that levels of H3K27me3 decreased from the 1-cell to the morula stage, whereit reached the lowest,with an increase of H3K27me3 levels in hatched

blastocysts. The present study together with the data reported by Gao et al. (Gao *et al.*, 2010) provide evidence that the H3K27me3 mark begins to appear in porcine embryos at later stages of development. Conversely, intense fluorescence was found in bovine (Ross *et al.*, 2008) and murine (Erhardt *et al.*, 2003) blastocysts.

Approximately 10% of the blastocysts analyzed in this study displayed a distinct pattern of H3K27me3 signal between the ICM and the trophectoderm. In all of them,only the ICM presented the H3K27me3 mark. A similar condition was reportedin murine embryos, with the pluripotent cells of ICM showing an intense fluorescent signal and the differentiated trophectoderm (TE) cells displaying a weak staining pattern (Erhardt *et al.*, 2003). Conversely, Gao et al. (Gao *et al.*, 2010) observed that the expression of H3K27me3 in porcine hatched blastocysts was higher in the trophectoderm compared to the ICM. These contradictory data might be due to different culture conditions of both studies, considering that the study performed by Gao's team investigated epigenetic marks in in vivo-generated embryos and the present study was performed with investigated IVP embryos. Alternatively, there could be an inversion of the ICM and trophectoderm staining patters in the later stages of embryogenesis, considering that the present study analyzed mostly D8 expanded unhatched blastocysts and Gao's study analyzed hatched embryos. In bovine embryos a difference in the H3K27me3 mark between ICM and TE cells was not observed, indicating that maybe in this species such difference is expressed at later stages of development (Ross *et al.*, 2008).

Monomethylated H3K27 was detectable in GV and MII-stage oocytes, in agreement with other observations in porcine embryos (Park *et al.*, 2009). However, in this study we found that H3K27me1 was present in only one PN, contradicting the data obtained by Park et al (Park *et al.*, 2009), who reported intense fluorescentH3K27me1 signal in both PN. This unconformity could be explained by different times of embryo fixation. The authors affirmed that H3K27me1 is initially detectable only in DNA within the maternal PN and becomes

detectable in the male pronucleus several hours after fertilization. In the referred study the embryos were collected 20 h after IVF, while in the present studythe embryos were fixed at 18 hpf. In mice embryos, data regarding H3K27me1 in PN are also controversial. Santos et al. (Santos *et al.*, 2005) reported that the male PN is also positive for H3K27me1, and the signal increased during pronuclear maturation. Conversely, Erhardt et al. (Erhardt *et al.*, 2003) found that this mark was strongly associated with the female PN at the onset of development, but staining of the paternal PN was observed hours later. Van der Heijden et al. (Van Der Heijden *et al.*, 2005), in turn, analyzed mouse zygotes until 210 min after fertilization and did not observe this mark in the paternal chromatin at this time window. The H3K27me1signal observed in this study from 2C until the blastocyst stage was also observed by Park et al. (Park *et al.*, 2009) in porcine embryos.

The H3K27me2 mark was not observed in PN stage zygotesin thepresent study. This disagrees with data obtained from murine embryos in which this mark was highly present in the feminine PN and observed several hours later in the male PN (Erhardt *et al.*, 2003). However, our findings regarding the presence of H3K27me2 in blastocysts are in agreement with the data reported in mice(Erhardt *et al.*, 2003).

H3K27acsignal was intensely observed in oocytes, in both PN and in all stages of embryo development analyzed, except for the 8C-stage embryos, which presented with a weak signal. A similar scenario was observed by Zhou et al. (Zhou *et al.*, 2014) in IVF porcine embryos, with a strong signal at the pronuclear stage and a decrease in the signal intensity during the 4-cell to 8-cell stages, the period of major genomic activation. The signal increased again at morula and blastocyst stages.

H3K27 methylation is catalyzed by the transcriptional repressors Polycomb group (PcG) proteins, which form two chromatin modifying complexes: Polycomb-Repressive

Complex 1 and 2 (PRC1 and PRC2) (O'meara e Simon, 2012; Williams *et al.*, 2014). Removal of this mark can make undifferentiated normal (Patel *et al.*, 2012) or cancerous cells (Gannon *et al.*, 2013; Ciarapica *et al.*, 2014) susceptible to differentiation.

Acetylation of lysine side chains has been reported to be biochemically incompatible with their methylation. H3K27me3 is a Polycomb repressed state, which is functionally opposed by actively transcribed chromatin (O'meara e Simon, 2012). H3K27ac is generally enriched in promoters of active genes (Tie *et al.*, 2009; O'meara e Simon, 2012) and can antagonize PcG activity by competing with the placement of the H3K27me3 mark (Schwartz *et al.*, 2010). Consistent with this, it has been shown that H3K27ac and H3K27me3 have dynamic and complementary temporal profiles during embryogenesis. H3K27ac is present at high levels in early embryos and decrease in later stages; during this same period, H3K27me3 levels increase (Tie *et al.*, 2009). Our findings seem to be in accordance with this information. H3K27ac was strongly observed during pre-implantation stages, while H3K27me3 signal was weak or absent. H3K27me3 mark seems to increase in some of the most developed blastocysts, period during which this transition might take place in porcine embryos.

In summary,the present study investigated the changes in H3K27 acetylation and mono-, di- and trimethylation during oocyte maturation and preimplantation development of IVP porcine embryos. Our observations evidence the differences in global patterns of epigenetic marks of the histone H3 at lysine 27 during embryo development. Low levels of H3K27 methylation and high levels of H3K27 acetylation during early cleavage stages indicate a state of open chromatin during preimplantation embryonic development.

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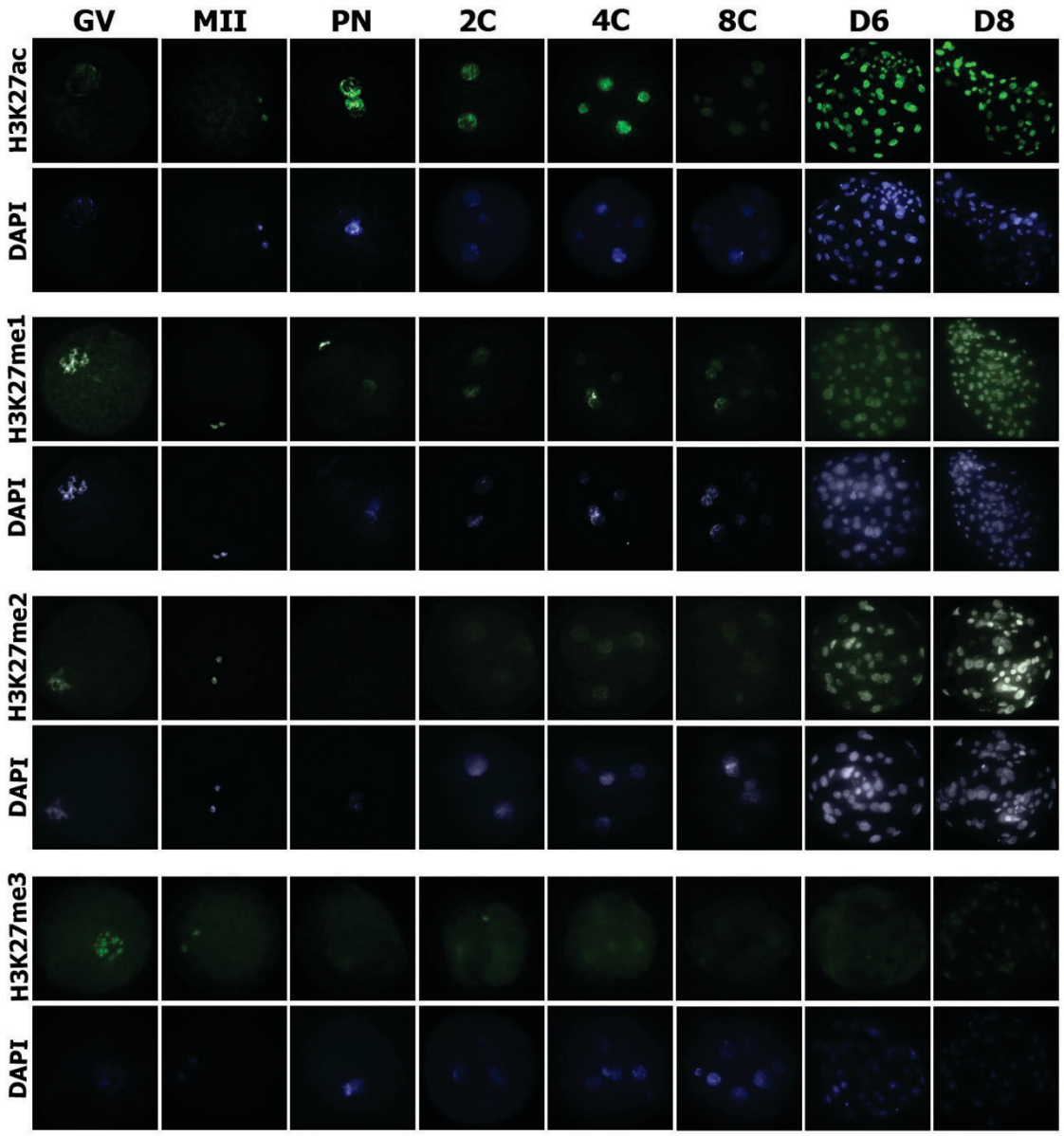


**Figure 1.** Distribution ofacetylated, mono-, di- and trimethylatedH3K27 in porcine oocytes and embryos. Images of oocytes at germinal vesicle (GV) and metaphase II (MII) stages, zygotes at pronuclear stage (PN), two-cell embryos (2C), four-cell embryos (4C), D6 blastocysts (D6) and D8 blastocysts (D8) are shown.

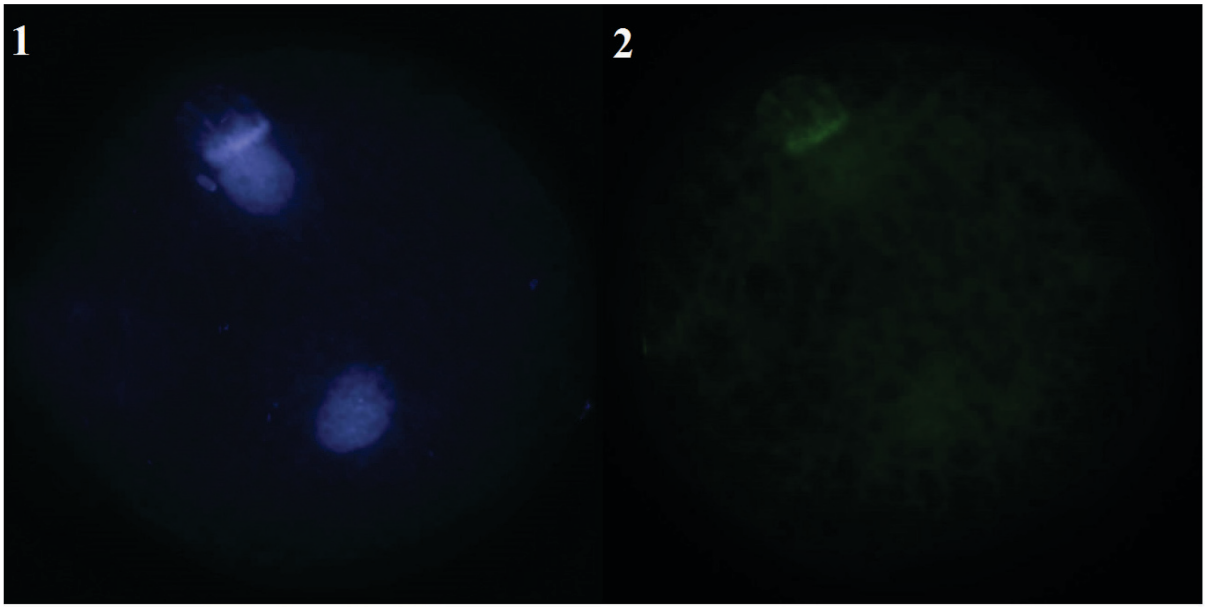


**Figure 2.** H3K27me3 immunofluorescence analysis of a polyspermic in vitro-produced porcine embryo at 18 hours after fertilization (1) DNA of three pronuclei stained with DAPI. (2) Fluorescent H3K27me3 signal only in one pronucleus.

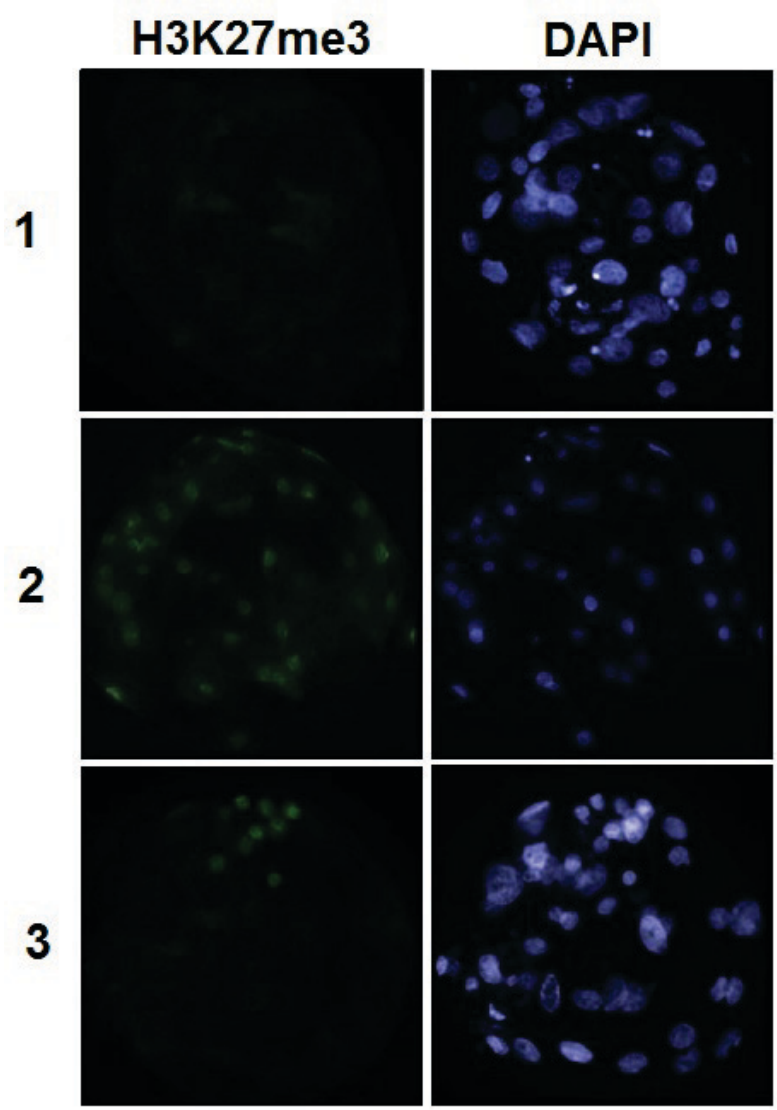


**Figure 3.**H3K27me3 immunofluorescence analysis of in vitro-produced porcine blastocysts at day 8 of development. (1) Embryo with no stained cells (24.1%). (2) Embryo with all blastomeres stained (65.5%). (3) Embryo with fluorescent signal only in the ICM cells (10.3%).

# ARTIGO 2

## PHARMACOLOGICAL DISRUPTION OF H3K27 TRIMETHYLATION BY DZNEP ALTERS MRNA EXPRESSION OF POLYCOMB ENZYMES AND IMPAIRS DEVELOPMENT OF IVP BOVINE EMBRYOS

# Pharmacological disruption of H3K27 trimethylation by DZNepalters mRNA expression of Polycomb enzymesand impairs development of IVP bovine embryos

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**Abstract**

Trimethylation of histone H3 on lysine 27 (H3K27me3) is established by Polycomb repressive complex 2 (PRC2) and is associated with stable and heritable gene silencing. In pluripotent cells, genes associated with development and cell differentiation are maintained repressed by H3K27me3; however, this process is not fully understood. This study used 3-Deazaneplanocin A (DZNep) to inhibit PRC2 and investigate the role of H3K27me3 in early development of bovine embryos. Cleaved embryos were exposed to DZNep from days 3 to 5 (DZNep D3-D5), from days 3 to 8 (DZNep D3-D8), from days 5 to 8 (DZNep D5-D8) or cultured without DZNep (Control). Blastocyst rates and total cell number were evaluated on day 8 and mRNA expression of the genes EZH2, EED, SUZ12, NANOG, OCT4 and CDX2 was assessed in blastocysts. DZNep D3-D8 had lower blastocyst rate (6.9%) than DZNep D3-D5 and DZNep D5-D8 (16.7 and 14.3%, respectively; $P< 0.05$), which in turn were lower than the rate of Control group (33.9%). Mean cell number was also reduced from 118.2 ± 5.6(Control group) to70.4 ±4.3 (DZNep D3-D5), 51.5 ± 2.5 (DZNep D5-D8) and 36.3 ± 1.6 (DZNep D3-D8; $P < 0.01$). Embryos treated with DZNep also had developmentdelayed, evidenced by fewer embryos at later stages of development. DZNep did not influence mRNA expression of SUZ12, NANOG, OCT4 and CDX2. However, DZNep D3-D8 had higher mRNA levels of EZH2 and EED than the Control group. These results demonstrate that PRC2 is important for bovine embryo development, considering that DZNep decreased blastocyst rates, embryo quality and speedof development. Also, exposure of embryos to DZNep from days 3 to 8 increased mRNA expression of the two Polycomb enzymes EZH2 and EED.

Keywords: Epigenetics, Polycomb, H3K27me3, EZH2, embryo, bovine.

## Introduction

Reprogramming of histone post-translational modifications (PTMs) established in gametes is required for proper embryonic development, especially during the onset of embryonic genome activation (Beaujean, 2014). Histone PTMs function as chromatin-based "on/off" switches that control DNA accessibility and the consequent gene-expression (Zentner e Henikoff, 2013). These marks also play important roles in DNA replication, repair and recombination (Bannister e Kouzarides, 2011).

Trimethylation of histone H3 at lysine 27 (H3K27me3) is an epigenetic mark strongly associated with stable and heritable gene silencing (Cao *et al.*, 2002). Pluripotent cells possess a distinctive chromatin structure that is characterized by H3K27me3 marks in the promoter regions of lineage-specific genes, resulting in a repressed state of genes associated with embryo development and cell differentiation (Bernstein *et al.*, 2006; Rada-Iglesias *et al.*, 2011).

A key transcriptional regulator in animals is the Polycomb Repressive Complex 2 (PRC2), which contains the enhancer of zeste homolog 2 (EZH2), a histone methyltransferase (HMTase) that specifically methylates H3 at lysine 27 (Cao *et al.*, 2002). Although EZH2 contains the HMTase activity, the other PRC2 components, embryonic ectoderm development (EED) and suppressor of zeste 12 homolog (SUZ12), are also required for this activity (Pasini *et al.*, 2004; Montgomery *et al.*, 2005) and vital for embryonic development (Faust *et al.*, 1995; O'carroll *et al.*, 2001; Pasini *et al.*, 2004).

The activity of PRC2 can be disrupted by the S-adenosylhomocysteine(AdoHcy) hydrolase inhibitor 3-Deazaneplanocin A (DZNep)(Ciarapica *et al.*, 2014; Girard *et al.*, 2014). DZNep appears to be a unique chromatin remodeling compound that can deplete cellular levels of PRC2 components and inhibit associated histone H3K27 trimethylation

(Vella *et al.*, 2013; Ciarapica *et al.*, 2014). Interestingly, DZNepeffectively induced cell death and prevented tumor progression in cancers in which EZH2 overexpression led to the inactivation of tumor suppressor genes (Tan *et al.*, 2007; Benoit *et al.*, 2013). Moreover, pharmacological inhibition of EZH2 using DZNep produced the same phenotypical result as the genetic knockdown of EZH2, leading to myogenic differentiation both in vivo and in vitro (Ciarapica *et al.*, 2014).

Although DZNep has been widely studied in cancer cells, its use in mammalian embryos has never been described. Blocking the activity of PRC2 withDZNep could be an interesting strategy to study the role of the Polycomb enzymes and H3K27me3 in embryonic development, particularly in cell pluripotency.

Early development of mammalian embryos highly depends on the influence of epigenetic control. Events that occur during the preimplantation period and determine embryonic survival such as genome activation, blastocoel formation and establishment of cell lineages require the correct epigenetic signaling for the activation of genes required for overcoming each phase(Bedzhov *et al.*, 2012; Graf *et al.*, 2014).

The aim of this study was to investigate the effect of DZNep added to embryo culture media at different periods of in vitro culture on embryo development and mRNA expression of the genes EZH2, EED and SUZ12, encoding the PRC2 enzymes; NANOG and OCT4, related to cellular pluripotency; and CDX2, involved in cell differentiation, of bovine in vitro-produced embryos.

## Materials and Methods

Unless otherwise indicated, chemical and reagents were purchased from Sigma Chemical Company (Sigma-Aldrich).

*Experimental design*

Oocytes were fertilized and cultured in vitro. On day 3 (D3), the cleaved embryos were separated into four treatments: embryos cultured with 5 μM 3-Deazaneplanocin A (DZNep) from days 3 to 5 (DZNep D3-D5), from days 3 to 8 (DZNep D3-D8), from days 5 to 8 (DZNep D5-D8) or without DZNep (Control group). The experiment was performed in 15 replicates and blastocyst ratesand evaluation of cell numberwere assessed at D8 of culture. Investigation of mRNA expression of the genes EZH2, EED, SUZ12, NANOG, OCT4 and CDX2 was also performed in D8 embryos.

*Oocyte recovery and in vitro maturation (IVM)*

Bovine abattoir-derived ovaries were collectedat a local abattoir, stored in saline solution at 25 °C to30 °C and transported to the laboratory for approximately half an hour. Briefly, each follicle was punctured witha disposable hypodermic needle(21-gauge 1 ¼ ’’) connected to a 10-mL syringe. The selected oocytes (n = 3,666) were matured for24 hours in 100 mL drops of TCM-199 (Gibco BRL; Grand Island, NY, EUA)supplemented with 10%

FCS (CripionBiotecnologia, Andradina, SP, Brazil), 1 µg/mL FSH (Pluset, HertapeCalier, Juatuba, MG, Brazil), 50 µg/mL LH (Vetecor, HertapeCalier), 1 µg/mL estradiol, 0.2 mM sodium pyruvate, and 83.4 mg/mL amikacin (Novafarma, Anápolis, GO, Brazil) under mineral oil at 38.5°C, 5%atmospheric $CO_2$ (15–20 oocytes per microdrop) and saturated humidity.

*In vitro fertilization (IVF) and in vitro culture (IVC)*

Frozen-thawed spermfromsires of knownfertility were used.Straws were thawed for 30 seconds in a 35 °C water bath(Sanches *et al.*, 2013). Semen (100 µL)wascarefully placed onto the top of a Percoll gradient of 45 and 90% (300 µL of each). Tubes were centrifuged at 13,400 rpm (12,100 x g) for 2 min. The supernatant was discarded and 300 µL of fertilization medium was added to the pellet of approximately 40 µL. The sperm were centrifuged again at 9,000 rpm (8,127 x g) for 45 seconds. After visual assessment of motility, the sperm concentrationwasadjusted to 1.5 x $10^6$ motile sperm/mL. IVF medium consisted of TALP-IVF medium thatwas supplemented with 10 g/mL heparin, 18 M penicillamine,10Mhypotaurine, and 8Mepinephrine. Sperm and oocytes were incubated for 18 to 22 hours in 100 µL drops (15–20 oocytes per microdrop)under mineral oil at 38.5°C and 5%atmospheric $CO_2$.

Presumptive zygotes had their cumulus cells removed (n = 3,240) and were transferred to 100 µL drops of embryo culturemedium (SOFaa BSA containing 0.5% BSA and 2.5% FCS)under the same temperature and gaseous atmosphericconditions that were used for IVF. After 3 days (D3) of culture, cleavage rates were evaluated and the cleaved embryos were transferred to plates containing new culture media. The embryos from groups DZNep D3-D5 and DZNep D3-D8 were placed in media containing 5 µM DZNep. At D5, all the embryos

were transferred to other plates containing new culture media. At this point, the embryos from groupDZNep D3-D8 were maintained in media containing DZNep and the embryos from group DZNep D5-D8 were transferred to media containing DZNep. The embryos from group DZNep D3-D5 were washed three times in culture media without DZNep and subjected to culture without DZNep until D8.

*Evaluation of cell number*

Total cell number was estimated by counting of cell nuclei stained with 4',6-diamidino-2-phenylindole(DAPI).At D8 of development, embryos were rinsed in PBS, fixed in 4% paraformaldehyde for 15 to 20 min and stored at 4°C in PBS with 0.2% TritonX-100 and 0.3% BSA. The fixed embryos were then exposed for 20 min to 10 µg/mL DAPI diluted in PBS for DNA staining, washed in PBS and mounted on microscope slides using a drop of Vectashield mounting media (Vector Labs, Burlingame, CA, United States). Nuclear counting was performed withepifluorescence microscopy (Axio Imager A1, Zeiss, Jena, TH, Germany).

*RNA extraction and qRT-PCR*

Total RNA was extracted from groups of 10D8-embryos (three replicates per treatment) using the PicoPure RNA Isolation Kit(Life Technologies, Burlington, ON, Canada) according to the manufacturer's instruction.RNA was treated with DNase I (Qiagen, Toronto, ON, Canada) and reversetranscribed usingSuperScript VILO cDNA Synthesis Kit

(LifeTechnologies). Quantitative real-time PCR reaction was performedusing a CFX384 Real-Time Detection System (Bio-Rad, Mississauga, ON, Canada),iQ SYBR Green Supermix (Bio-Rad), 400 nM of primers, and 2µl of cDNA. Primers (Table 1) were designed using Primer-Blast,and specificity was confirmed using BLAST (NCBI). Commonthermal cycling parameters (5 min at 95 °C and 40 cycles of 15s at 95 °C and 30 s at 60 °C) were used to amplify each transcript,and melting-curve analysis was used to verify the specificityof reaction products. Samples were run in duplicates, standard curve method was used to determine the abundance of mRNA for genes EZH2, EED, SUZ12, NANOG and OCT4, and expression was normalized to the abundanceof the housekeeping genesactin beta (ACTB) and 18S ribosomal RNA (18S rRNA).All reactions used for quantificationhad efficiency between 90 and 110%, $R2 \geq 0.98$ and slopevalues from −3.6 to −3.1.

*Statistical analysis*

For analysis of cleavage rates, blastocyst rates and rate of developmental stages at D8, the variable responses were presented as percentage and subjected to a logistic regression test using the Car statistical package of "R" software. The average numbers of cellswere presented as mean and standard error. Analyses concerning the average numbers of cells and mRNA levels were performed using the JMP Software (SASInstitute, Inc). Data were analyzed using ANOVA and the means were compared by Dunnett's test. Differences were considered statistically significant if $P< 0.05$.

## Results

### *Embryo development*

Total cleavage rate was 70.3% (2,277/3,240). The Control group had a higher blastocyst rate than the groups DZNep D3-D5, DZNep D3-D8 and DZNep D5-D8 (all *P* values < 0.01; Table 2).

Considering all blastocysts produced in each treatment, the Control group had proportionally more hatched embryos at D8 than DZNep D3-D5 and DZNep D5-D8 (both *P* values < 0.01); DZNep D3-D8 had no hatched blastocysts. Also, the Control group had fewer early blastocysts at D8 than DZNep D3-D8 (*P* = 0.016) and DZNep D5-D8 (*P* = 0.025; Table 3).

The embryos from the Control group had higher number of cells than the embryos from groups DZNep D3-D5, DZNep D3-D8 and DZNep D5-D8 (all *P* values < 0.01). Results of cell numbers are described in Table 4. Representative pictures of embryos from Control, DZNep D3-D5, DZNep D3-D8 and DZNep D5-D8 groups are shown in Figure 1.

### *mRNA expression of genes EZH2, EED, SUZ12, NANOG, OCT4 and CDX2*

Treatments with DZNep did not influence mRNA levels of genes SUZ12, NANOG, OCT4 and CDX2. However, exposure to DZNep from days 3 to 8 increased mRNA levels of

genes encoding the Polycomb enzymes EZH2 and EED. The results of relative mRNA abundance are described in Figure 2.

**Discussion**

Findings of this study show that culture of bovine embryos with DZNep, a well-known inhibitor of PRC2, strongly impairs embryo development and alters gene expression of EZH2 and EED, two enzymes required for trimethylation of H3K27. To the best of our knowledge, this is the first report of exposure of mammalian embryos to DZNep.

Treatment with DZNep drastically reduced blastocyst rates and total cell number. Also, DZNep delayed embryo development, especially when embryos were treated from days 3 to 8. Likewise, zebrafish embryos cultured with 10 μM DZNep had increased mortality rates and morphological defects (Ostrup *et al.*, 2014). In cultured cells, although DZNep induced death of several cancer cell lines, it appears to exert no harmful effect in normal cells (Tan *et al.*, 2007; Hung *et al.*, 2013), except for a slight decrease in growth of chondrocytes exposed for seven days to 1 μM DZNep(Girard *et al.*, 2014). Perhaps DZNepselectively induces cytotoxicity in undifferentiated cells types.

In accordance with reports in cultured cells (Azghadi e Clark, 2011; Gannon *et al.*, 2013), zebrafish embryos cultured with DZNep displayed a dose-dependent decrease in H3K27me3 (Ostrup *et al.*, 2014). Even though thereferred study has been conducted in a different species,Polycomb group proteins are highly conserved from *Drosophila* to humans (Kennison, 2004; Kerppola, 2009), indicating that the same effect could be expected in bovine embryos cultured with DZNep. Interestingly, DZNep did not reduce levels of the EZH2

protein in zebrafish embryos, despite its reported effect on cell lines (Zhou *et al.*, 2011; Au *et al.*, 2013).

DZNep did not alter the transcript levels of genes SUZ12, NANOG, OCT4 and CDX2. Conversely, levels of genes encoding EZH2 and EEDwere increased in embryos cultured with DZNep from days 3 to 8, demonstrating that a signal for increasing the production of Polycomb enzymes was issued by the embryos. Although a significant difference among treatments regarding the mRNA expression ofSUZ12 was not detected, all Polycomb enzymes analyzed displayed a similar pattern of response to the treatments. In all cases, the relative mRNA abundance numerically increased with the exposure of the embryos to DZNep from days 3 to 5 and 5 to 8, and displayed even higher levels when the embryos remained under the effect of DZNep for the longest period. This may indicate that all the PRC2 complex is affected by DZNep in a similar way. DZNep did not exert a pronounced effect on gene expressionof zebrafish embryos, indicating that the loss of this silencing mark does not necessarily influence transcription rates of embryos at early development stages (Ostrup *et al.*, 2014). However, in the referred study the zebrafish embryos were exposed to DZNep from the 2-cell stage until 24 hours postfertilization. Conversely, in the present experiment, embryos were treated with DZNep with at least 48 hours.

The genes NANOG and OCT4 are key regulators and markers of cell pluripotency, and CDX2 plays an essential role in trophectoderm differentiation (Chawengsaksophak *et al.*, 2004; Wang *et al.*, 2013). However, none of these three genes appeared to have been affected by DZNep. It is important to consider that all embryos analyzed were already at the blastocyst stage. We believe that the embryos that were able to reach this stage could somehow, at least in parts, bypass the effect of DZNep.

Studies with cancerous cells reported that mRNA levels of all the PRC2 proteins remained unchanged after DZNep treatment, indicating that the decrease in PRC2 proteins observed in cells treated with DZNep is a result of a post-transcriptional mechanism (Tan *et al.*, 2007; Fujiwara *et al.*, 2014; Girard *et al.*, 2014). DZnep is a 3-Deazaadenosine analog and one of the most potent inhibitors of AdoHcy hydrolase (Glazer *et al.*, 1986). Inhibition of AdoHcy hydrolase causes accumulation of AdoHcy within the cells, resulting in an indirect inhibition of various S-adenosyl-L-methionine-dependent methyltransferases(Chiang, 1998).

Studies performed with different cell types have demonstrated that DZNep depletes the PRC2 proteins levels through increased protein degradation (Tan *et al.*, 2007; Fiskus *et al.*, 2009; Miranda *et al.*, 2009). Also, it is known that downregulation of one PRC2 component can lead to the downregulation of the other two components, resulting in the instability of the hole complex and subsequent inhibition of H3K27me3 (Tan *et al.*, 2007; Fiskus *et al.*, 2009).However, the exact mechanism by which the levels of PRC2 proteins are decreased upon DZNep treatment remain unclear.

In the present study, exposure to DZNep from days 3 to 8 was the treatment that mostly impaired embryo development. During this period, embryos undergo events that are crucial for proper development. In bovine embryos, major embryonic genome activation (EGA) takes place during the fourth cycle (transition between the 8- and16-cell stages) (Kopecny *et al.*, 1989). As a consequence, major changes in bovine embryos occur during the transition from the fourth to the fifth cycle (from 8- to 32-cell stages; around D4 and D5 for in vitro-produced embryos (Grisart *et al.*, 1994; Holm e Callesen, 1998). Compaction and cavitation are also vital events in the embryonic early life. Compaction is the development of tight intercellular junctions that occurs around 32-cell stage in the late morula(D5 to D6), which is a prerequisite to the formation of trophectoderm (TE) (Van Soom *et al.*, 1997). Cavitation is the formation of the fluid-filled cavity found in the blastocyst, which

accompanies the formation of the first two embryonic cell lines, inner cell mass (ICM) and TE (Wu *et al.*, 2010). The pluripotent cells in the ICM will give rise to allsomatic lineages of the embryo and the germline, and the TE differentiates into trophoblastcells to construct the placenta(Marikawa e Alarcon, 2012; Tee e Reinberg, 2014). Both compaction and cavitation depend on transcription of appropriate embryonic genes and their sequential activation (Watson *et al.*, 1999; Cui *et al.*, 2007). It is possible that inhibition of H3K27me3 by DZNep has altered transcript or protein levels of genes that regulate these and other events, thus harming blastocyst development.

It was not expected that exposure to DZNep from days 3 to 5 would damageembryo development. In bovine IVP embryos, EZH2 was expressed at all stages while EED and SUZ12 were only evident at morula and blastocyst stages (Ross *et al.*, 2008). Considering that the absence of EED and SUZ12 proteins would result in the absence of H3K27 trimethylation activity (Ross *et al.*, 2008; Fiskus *et al.*, 2009), PRC2 was not likely to play an important role in this period. Moreover, other repressive modifications like DNA methylation (Dean *et al.*, 2001) and H3K9me2 (Santos *et al.*, 2003) displayed a similar pattern by the time of EGA, suggesting that removal of repressive epigenetic marks are required for proper genome activation (Ross *et al.*, 2008). However, disruption of PRC2 from days 3 to 5 decreased blastocyst rate, total cell number and promoted a delay in embryo development.

Exposure to DZNep from days 5 to 8 also decreased blastocyst rate and embryo quality, but in a milder way when compared to group DZNep D3-D8. It is possible that a part of the embryos are relatively capable of overcoming important events like compaction and cavitation without the need of PRC2. According to Ostrup(Ostrup *et al.*, 2014), embryonic cells display features that were also observed in DZNep-insensitive cancer cell lines, like repressed activity of tumor suppressor p53 and high levels of anti-apoptotic Bcl-2.

Although cell death was not specifically assessed in this experiment, the DNA staining showed that the embryos exposed to DZNep appear to have fragmented DNA, a classic feature of apoptosis (Hardy, 1997). Apoptosis plays a particularly important role in eliminating unwanted cells during cell differentiation, thus avoiding inappropriate expression during cell line establishment (Hardy, 1997). It is possible that inhibition of H3K27me3 by DZNep produced blastomeres with an aberrant transcriptome, implying the need for elimination of these cells.

However, DZNep might not specifically target PRC2. Despite reports describing a EZH2-selective effect of this drug (Tan *et al.*, 2007), DZNeptreatment seems to also decrease H4K20 monomethylation(Tan *et al.*, 2007) and increase H3K9me3 (Hung *et al.*, 2013). Furthermore, it was suggested that DZNep decreases both repressive and active histone modifications in a non-selective manner (Girard *et al.*, 2014). The controversial reports about the specificity and selectivity of DZNep highlight the need for further information that clarifies the action of this drug.A better understanding of inhibition of PRC2 by DZNep might have a practical application in inducing differentiation of stem cells, increasing the efficiency of the results currently obtained with this biotechnology.

In summary, the present study examined the effects of exposing bovine IVP embryos to DZNep in different periods of culture on embryo production, embryo quality and mRNA expression. The results of these analyses indicate that PRC2 exerts an important role in early embryo development of bovine in vitro-produced embryos. Culture with DZNep, mainly from days 3 to 8, decreased blastocyst rates, mean cell number and delayed embryo development. Moreover, exposure of embryos to DZNep from days 3 to 8 increased mRNA expression of the two Polycomb enzymes EZH2 and EED.

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**Table 1.**Primers used for quantitative real-time PCR of in vitro-produced bovine embryos exposed or not to DZNep.

| Gene | Forward primer (5′ → 3′) | Reverse primer (5′ → 3′) | Accessionnumber |
|---|---|---|---|
| EZH2 | GGGCACAGCAGAAGAGCTAA | CTCTGGACAGACTTGGCGTT | NM_001193024.1 |
| EED | ACGAGAATAGCAACCCGGAC | GGTGTATCAGGGCGTTCTGT | NM_001040494.2 |
| SUZ12 | CCCTGAACTGCCGCAAACT | ATCCTAGCACCTTTTGGATGATAAA | NM_001205587.1 |
| OCT4 | GGAGAAAGACGTGGTCCGAG | CCAGAGGAGAGGATACGGGT | NM_174580.2 |
| NANOG | TTCCCTCCTCCATGGATCTG | ATTTGCTGGAGACTGAGGTA | NM_001025344.1 |
| CDX2 | GGCAGCCAAGTGAAAACCAG | CCTCCGGATGGTGATATAGCG | NM_001206299.1 |
| ACTB | TGTGGATCAGCAAGCAGGAGTA | TGCGCAAGTTAGGTTTTGTCA | NM_173979.3 |
| 18SrRNA | GGACGTGAAGGACGGGAAAT | GTGGGCCCGAATCTTCTTCA | NM_001033614.2 |

**Table 2.**Developmental rates of embryos cultured without DZNep (Control), with 5 μM DZNepfrom day 3 to 5 (DZNep D3-D5), from day 3 to 8 (DZNep D3-D8) and from day 5 to 8 (DZNep D5-D8).

| Group | Total | Blastocysts | |
|---|---|---|---|
| | | (n) | (%) |
| Control | 558 | 189 | 33.9[a] |
| DZNep D3-D5 | 604 | 101 | 16.7[b] |
| DZNep D3-D8 | 553 | 38 | 6.9[c] |
| DZNep D5-D8 | 546 | 78 | 14.3[b] |

[a,b,c] - different letters in the same column indicate a significant difference ($P< 0.05$).

**Table 3.**Developmental stages of D8 bovine blastocysts cultured without DZNep (Control), with 5 μM DZNep from day 3 to 5 of in vitro culture (DZNep D3-D5), from day 3 to 8 (DZNep D3-D8) and from day 5 to 8 (DZNep D5-D8).

| Developmentalstage | Hatched blastocysts % (n) | Expanded blastocysts % (n) | Blastocysts % (n) | Early blastocysts % (n) | Total (n) |
|---|---|---|---|---|---|
| Control | 30.2[a] (57) | 46.0[bc] (87) | 20.6[c] (39) | 3.2[b] (6) | (189) |
| DZNep D3-D5 | 3.0[b] (3) | 60.4[a] (61) | 31.7[b] (32) | 4.9[ab] (5) | (101) |
| DZNep D3-D8 | 0 (0) | 31.6[c] (12) | 55.3[a] (21) | 13.2[a] (5) | (38) |
| DZNep D5-D8 | 1.3[b] (1) | 53.8[ab] (42) | 34.6[b] (27) | 10.3[a] (8) | (78) |

[a,b,c] - different letters in the same column indicate a significant difference ($P< 0.05$).

**Table 4.** Total cell number of D8 bovine blastocysts cultured without DZNep (Control), with 5 μM DZNep from day 3 to 5 of in vitro culture (DZNep D3-D5), from day 3 to 8 (DZNep D3-D8) and from day 5 to 8 (DZNep D5-D8).

| Group | n | D8 cellnumber (mean ± SE) |
|---|---|---|
| Control | 117 | 118.2 ± 5.6 |
| DZNep D3-D5 | 73 | 70.4 ± 4.3* |
| DZNep D3-D8 | 32 | 36.3 ± 1.6* |
| DZNep D5-D8 | 68 | 51.5 ± 2.5* |

* Asterisks indicate treatments that differ from the Control group ($P < 0.05$).

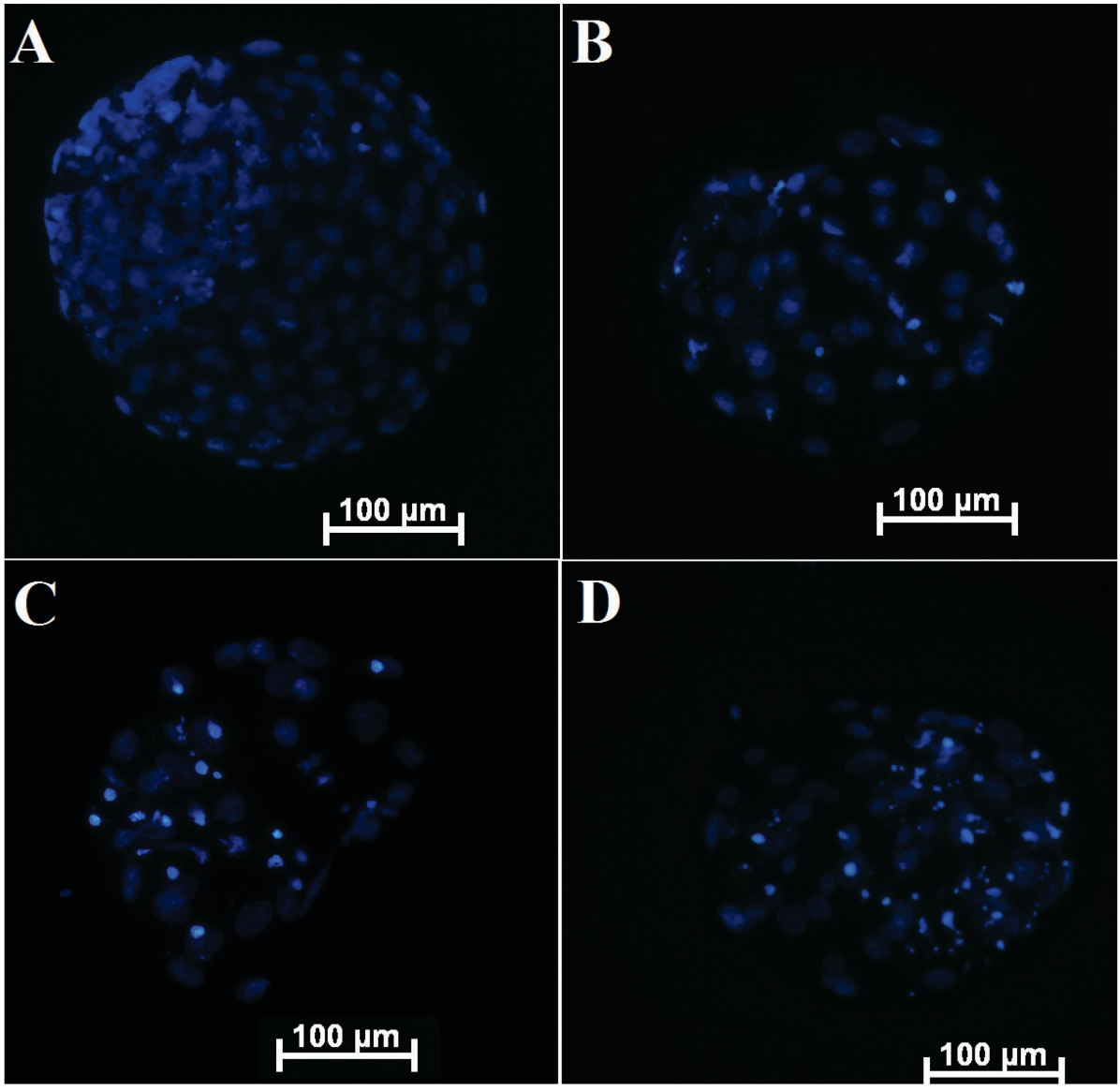


**Figure 1.** In vitro-produced bovine embryos cultured without DZNep (A), with 5 µM DZNep from day 3 to 5 (B), from day 3 to 8 (C) and from day 5 to 8 (D) stained with DAPI.

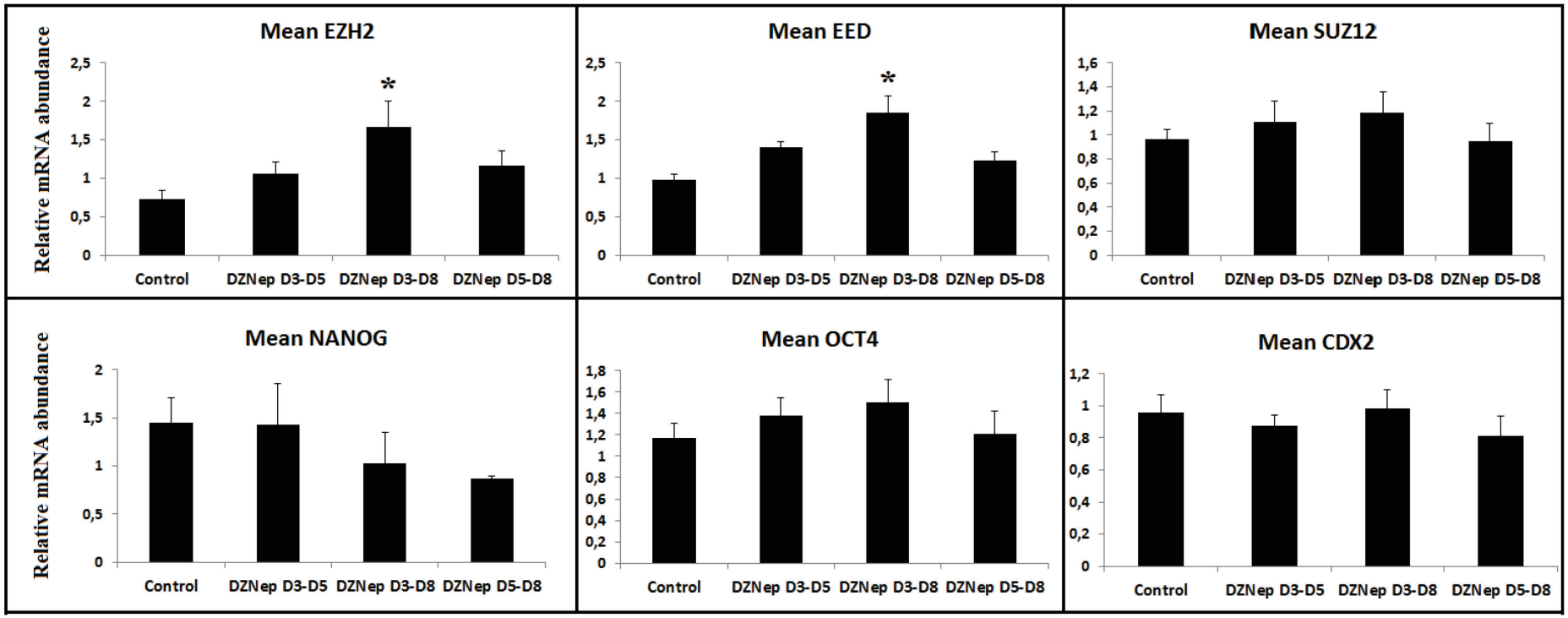


**Figure 2.** Relative mRNA abundance of genes encoding the PRC2 enzymes (EZH2, EED and SUZ12), genes related to pluripotency (NANOG and OCT4) and the homeobox gene CDX2 of bovine in vitro-produced embryos cultured without DZNep (Control) or with 5 µM DZNep from day 3 to 5 (DZNep D3-D5), from day 3 to 8 (DZNep D3-D8) and from day 5 to 8 (DZNep D5-D8).Asterisks indicate treatments that differ from the Control group ($P < 0.05$).

# 6 CONCLUSÕES

A partir destes resultados, é possível concluir que:

1) A acetilação da H3K27 está presente na maior parte dos estágios de desenvolvimento de embriões suínos PIV e apresenta um perfil dinâmico, conforme evidenciado pela variação na intensidade do sinal de fluorescência durante o desenvolvimento inicial;
2) A mono-, di- e trimetilação da H3K27 são altamente remodeladas em oócitos e embriões suínos produzidos *in vitro,* com períodos de sinal fluorescente fraco ou ausente e períodos de sinalização evidente;
3) A adição do DZNep ao meio de cultivo embrionário resultou em baixas taxas de blastocisto, diminuição do número de células dos embriões produzidos e atraso no desenvolvimento de embriões bovinos produzidos *in vitro*;
4) O cultivo de embriões bovinos PIV com DZNep resultou em aumento da expressão de mRNA das enzimas Polycomb EZH2 e EED e não alterou a expressão de mRNA dos genes SUZ12, OCT4, NANOG e CDX2.

# 7 CONSIDERAÇÕES GERAIS

A proposta deste trabalho surgiu em decorrência de uma prévia colaboração entre a Universidade Estadual de Londrina (UEL) e a *McGillUniversity*, iniciada em 2006 através da ida do Prof. Dr. Marcelo Seneda ao *Macdonald Campus* para a realização do pós-doutorado. Desde então, visitas mútuas entre pesquisadores e alunos de ambas as instituições proporcionaram condições favoráveis ao período sanduíche no exterior já pretendido desde o início do doutorado.

A opção pela espécie suína, no primeiro experimento, decorreu de ser este o principal modelo animal utilizado no Departamento*Animal Science*. Informações importantes a respeito das modificações epigenéticas nesta espécie haviam sidopreviamente relatadas pela equipe do Dr.Bordignon. Entretanto, permaneciam dúvidas quanto ao papel da H3K27 no controle do desenvolvimento embrionário, fato que nos motivou a estudar as alterações epigenéticas desta lisina.

A constatação de como as modificações epigenéticas são cruciais nos motivou a considerar a proposta deste segmento da ciência - a epigenética - em uma área bastante importante para o Brasil, no cenário da Biotecnologia da Reprodução: a PIVE. Existiu ainda um interesse especial por parte da nossa equipe em investigar mais especificamente os animais *Bosindicus*, considerando a grande importância dessa subespécie no Brasil, devida principalmente à rusticidade e adaptabilidade ao clima tropical.

O desafio inicial consistiu em aprimorar os resultados da produção *in vitro* de embriões suínos para dar início ao primeiro experimento.Utilizando um *pool* com sêmen de aproximadamente três reprodutores, que variavam conforme o dia da coleta, os resultados apresentavam-se extremamente instáveis, variando de 0% de taxa de fecundação a quase

100% de poliespermia em uma mesma semana. Após a superação deste entrave, os resultados tornaram-se estáveis, viabilizando o início do projeto.

No Artigo 1, objetivamos avaliar o perfil de acetilação e metilação da H3K27 durante o desenvolvimento de embriões suínos PIV. Este trabalho mostrou uma variação na intensidade das marcas epigenéticas nos diferentes estágios de desenvolvimento embrionário, sugerindo um padrão dinâmico de estabelecimento e de remoção, quer seja ativa ou passiva, da acetilação e metilação da H3K27. Mostrou também, pela primeira vez, o perfil da H3K27me2 durante o desenvolvimento inicial de embriões suínos. Houve uma predominância de marcações que estimulam a transcrição gênica sobre as marcações repressivas, evidenciada por forte sinal da H3K27ac durante a maior parte do desenvolvimento, enquanto os sinais da mono-, di- e trimetilação apresentaram-se fracos ou inexistentes em grande parte dos estágios. Foram demonstrados ainda diferentes padrões da H3K27me3 em embriões em D8, indicando uma variação no perfil desta alteração em blastocistos suínos produzidos *in vitro*.

No momento do início da execução do referido experimento, os protocolos de produção *in vitro* de embriões suínos e de imunofluorescência já estavam bem estabelecidos no laboratório do Dr. Bordignon. Adicionalmente, a boa estruturação do laboratório e dos laboratórios vizinhos, com quem o Dr. Bordignon possui estreito relacionamento, contribuíram para a realização do experimento sem maiores entraves. Portanto, o principal obstáculo a ser superado foi o aprendizado, no curto período de cinco meses, das particularidades da produção de embriões da espécie suína e das técnicas de imunofluorescência e captação de imagens.

O segundo desafio se deu após a volta ao Brasil, com a necessidade de estruturar todo o laboratório de PIVE no então recém-inaugurado Laboratório de Reprodução Animal da UEL – ReproA. Inicialmente, os embriões eram produzidos no Laboratório de Virologia da

UEL, levando a equipe a adaptar a produção dos embriões às condições ótimas para o cultivo de tecido infectado. Posteriormente, tornou-se necessária a compra de equipamentos, reagentes, adaptações das instalações do ReproA, e treinamento das pessoas envolvidas no experimento. Uma vez superados os entraves típicos de uma proposta desta natureza, as condições tornaram-se favoráveis para a execução do experimento que originou o segundo artigo.

No Artigo 2, foi investigado o efeito da adição do DNZep, inibidor do complexo repressivo Polycomb 2, ao meio de cultivo *in vitro* em diferentes períodos do desenvolvimento embrionário. Em todos os períodos testados, o DZNep reduziu as taxas de blastocisto, o número total de células e promoveu um atraso no desenvolvimento embrionário, restringindo a porcentagem de embriões eclodidos. Entretanto, o efeito mais pronunciado foi observado quando os embriões estiveram em contato com o DZNep pelo período mais longo (D3 a D8), de forma que mais eventos decisivos para o desenvolvimento embrionário estiveram sob a influência do inibidor. A adição de DZNep do D3 ao D8 aumentou a expressão das enzimas Polycomb EZH2 e EED, indicando a reação das células embrionárias a umapossível redução nos níveis destas proteínas, conforme observado em outros estudos. Os resultados deste trabalho sugerem grande importância da ação do complexo Polycomb2 e da trimetilação da H3K27 no desenvolvimento inicial de embriões bovinos, uma vez que a sua inibição resultou em redução na taxa de blastocisto, atraso no desenvolvimento embrionário, redução da qualidade dos blastocistos produzidos e alterações na expressão de mRNA dos embriões.

Dentre os aspectos favoráveis à execução do segundo experimento, pode-se destacar o bom desempenho e sincronia da equipe de produção dos embriões, uma vez capacitada pelo treinamento. Além disso, a possibilidade de retornar à *McGill* para executar as análises de expressão gênica por PCR em tempo real contribuiu para obtenção dos resultados referentes a

essa análise em um curto espaço de tempo. Os protocolos utilizados no laboratório do Dr. Bordignon permitiram a obtenção de resultados com amostras compostas por um número muito limitado de embriões, viabilizando as análises.

Por outro lado, a inexistência de outros trabalhos realizados com o DZNep em embriões de mamíferos gerou a necessidade de realização de testes prévios ao início do estudo.

Os resultados apresentados nos Artigos 1 e 2agregam ao atual cenário científico informações relevantes a respeito do dinâmico perfil de alterações epigenéticas da H3K27 durante o desenvolvimento de embriões suínos e da importância do complexo repressivo Polycomb 2 no desenvolvimento inicial de embriões bovinos. De forma similar, a experiência e o conhecimento adquiridos neste período de doutorado contribuíram muito para impulsionar a perspectiva de novos projetos de pesquisa, viabilizando a continuidade de investigações a respeito das alterações de histonas em embriões de mamíferos em um contexto mais aplicado.

# ANEXOS - PRINCIPAIS PUBLICAÇÕES GERADAS DURANTE O DOUTORADO

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# Cryosurvival and pregnancy rates after exposure of IVF-derived *Bos indicus* embryos to forskolin before vitrification

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ABSTRACT

*In vitro*–produced (IVP) bovine embryos are more sensitive to cryopreservation than their *in vivo* counterparts due to their higher lipid concentrations, whereas *Bos indicus* IVP embryos are even more sensitive than *Bos taurus* IVP embryos. To examine the effects of a lipolytic agent, before vitrification of *Bos indicus* IVP embryos, on embryo survival, viability, and pregnancy rates, two experiments were conducted. In experiment 1, *Bos indicus* (Nelore) embryos were produced from abattoir-derived ovaries and allocated into two groups. In the treatment group, 10 μM of forskolin was added to the *in vitro* culture medium on Day 5 and incubated for 48 hours. On Day 7 of culture, IVP-expanded blastocysts from both the control (n = 101) and treatment (n = 112) groups were vitrified with ethylene glycol and DMSO via the Cryotop procedure. Although there was no significant difference between the rates of blastocoel reexpansion and hatching of the embryos exposed to forskolin (87.5% and 70.5%, respectively) compared with the control embryos (79.2% and 63.3%, respectively), the numerically superior rates of the embryos exposed to forskolin led to another experiment. In experiment 2, blastocysts produced from the ovum pick up were exposed or not exposed to the lipolytic agent and vitrified as in experiment 1. Embryos treated with forskolin had higher pregnancy rates than the control group (48.8% vs. 18.5%). In view of these results, 1908 *Bos indicus* embryos were produced from ovum pick up, exposed to the lipolytic agent, and blastocysts were transferred to recipients, and the pregnancy rates of the embryos of various breeds were compared. The mean pregnancy rate obtained was 43.2%. All data were analyzed by chi-square or by binary logistic regression ($P \leq 0.05$). In conclusion, treatment with forskolin before vitrification improved cryotolerance of *Bos indicus* IVP embryos, resulting in good post-transfer pregnancy rates.



## 1. Introduction

*Bos indicus* accounts for the majority of Brazilian cattle herds, thereby contributing to this country's place at the forefront of *in vitro* embryo production (IVEP) [1]. However, the availability of recipients is typically less than needed (more embryos are produced than are implanted). Thus, considerable effort has been invested in the development of an efficient protocol for bovine embryo cryopreservation. Low rates of pregnancy after warming are associated with low numbers of embryos that are produced *in vitro* and subjected to cryopreservation worldwide [2]. The main problems include the lack of consistency in results [3] and

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differences in the survival and developmental rates after warming across species, developmental stages, and quality of embryos [4].

Most cryopreservation methods are based on two factors, the use of cryoprotectants and cooling rate [5]. Vitrification is a widely used method [6]. The minimal-volume approach of the Cryotop method increases the rates of cooling, and especially warming (up to 40,000 °C/minute), which may contribute to consistent, improved survival rates and improved rates of development, both *in vitro* and *in vivo*. Cryotop technology has been successfully used for cryopreservation of oocytes from various species [7,8] and *in vitro* fertilization (IVF)-derived embryos [9], reconstructed embryos with somatic cell nuclei [10], blastocysts produced by parthenogenic activation, or somatic cell nuclear transfer from delipidated *in vitro*-matured oocytes [10] and embryos been derived from intracytoplasmic sperm injection [11].

Vitrification is the most efficient cryopreservation method for embryos produced *in vitro* [6], which are more sensitive to cryoinjuries than their *in vivo* counterparts [12] because they contain more intracellular lipid droplets [13]. In addition, sensitivity of *Bos indicus* IVP embryos is higher than that of *Bos taurus* embryos [14]. The extent of cryoinjury is dependent on the size and shape of the embryonic cells, as well as on membrane permeability and quality and sensitivity of the embryos [5]. Triacylglycerols make up most of the intracellular lipids of embryos [15], and lipolysis can be induced by lipolytic agents, such as norepinephrine, dibutyryl cAMP, isoproterenol, forskolin, and theophylline [16,17]. The diterpene forskolin, which is derived from the roots of *Coleus forskohlii* [17], is used to induce chemical delipidation of IVP-derived embryos [18,19].

Data concerning the effect of forskolin on *Bos indicus* IVP embryos are scarce, and the effect of forskolin on pregnancy rates of cryopreserved IVP bovine embryos is currently unknown. Addition of forskolin to culture medium of *Bos indicus* IVP embryos before vitrification could improve survival rates, providing satisfactory pregnancy rates after the transfer of these embryos.

The aim of this study was to evaluate the effect of forskolin added during *in vitro* embryo culture on cryosurvival by assessing the rates of blastocoel reexpansion and hatching after vitrification of *Bos indicus* IVP embryos; in addition, its effect on pregnancy rates following embryo transfer was assessed and compared among zebu breeds.

## 2. Materials and methods

All chemicals used in this study were purchased from Sigma-Aldrich (St Louis, MO, USA), unless stated otherwise.

### 2.1. Experimental design

In experiment 1, conducted in two replications, the blastocysts produced from abattoir-derived ovaries were cultured until Day 7 (fertilization = Day 0) when they were vitrified and rewarmed at the expanded blastocyst stage by the Cryotop procedure. On Day 5, the embryos were separated into two groups, those that were exposed and not exposed to 10 μM forskolin (7β-acetoxy-8,13-epoxy-lα,6β,9α-trihydroxylabd-14-en-11-one, $C_{22}H_{34}O_7$) for 48 hours before vitrification. Following rewarming, the blastocysts were cultured for 24 hours to assess cryosurvival. Embryos that survived vitrification were cultured for an additional 48 hours to assess hatching ability. For both the assessments, nonvitrified fresh blastocysts were used as controls.

In experiment 2, embryos produced from ovum pick up were cultured with or without exposure to forskolin, as in experiment 1. Embryos were transferred at the blastocyst stage to female recipients (two replications) and the pregnancy rates were compared. Results indicating the forskolin-induced embryo survival after vitrification led to the production of 1908 *Bos indicus* embryos after ovum pick up, all of them exposed to forskolin and vitrified. These embryos were transferred to recipients, and the pregnancy rates obtained from the embryos of various breeds were compared.

### 2.2. Oocyte recovery

#### 2.2.1. In vitro

Abattoir-derived ovaries from Nelore cows were collected at a local abattoir stored in saline solution at 25 °C to 30 °C and transported to the laboratory within 25 minutes after slaughter. Briefly, each follicle was punctured with a disposable 19-gauge 1/2″ hypodermic needle (Becton Dickinson, Curitiba, PR, Brazil) connected to a 20-mL syringe.

#### 2.2.2. In vivo

Follicular aspiration was performed as described [20,21]. Briefly, each visible follicle ≥2 mm in diameter was aspirated using a real-time B-mode ultrasound scanner (Scanner 200 Vet, 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 × 12 mm hypodermic needle (Becton Dickinson, Curitiba, PR, Brazil) connected to a 50-mL conical tube (Corning, Acton, MA, USA) via a silicon tube (0.8 m long; 2 mm internal diameter). Aspiration was performed using a vacuum pump (Cook Veterinary Products, Queensland, Australia) with a negative pressure of 10 to 12 mL of water/minute. The collection medium was TCM-199 (Gibco Life Technologies, Grand Island, NY, USA), supplemented with 25 mM HEPES (Sigma H-0763), 5% fetal calf serum (FCS), 50 μL/mL gentamycin sulfate (Schering-Plough, São Paulo, SP, Brazil), and 10,000 IU/L sodium heparin (Sigma H-3149).

### 2.3. In vitro maturation

Oocytes with at least three layers of compact cumulus cells were classified as grade 1 [20] and were matured for 24 hours in 100 μL drops of TCM-199 (Gibco Life Technologies) that were supplemented with 10% FCS (Gibco Life Technologies), 1 μg/mL FSH (Folltropin, Bioniche Animal Health, Belleville, ON, Canada), 50 μg/mL hCG(Profasi, Serono, São Paulo, SP, Brazil), 1 μg/mL estradiol (estradiol-17β), 0.2 mM sodium pyruvate, and 83.4 μg/mL amikacin

(Instituto Bioquímico, Rio de Janeiro, Brazil) under mineral oil (D'Altomare, Santo Amaro, SP, Brazil) at 39 °C and 5% atmospheric $CO_2$ (25–30 oocytes per microdrop). Before *in vitro* maturation, cumulus oocyte complexes were washed three times in TCM-199 HEPES that was supplemented with 10% FCS, 0.20 mM sodium pyruvate, and 83.4 μg/mL amikacin.

### 2.4. IVF and in vitro culture

Freeze-thawed sperm ($2 \times 10^7$/dose) from sires of known fertility (based on previous utilization for IVF) were used. Straws were thawed for 30 seconds in a 35 °C water bath. Sperm were washed twice by centrifugation at 200 × *g* for 5 minutes in 2 mL TALP medium that was supplemented with 10 mM HEPES (Gibco Life Technologies), 0.2 mM sodium pyruvate, and 83.4 g/mL amikacin. Sperm were capacitated using heparin, and motility was stimulated by the addition of penicillamine, hypotaurine, and epinephrine.

After visual assessment of motility, the sperm concentration was adjusted to $25 \times 10^6$ motile sperm/mL, and each fertilization drop containing 90 μL TALP-IVF medium that was supplemented with 10 g/mL heparin, 18 M penicillamine, 10 M hypotaurine, and 8 M epinephrine received 4 μL of sperm (final concentration of $1 \times 10^5$ sperm per drop) [20]. After maturation, the cumulus oocyte complexes were washed three times in TCM-199 prefertilization medium that was supplemented with 25 mM HEPES and 0.3% BSA and washed once in TALP fertilization medium that was supplemented with 10 μg/mL heparin and 160 μL of penicillamine, hypotaurine, and epinephrine solution [22,23].

Presumptive zygotes had their cumulus cells removed and were transferred to 100 μL drops of embryo culture medium (SOFaa BSA containing 0.5% BSA and 2.5% FCS) under the same temperature and gaseous atmospheric conditions that were used for IVF. After 3 days (D3) and 5 days (D5) of culture, 50% of the culture medium was replaced with fresh medium (feeding) from a stock of the same medium that was used at the beginning of the culture. The cleavage and blastocyst production rates were recorded at D3 and D7 of culture, respectively.

### 2.5. Vitrification and warming

In experiment 1, embryos cultured with (n = 112) or without (n = 101) 10 μM forskolin were subjected to Cryotop vitrification, as described [24]. Expanded blastocysts of excellent quality were equilibrated with 10% ethylene glycol (WakoPure Chemical Industries Co., Osaka, Japan) and 10% DMSO (WakoPure Chemical Industries Co.) in TCM-HEPES base medium (TCM-199, 25 mM HEPES that was supplemented with 20% FCS) for 1 minute at room temperature. Then, the embryos were transferred into a vitrification solution consisting of 20% ethylene glycol, 20% DMSO, and 0.5 M sucrose in the base medium and incubated for 20 seconds at room temperature. During this incubation, blastocysts were loaded onto the top of the polypropylene strip of a Cryotop (three to five embryos; Kitazato BioPharma Co., Shizuoka, Japan) with a minimal amount of vitrification solution. They were then quickly immersed in liquid nitrogen ($N_2$). As a control, randomly selected fresh embryos (n = 96) were evaluated at D7, D8, and D9 of culturing. In experiment 2, embryos were vitrified using the same protocol.

### 2.6. Postwarming assays

After storage for >2 hours in liquid $N_2$, blastocysts were exposed to air for 4 seconds, warmed by immersing the polypropylene strip of a Cryotop into the base medium (TCM-HEPES and sucrose) at ~35 °C and then held for 1 minute. Then, the blastocysts were transferred to the base medium at room temperature in a stepwise manner (0.3 and 0.15 M sucrose for 5 minutes each) [25,26]. In experiment 1, the blastocysts were cultured in 100 μL drops of SOF medium containing 2.5% FCS and 0.5% BSA under mineral oil at 39 °C and 5% atmospheric $CO_2$. Cryosurvival was assessed by reexpansion of the blastocoel after 24 hours of culturing. Thereafter, the surviving embryos and the corresponding fresh control D9 embryos were randomly assigned to further 48-hour cultures to determine their abilities to hatch. Embryos with more than half of their embryonic cells emerging from the zona pellucida were defined as hatched. In experiment 2, the embryos were transferred to recipients after warming.

### 2.7. Protocol for embryo transfer

In experiment 2, all vitrified embryos were transferred to Nelore and Nelore crossbred recipients after warming. A fixed-time embryo transfer protocol was used to ensure recipient estrus synchrony. Each recipient received an intravaginal progesterone implant (CIDR, Pfizer, Hamilton, New Zealand) and 2 mg of estradiol benzoate (Estrogin, Farmavet, São Paulo, SP, Brazil) on Day 0. The progesterone implants were removed on Day 8, at which time the animals were given 300 IU of eCG (Novormon, Syntex, Buenos Aires, Argentina), 150 μg of D-cloprostenol (Preloban, Intervet, São Paulo, SP, Brazil), and 1 mg of estradiol cypionate (Pfizer, Guarulhos, SP, Brazil). No detection of estrus was performed; Day 10 was considered the day of estrus. One embryo was transferred per recipient on Day 17. Before embryo transfer, each recipient was subjected to a transrectal ovarian examination (Aloka SSD 500, 5 MHz linear transducer, Tokyo, Japan) to confirm the presence and size of CLs. Only recipients with a CL ≥ 13 mm received an embryo. Pregnancy status was determined by ultrasonography on Day 30.

### 2.8. Statistical analyses

Cleavage, blastocyst, and pregnancy rates were compared between forskolin and control groups by a binary logistic regression model in a two-factorial design (treatment and replicate). Pregnancy rates were compared between different breeds by the chi-square test. For all analyses, $P \leq 0.05$ was considered statistically significant.

## 3. Results

In experiment 1, there were no differences in blastocyst rates across groups ($P > 0.05$). Out of the 314 IVF-derived

**Table 1**
Cryosurvival and hatching abilities of IVP *Bos indicus* blastocysts after treatment with or without forskolin for 48 hours in culture.

| Treatment | Oocytes, no. | Cleavage rate, no. (%) | Blastocyst rate, no. (%) | Reexpanded blastocysts 24 h, no. (%) | Hatched blastocysts 48 h, no. (%) |
|---|---|---|---|---|---|
| No forskolin vitrification | 348 | 253 (72.7) | 139 (39.9) | 80 (79.2)[a] | 64 (63.3)[b] |
| 10 μM forskolin vitrification | 314 | 247 (78.8) | 142 (45.3) | 98 (87.5)[a] | 79 (70.5)[b] |
| Nonvitrified (fresh) control | 331 | 241 (72.7) | 132 (39.9) | - | 82 (85.4)[a] |

[a,b] Within a column, rates without a common superscript differed ($P \leq 0.05$).

zygotes cultured with forskolin, 247 (78.8%) cleaved on Day 3, whereas 142 (45.3%) developed to expanded blastocysts on Day 7. Out of the 348 IVF-derived zygotes cultured without forskolin, 253 (72.7%) cleaved on Day 3 and 139 (39.9%) developed to expanded blastocysts on Day 7 (Table 1).

There were no differences between the rates of blastocoel reexpansion and hatching of the embryos exposed to forskolin (87.5% and 70.5%, respectively) compared with embryos that were not treated with the lipolytic agent before vitrification (79.2% and 63.3%, respectively). Hatching rates of both groups were still lower ($P < 0.05$) than those in the fresh control embryos (85.4%; Table 1).

In experiment 2, the average cleavage rate was 78% and the average blastocyst rate was 42%. Pregnancy rates were higher for the embryos that were exposed to forskolin (48.8%) than for the embryos that were not (18.5%; Table 2). After transfer of the 1908 vitrified-warmed embryos that had been treated with forskolin, pregnancy rates were not different ($P > 0.05$) among various zebu breeds (rates varied from 40.3% to 45.5%, with an average rate of 43.2%; Table 3).

## 4. Discussion

To our knowledge, this was the first study to report pregnancy rates from vitrified *Bos indicus* embryos treated with a stimulator of lipolysis. Herein, we report that exchanging genetic material for research or commercial purposes was possible with the use of the lipolytic agent forskolin before embryo vitrification using the Cryotop procedure. These data should be of great interest to the embryo industry, as reasonable pregnancy rates were obtained following cryopreservation of IVP zebu embryos.

There is a scarcity of literature regarding the pregnancy rates of *Bos indicus* IVP embryos, and even less information describing cryopreserved embryos. Our mean pregnancy rate with forskolin (43.2%) seemed much higher than those that had been reported by Block et al. [27] (27.7%) and Stewart et al. [28] (25.7%) with vitrified IVP Holstein and Holstein and Jersey embryos, respectively. It was also higher than that obtained by Dochi et al. [29] with frozen IVP Holstein embryos (29.5%). Interestingly, the pregnancy rates that were obtained in this study are very similar to or even higher than those obtained by our team after transfer of fresh IVP *Bos indicus* embryos (33.5% [30] and 36.57% [31] with Nelore cattle and 37% to 40% with Holstein, Gir, and Holstein-Gir crossbred females [32]). It is important to emphasize that achievement of such encouraging pregnancy rates requires only cryopreservation of embryos of excellent quality.

Few studies have reported the use of forskolin for chemical delipidation of *Bos indicus* embryos before cryopreservation and its effect on cryosurvival of vitrified embryos. In a recent study [33], rates of blastocoel reexpansion in IVP Nelore-sired expanded blastocysts that had been cultured with or without 10 μM forskolin (81.09% vs. 75.07%) did not differ from rates of expansion of *in vivo*–derived Nelore embryos (86.40%). Although those rates are similar to our results (87.5% vs. 79.2%), the duration of exposure to forskolin was different (12 vs. 48 hours of culture). Additionally, in agreement with our study, there was no significant improvement in reexpansion rates after cryopreservation after exposure to forskolin. Similarly, Pryor et al. [19] reported no differences between *Bos indicus* embryos that had been cultured with or without forskolin in terms of survival to freezing (59.4% vs. 49.0%) and blastocyst hatching rates (29.1% vs. 48.5%) when forskolin was added on Day 6 of culture. In a study by Paschoal et al. [34], forskolin was also added on Day 6; it did not improve reexpansion rates after vitrification, but apparently reduced the number of damaged cells after cryopreservation when serum was used. In the present study, the addition of 10 μM forskolin on D5 of culture, which was 48 hours before vitrification, did not significantly improve rates of blastocoel reexpansion and hatching (89.5% vs. 79.2% and 70.5% vs. 63.3%, respectively). Nevertheless, numerically superior rates obtained with this treatment encouraged us to investigate the effects of exposure to forskolin before vitrification on pregnancy rates of IVP Nelore-sired embryos.

There were no significant differences in blastocyst rates for embryos that were treated with forskolin (45.3%) compared with those that were observed in the vitrified control embryos (39.9%). These results were in accordance with the study by Paschoal et al. [33], who reported blastocyst rates of 46.3% versus 46.8% for Nelore-sired IVP embryos that were cultured with or without forskolin before vitrification. Paschoal et al. [34] also reported no differences between blastocyst rates of embryos that were cultured with (46.3% with FCS and 34.2% with no serum) or without forskolin (46.8% with FCS and 33.3% with no serum).

**Table 2**
Pregnancy rates of IVP *Bos indicus* embryos after treatment with or without forskolin for 48 hours in culture before vitrification.

| Treatment | Transferred embryos (no.) | Pregnancies (no.) | Pregnancy rate (%) |
|---|---|---|---|
| Control | 65 | 12 | 18.5[b] |
| Forskolin | 80 | 39 | 48.8[a] |

[a,b] Within a column, rates without a common superscript differed ($P \leq 0.05$).

**Table 3**
Pregnancy rates of IVP *Bos indicus* embryos treated with forskolin for 48 hours in culture and submitted to vitrification.

| Breed | Transferred embryos (no.) | Pregnancies (no.) | Pregnancy rate (%) |
|---|---|---|---|
| *Taurus-indicus* | 87 | 37 | 42.5 |
| Gir | 701 | 314 | 44.8 |
| Guzera | 680 | 274 | 40.3 |
| Nelore | 440 | 200 | 45.5 |
| Total | 1908 | 825 | 43.2 |

No effect ($P > 0.05$) of breed on pregnancy rate.

*Bos indicus* IVP embryos have higher levels of intracellular lipids than those of *Bos taurus*, which increases their sensitivities to conventional freezing methods [14]. The use of lipolytic agents is a noninvasive approach to achieve the metabolic reduction of intracellular lipids, high levels of which are largely responsible for the exceptional sensitivity of these cells to cryopreservation. Lipolytic agents act on various components of the lipolytic signal transduction pathway, stimulating hydrolysis of intracellular lipids. Forskolin stimulates lipolysis by directly activating adenylate cyclase, increasing the levels of cAMP and activating lipases [35–37]. In porcine embryos, forskolin was responsible for reported reduction in levels of intracellular lipids [18]. In bovine IVP embryos, however, controversial effects were observed, whereas forskolin was able to decrease cellular lipid concentrations [38] or not [39]. Nevertheless, it is possible that forskolin may induce effects on the embryonic cells that are still unknown. By increasing cAMP concentrations, it might alter embryo metabolism or gene expression, leading to altered development. Although embryos and the offspring seemed normal, the effects of forskolin on metabolism and development were not thoroughly investigated in this study.

There are other substances that seem capable of decreasing embryo lipid concentrations, but their effects have not been well established. Pereira et al. [40,41] reported that the addition of an isomer of conjugated linoleic acid (CLA) to culture media decreased lipid concentrations and increased cryotolerance of IVP bovine embryos. However, no pregnancy rates were reported. The t10, c12 CLA seemed to act mainly by reducing the uptake and synthesis of fatty acids [42,43]. Conversely, Darwich et al. [44], reported no improvement in cryopreserved embryo survival after addition of t10, c12 CLA.

This is apparently the first report of pregnancy rates from IVP bovine embryos that were vitrified after treatment with forskolin. This represents great progress in IVEP, enabling the commercial use of the technique. Until recently, surplus embryos obtained after embryo production programs have been discarded because there was no protocol allowing for reasonable pregnancy rates after cryopreservation of these embryos.

The optimal pregnancy rates that were obtained after the use of forskolin and vitrification with the Cryotop method may simplify transport of embryos over long distances, the exportation of embryos, and the conservation of species by storing genetic material. Additionally, the surplus embryos that are obtained from IVEP programs can now be stored for subsequent use during strategic periods.

In conclusion, adding forskolin before vitrification of IVP-derived embryos improved *Bos indicus* embryo cryotolerance, resulting in optimal pregnancy rates after transfer of cryopreserved *Bos indicus* IVP embryos.

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# Resynchronization of estrous cycle with eCG and temporary calf removal in lactating *Bos indicus* cows

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ABSTRACT

The aim of this study was to compare four methods of estrus resynchronization performed 23 days after timed artificial insemination (TAI) plus estrus observation in *Bos indicus* cows. Eight hundred fourteen lactating Nelore cows were submitted to TAI and then randomly assigned to one of the five following treatments: R23 (resynchronization without eCG), R23/200 (resynchronization with 200 IU of eCG), R23/300 (resynchronization with 300 IU of eCG), R23/TCR (resynchronization with temporary calf removal [TCR]), and a control group, with estrus observation followed by AI (with no resynchronization). Treatment consisted of a progesterone device plus administration of estradiol benzoate on Day 0; on Day 8, the device was removed and cloprostenol was applied, together with estradiol cypionate. Also on Day 8, either eCG was administered or TCR was performed in the resynchronized groups, except for R23. The females were inseminated 48 hours after device removal or TCR (33 days after the first TAI). The control group was kept under estrus observation from 18 to 23 days after the first TAI and was inseminated 12 hours after detection of estrus. The first pregnancy evaluation was performed using ultrasound examination 31 days after the first TAI. After 30 days of the resynchronization, a second pregnancy evaluation was performed and the animals in the R23/300 and R23/TCR groups achieved the highest conception rates, 76.6% and 74.0%, respectively ($P < 0.05$). There were no differences between the conception rates of the animals in the R23/200 (63.3%), R23 (61.3%), and control (54.3%) groups ($P > 0.05$). These results suggest that estrus resynchronization at 23 days after TAI can effectively improve the conception rate of lactating *Bos indicus* cows in a short time period. Furthermore, resynchronization with 300 IU of eCG or with TCR provided the best results.



## 1. Introduction

The optimization of reproductive efficiency is an important way of maximizing the profitability of beef herds. For this purpose, there are different reproductive strategies. Organized breeding programs involving synchronization of the estrous cycle and timed artificial insemination (TAI) have been widely applied to enhance the reproductive efficiency of cattle. Therefore, female animals that do not conceive from the first service need to be detected and reinseminated as soon as possible. The failure to inseminate nonpregnant cows in a short time period results in prolonged intervals between the services and in the delayed reestablishment of pregnancies.

The detection of estrus in *Bos indicus* female animals is particularly demanding and to maximize the number of cows that become pregnant from AI early in the breeding season, the estrous cycle of cows that failed to conceive from the TAI can be resynchronized. Estrus resynchronization can increase the percentage of nonpregnant cows that are submitted for a second insemination [1]. In addition, it

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can be especially convenient when the number of bulls is insufficient to mate with all of the cows that did not become pregnant after the first TAI and return to estrus in a synchronous way. Most of the cows that fail to conceive from the TAI are not diagnosed as nonpregnant until approximately 30 days afterward, when transrectal ultrasonography can be performed [2,3]. A new hormonal protocol could be initiated earlier without undermining the pregnancy rate obtained with the first TAI.

Zebu breeds have a shorter duration of estrus compared with that of European cattle, and a high incidence of estrus at night [4,5]. Furthermore, *Bos indicus* cows have a high incidence of postpartum anestrus [6]. These features make it difficult to detect the estrus of *Bos indicus* cows and can impair the AI efficiency of Nelore cattle. Therefore, one method to improve the service rates of zebu cows is to use hormonal treatments that stimulate follicular development and synchronize ovulation, allowing for TAI.

One of the most common treatments for TAI or estrus resynchronization in cattle involves the use of an intravaginal progesterone (P4) device with estradiol benzoate or estradiol cypionate [7,8]. However, temporary calf removal (TCR) has also been used successfully to improve the reproductive performance of zebu cows [9]. Temporary calf removal is performed to avoid any sensory interactions between the cows and their calves because these stimuli have been associated with the inhibition of the normal pattern of LH release [10].

The objective of this study was to evaluate the effect of using different treatments, including eCG administration and TCR, for estrus resynchronization 23 days after the TAI of Nelore cows of unknown pregnancy status on the rates of conception and pregnancy in a time interval of 33 days.

## 2. Materials and methods

### 2.1. Location and animals

The experiment was conducted during the breeding season (November–March) in South America, latitude 23° 24′ 23″ and longitude 57° 26′ 4″. The climate in this region is tropical, with an average temperature of 24 °C and a rainy season from November to January.

A total of 814 Nelore (*Bos indicus*) multiparous, lactating cows were used. The animals had a mean age of 6.6 years (range, 6–7) and were, on average, 77 (64–90) days postpartum, with a body condition score of between 2.5 and 3.5 (on a scale of 1–5).

### 2.2. Experimental design

For the first TAI, all of the cows were synchronized with the same protocol, using an intravaginal device containing 1 g of P4 (DIB; Syntex, Buenos Aires, Argentina) and administration of 2 mg estradiol benzoate (Syntex; Syntex) on Day 0. On Day 8 the device was removed, and 500 μg of cloprostenol (DL Cyclase; Syntex), 300 IU of eCG (Novormon; Syntex) and 1 mg of estradiol cypionate (ECP; Pfizer, São Paulo, Brazil) were applied im. The cows were inseminated 48 hours after device removal.

Twenty-three days after the first TAI, the 814 cows were allocated, based on their body condition score, into one of the five following resynchronization treatment groups: the R23 group, composed of 159 cows that were subjected to a hormonal protocol without the administration of eCG; the R23/200 group, composed of 142 cows that were subjected to a hormonal protocol with the administration of 200 IU of eCG; the R23/300 group, composed of 150 cows that were subjected to a hormonal protocol consisting of the administration of 300 IU of eCG; the R23/TCR group, composed of 177 cows that had their calves temporarily removed from the moment of device withdrawal until the TAI; and the control group, composed of 186 cows that were kept under estrus observation from 18 to 23 days after the TAI and were inseminated 12 hours after the detection of estrus.

The hormonal protocol used in the second TAI was similar to the one used in the first, except for the differences in eCG administration and the TCR. The treated females were inseminated 48 hours after device removal or TCR (33 days after the first TAI) by three experienced inseminators with the semen of four bulls.

The pregnancy diagnosis was performed using ultrasonography 31 days after the first TAI and led to the resynchronization of 73 of the nonpregnant cows from the R23 group, 70 of the nonpregnant cows from R23/200 group, 74 of the nonpregnant cows from the R23/300 group, and 79 of the nonpregnant cows from the R23/TCR group, and to the estrus observation of 97 of the nonpregnant cows from the control group. Other pregnancy diagnoses using ultrasonography was performed 30 days after the second TAI. A schematic representation of all of the resynchronization treatments is illustrated in Figure 1.

### 2.3. Statistical analysis

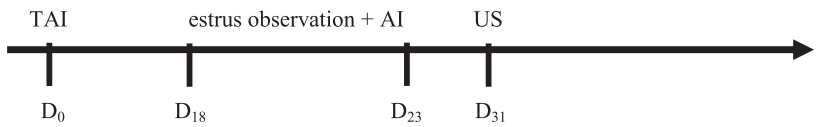
The data are presented as proportions for the descriptive statistical analyses. The conception rates regarding the first TAI and the resynchronization were compared among groups, as were the pregnancy rates after the 33-day period. Conception and pregnancy rates were submitted to the Kolmogorov-Smirnov test to verify if the data presented parametric distribution. Variables with parametric distribution were subjected to analysis of variance and subsequent Tukey test. The effects of semen and inseminators were considered. The analyses were performed using SigmaStat Software Version 4.0, using 5% as the significance level.

## 3. Results

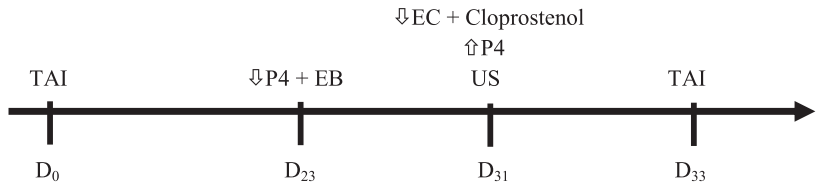
There were no differences between the conception rates after the first TAI among the groups, with the values ranging from 47.8% for the control group to 54.4% for the R23/TCR group ($P > 0.05$). The conception rates after the first and the second AI are shown in Table 1.

The pregnancy rates at the end of the 33-day period were higher in the R23/300 group (76.6%) than in the R23/200 (63.3%; $P = 0.019$), R23 (61.3%; $P = 0.006$), and control (54.3%; $P < 0.001$) groups, but similar to R23/TCR (74.0%). The pregnancy rate was also higher in the R23/TCR than in

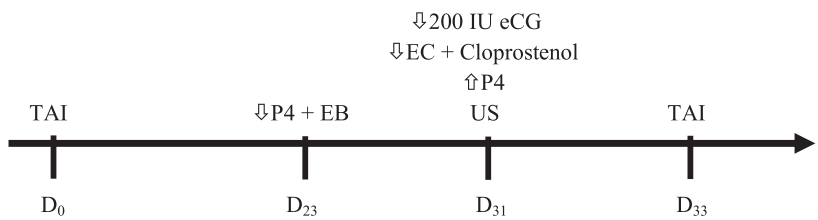
**Control group (N = 186)**



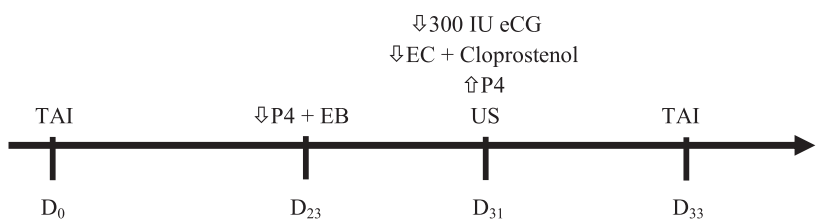

**R23 (N = 159)**




**R23/200 (N = 142)**




**R23/300 (N = 150)**




**R23/TCR (N = 177)**

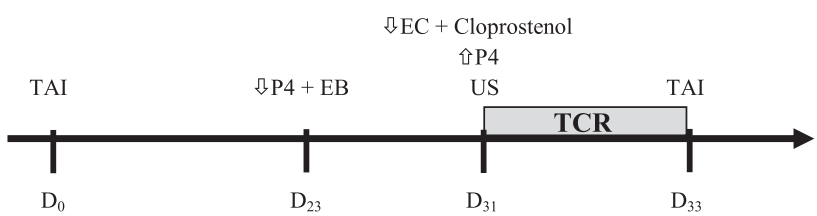



**Fig. 1.** Diagrammatic representation of the treatments used for the resynchronization of ovulation and timed artificial insemination (TAI) of multiparous lactating Nelore cows previously submitted to TAI. D, day; EB, estradiol benzoate; EC, estradiol cypionate; P4, progesterone; TCR, timed calf removal; US, ultrasound examination.

**Table 1**
Effect of the different resynchronization treatments performed 23 days after the TAI on the conception and pregnancy rates of lactating Nelore cows.

| Treatment group | Conception at the TAI, % (N/N) | Conception with estrus observation and AI, % (N/N) | Conception at the TAI after resynchronization, % (N/N) | Cows that became pregnant within 33 days, % (N/N) |
|---|---|---|---|---|
| Control | 47.8 (89/186)[a] | 80.0 (12/15)[a] | — | 54.3 (101/186)[b] |
| R23 | 54 (86/159)[a] | — | 16.4 (12/73)[c] | 61.6 (98/159)[b] |
| R23/200 | 50.7 (72/142)[a] | — | 25.7 (18/70)[c] | 63.3 (90/142)[b] |
| R23/300 | 50.6 (76/150)[a] | — | 52.7 (39/74)[b] | 76.6 (115/150)[a] |
| R23/TCR | 54.4 (98/179)[a] | — | 42.3 (33/78)[b] | 74 (131/177)[a] |

Cows were allocated into one of the five following treatment groups for estrus resynchronization 23 days after the TAI: the control group (estrus observation for 23 days, from 18 to 23 days after TAI and on AI 12 hours after detection of estrus); the R23 group (hormonal protocol with no eCG administration); the R23/200 group (hormonal protocol with 200 IU of eCG); the R23/300 group (hormonal protocol with 300 IU of eCG); and the R23/TCR hormonal group (calves were removed from the moment of device withdrawal until the TAI).
Abbreviation: TAI, timed artificial insemination.
[a–c] Rates with different superscript letters within the same column were significantly different ($P \leq 0.05$).

the R23/200 ($P = 0.049$), R23 ($P = 0.021$), and control ($P < 0.001$; Table 1) groups.

## 4. Discussion

Treatment for estrus resynchronization of lactating Nelore cows 23 days after the first TAI including 300 IU of eCG or TCR resulted in higher pregnancy rates than did the protocols of 200 IU of eCG, no eCG, or AI after estrus observation (the control group). After 33 days, 76.6% of the cows in the R23/300 group and 74.0% of the cows in the R23/TCR group were diagnosed pregnant.

Field data obtained with *Bos indicus* cows revealed different patterns of pregnancy rates concerning the first and second TAI, depending on the category of the resynchronized females. Primiparous and secundiparous cows, for instance, exhibited a decrease in the pregnancy rate from the second TAI (34.9%) compared with that from the first one (53.1%). However, multiparous cows and heifers presented similar pregnancy rates from first and second TAI, with 56.3% versus 52.7% for the cows and 58.4% versus 52.6% for the heifers, respectively [11]. Considering positive results with estrus resynchronization of *Bos indicus*, a third TAI (second resynchronization) was performed and pregnancy rates as high as 90% were obtained with the multiparous Nelore cows during the first 80 days of breeding season [11].

Studies have been performed to synchronize females' estrus, but not necessarily to promote an appropriate synchronization of ovulation, raising the need for estrus observation [2]. However, programs that allow a second TAI are particularly helpful for *Bos indicus* cattle. In addition to the shorter duration of estrus and the higher incidence of estrus that begin and end at night, relative to those of *Bos taurus* [4], *Bos indicus* females have other peculiarities. They have been reported to have greater serum P4 concentrations, smaller dominant follicles and CL diameters and a lower ovulation rate compared with those of *Bos taurus* heifers [12,13]. Moreover, there is evidence that *Bos indicus* cattle have higher circulating concentrations of IgF-I and insulin [14]. For these reasons, hormonal programs that successfully synchronize the estrus of European cows do not always provide the same desirable effects in Zebu cows. The hormonal protocol used in this study was effective in increasing the number of pregnant Zebu cows, particularly in groups R23/300 and R23/TCR. In addition, these programs are easily executable and require little animal handling.

Most of hormonal programs that aim to resynchronize the estrus of beef [2,15] and dairy cattle [3,16,17] do not use eCG. However, eCG treatment at the time that the P4 device is removed has been shown to increase the conception rates of suckling *Bos indicus* cows with an anestrus status [18,19]. In many cases, this drug has the effect of increasing the size of the ovulatory follicle and inducing single and multiple ovulations, therefore ensuring a higher concentration of P4 during the subsequent luteal phase [20]. Studies with indicus or crossbred indicus cows have shown that eCG increased pregnancy rates in the postpartum period, mainly in cows with medium or small follicles [18,21]. In both studies, however, the administration of this drug has increased plasmatic P4 concentrations without significantly affecting the size of the ovulatory dominant follicle and the CL.

Treatment with eCG has also been shown to induce cyclicity in females suffering from nutritional stress [22]. In the early postpartum period, cows in poor condition after calving might present small and hard ovaries, requiring a gonadotropic stimulus. Administration of eCG in these nutritionally deprived females can effectively increase estrus response [23]. Synchrony of estrus, preovulatory LH-surge and ovulation can also be improved in *Bos indicus* cows using treatment with eCG [24]. In the present study, treatment with 300 IU of eCG at the time of device removal has effectively improved the pregnancy rates of resynchronized Nelore cows.

Of the many factors that are known to affect the postpartum anestrus interval, the nutritional status and the presence of the calf are considered to be the most important [25]. Timed calf removal for 48 hours has been shown to increase the size of the dominant follicles and the ovulation rate in postpartum Nelore cows [26]. In this experiment, TCR for 48 to 54 hours resulted in a conception rate (42.3%) similar to that obtained from resynchronization with 300 IU of eCG (52.7%), and in a higher conception rate than that obtained from resynchronization with 200 IU of eCG (25.7%) or with no eCG (16.4%). Perea et al. [10] reported that 120 hours of TCR effectively induced a fertile estrus and reduced the interval between calving and conception in crossbred cows, compared with the values

for the control group, although hormonal treatment was more effective than TCR. Conversely, Pinheiro et al. [27] observed no effect of TCR on the pregnancy rates of Nelore cows 40 to 80 days postpartum. Timed calf removal is a natural and economical approach to induce the onset of estrus in suckling anestrus *Bos indicus* cows. Nevertheless, in Angus and Brangus females, TCR has stimulated follicle growth, but has failed to enhance pregnancy rates to TAI. Furthermore, it had a negative effect on subsequent calf performance, which was dependent on duration and age of the calf [28].

### 4.1. Conclusions

In summary, estrus resynchronization 23 days after TAI was feasible and effective in improving the reproductive efficiency of lactating Nelore cows. The R23/300 and R23/TCR treatments provided the best results, with approximately 75% of the cows becoming pregnant from the TAI and the subsequent insemination.

## Acknowledgments

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**Original Article**

# Effects of progestagen exposure duration on estrus synchronization and conception rates of crossbreed ewes undergoing fixed time artificial insemination

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**Synchronization of estrus and ovulation are of paramount importance in modern livestock improvement programs. These methods are critical for assisted reproduction technologies, including artificial insemination and embryo transfer, that can increase productivity. In the current study, subcutaneous implants containing norgestomet were placed for long (14 days), medium (9 days), and short (5 days) periods of time in 70 crossbred ewes undergoing fixed-time artificial insemination. The resulting effects on estrus synchronization and conception rates were subsequently evaluated. Among the synchronized ewes, 85.7% (60/70) underwent estrus over a period of 72 h after progestagen treatment ceased. The shortest mean interval between withdrawal of the device and onset of estrus (34.2 ± 8.9 h) was observed in the G14 days of P4 group ($p < 0.05$). The conception rate of the G14 days of P4 group was statistically higher than that of the other groups (83.3% *vs.* 60.9% *vs.* 47.8%; $p < 0.05$). In conclusion, 14 days of norgestomet treatment produced higher conception rates and a greater number of pregnancies at the beginning of the breeding season.**

**Keywords:** estrus synchronization, norgestomet, ovine, pregnancy, progesterone

## Introduction

Sheep production associated with the generation of milk, leather, wool, and mutton constitutes a major proportion of agricultural output in several countries worldwide. Seasonal reproductive physiology is the basis for characterizing various wool sheep breeds, and is influenced by habitat latitude as well as time of the year [3]. The mating period usually occurs during the fall when the number of hours of light per day is reduced [1]; this limits annual flock prolificacy.

The effects of reproductive seasonality can be minimized or reversed by the strategic use of reproductive technologies [5]. Hormone-based protocols have been widely employed to increase the reproductive performance and productivity of sheep. The use of fixed-time artificial insemination (FTAI) allows the synchronization of lambing periods, organization of lambs into batches suitable for meeting market demands, an earlier onset of puberty, and improved conception rates regardless of whether the estrus is observed or not [1]. The estrus cycle can be manipulated by maintaining the luteal phase using progesterone ($P_4$) and analogues (progestagens), and/or by interrupting the luteal phase with prostaglandin F2$\alpha$ or a synthetic analog [16,21]. The follicular phase can also be altered with equine chorionic gonadotropin (eCG) administered around the time that $P_4$ exposure ceases. This increases the occurrence and speed of follicular development and ovulation, thereby improving the fertility rate following insemination [2].

FTAI following estrus synchronization with progestagens has been reported to produce varying conception rates (84.6% [10], 71.4%, 80.4% [21], or 54.5% [7]). Many protocols involve long periods (12 to 14 days) of progestagen administration that provides good synchronization rates in cyclic and acyclic ewes but variable fertility rates [4,11]. It has also been found that

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long-term exposure to progestagens extends the phase of follicular dominance and leads to the ovulation of aged oocytes; this is less efficient than short-term progestagen treatment [22]. These inconsistencies in the literature and differences of opinions highlight the need for a consistent and efficient standard hormone-based protocol. Therefore, the aim of the current study was to evaluate the effects of norgestomet implants placed for long (14 days), medium (9 days), and short (5 days) periods on the estrus synchronization and conception rates of crossbred ewes undergoing FTAI during the breeding season in the southern hemisphere.

## Materials and Methods

### Experiment location and animals

This experiment was conducted in Brazil during April and May 2013, corresponding to the natural breeding season with an average of 11 h and 38 min of daylight. The local climate was subtropical, humid, and mesothermal. The study site was located at a longitude of 50°44'28" west and latitude of 23°43'40" south at 976 meters above sea level.

The study was performed in accordance with the ethical guidelines of Londrina State University (Brazil) animal welfare committee. Seventy non-pregnant crossbred adult sheep (Santa Inês x Texel) with body condition scores [18] ranging from 2.5 to 3.5 (on a scale of 1 ~ 5) were used. The sheep were raised in an extensive farming system, on pasture containing *Paspalum notatum* and *Brachiaria decumbens*, and had access to water and mineral salt (Nutristar Ovinos, Londrina, Brazil) *ad libitum*. During the night, the animals were housed in a pen.

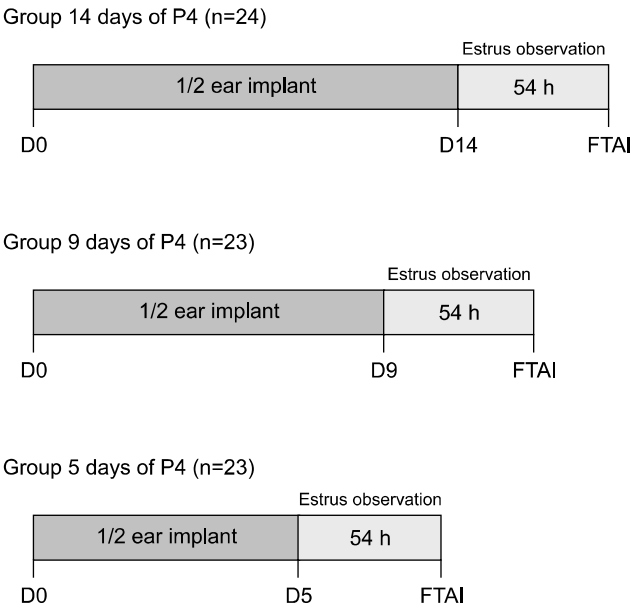


**Fig. 1.** Schematic representation of the fixed-time artificial insemination (FTAI) protocols used for crossbred sheep in Brazil. For G 14 days of P4 , the progestagen device (1.5 mg norgestomet, 1/2 Crestar) was implanted in the ear on the morning of day 0 (a random day of the estrous cycle) and remained in place until day 14 when 22.5 μg D-cloprostenol (Preloban) and 400 IU equine chorionic gonadotropin (eCG; Folligon) were administered. For Group 9 (G9 days of P4), the progestagen device remained in place until day 9 when sodic cloprostenol and eCG were administered at the same doses as in G14 days of P4. For G5 days of P4, the progestagen device remained in place until day 5 when sodic cloprostenol and equine chorionic gonadotropin were administered at the same doses as in G14 days of P4.

### Experimental design

Ewes with similar body condition and age were divided into three groups and subjected to the same procedure for estrus synchronization except that the length of time during which the norgestomet devices (Crestar; Intervet, The Netherlands) were implanted varied. The three groups had the implants for 14 (G14 days of P4, n = 24), 9 (G9 days of P4, n = 23), or 5 (G5 days of P4, n = 23) days.

**Protocol for estrus synchronization:** A schematic of the treatments administered in this experiment is shown in Fig. 1. The norgestomet implants were cut in half using scissors. One half (containing 1.5 mg norgestomet) was implanted subcutaneously in the ear of each ewe. At the time of implant removal, 400 IU of eCG (Folligon; Intervet) and 22.5 μg D-cloprostenol (Preloban; Intervet) were injected intramuscularly into every ewe. No implant was lost and no ear infections were observed.

**Estrus detection and distribution:** Four Santa Inês males that were marked with brisket paint (grating paint and mineral oil moisture) were used to detect estrus in the synchronized ewes. Observation for signs of estrus was conducted on the day that the implants were removed from 4:00 to 7:00 pm. The sheep were monitored for 2 days after the devices were withdrawn from 7:00 to 10:00 am and from 4:00 to 7:00 pm. We considered a ewe to be positive for estrus when the female was marked by the ram and was standing while the male mounted her. Heat duration was measured from the first positive sign recorded until the ewe started to reject the male. On the third day after FTAI, a final period of estrus observation was performed from 7:00 to 10:00 am. In total, estrus observation was conducted over a 72-h period. Even the ewes that did not show signs of estrus were inseminated.

**Artificial insemination (AI):** A pool of semen containing samples collected from one Texel, one White Dorper, and two Dorper rams (all 2 to 6 years old) was used for AI. Semen samples were collected using an artificial vagina (Walmur, Porto Alegre, Brazil) and combined. Sperm motility and vigor were then assessed with a light microscope (Nikon, Japan). Next, the concentration and percentage of abnormal sperm were determined using a

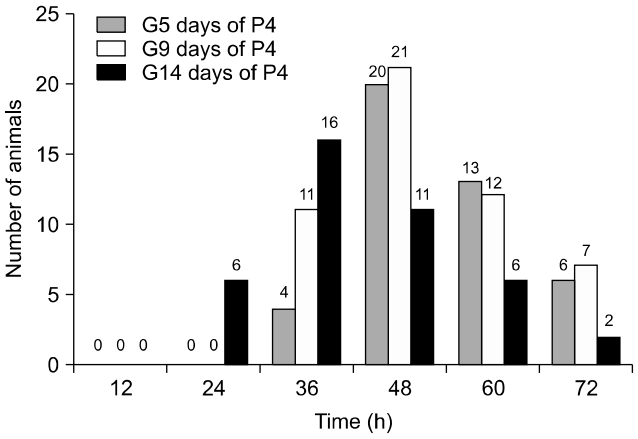


**Fig. 2.** Number of ewes coming in estrus during the 72-h observation period after synchronization with the norgestomet implant for 14, 9, or 5 days.

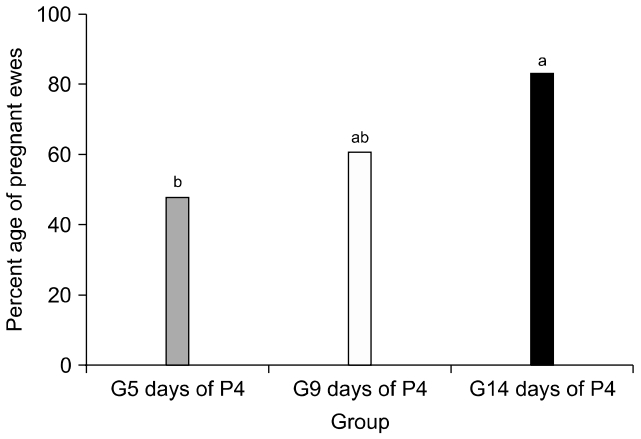


**Fig. 3.** Conception rates for ewes synchronized by hormonal protocols using norgestomet implants for 14, 9, or 5 days. Different letters over the columns indicate significant differences ($p < 0.05$).

Neubauer hemocytometer slides (AGB Scientific, Ireland) following standard operation procedures [10]. The semen was diluted using Tris-egg yolk extender (Trizma Base; Sigma, USA) to the concentration of $2 \times 10^8$ sperm/mL. The sperm used for AI had 80% progressive motility, a vigor score of 3, and 85% were normal sperm cells.

Fifty-four h after implant removal, AI was carried out at the entrance of the first cervical ring as previously described [14,15]. All AI procedures were performed by the same inseminator. In total, 0.5 mL corresponding to 160 million live sperm cells was delivered to each ewe.

**Pregnancy diagnosis:** Pregnancy was detected by transrectal ultrasonography (5.0 MHz, Aloka SSD-500; Aloka, Japan) 30 days after FTAI.

**Statistical analysis:** For this experiment, 70 ewes were randomly divided into three treatment groups. Differences in the rate of estrus onset during the 72-h observation period, the interval between the removal of the progestagen device and estrus onset, estrus duration, and conception rate were analyzed using Fisher's exact test (SigmaStat 3.5; Systat Software, USA). For all of the analyses, $p \leq 0.05$ was considered to be significant. Conception rates were analyzed using logistic regression with the "Car" statistical package of "R" software (R development core team 2013, Austria).

## Results

Among the synchronized ewes, estrus was noted in 85.7% (60/70) over the 72-h observation period following the removal of the norgestomet devices. No significant difference was observed in the percentage of ewes exhibiting estrus: 18/24 (75.0%) for G14 days of P4, 22/23 (95.7%) for G9 days of P4, and 20/23 (87.0%) for G5 days of P4. Differences in the dynamics of estrus associated with the various treatments were noted as presented in Fig. 2. The shortest average interval between implant removal and the onset of estrus was observed in the G14 days of P4 group (34.2 ± 8.9 h; $p < 0.05$). In contrast, this interval was not significantly different between ewes in the G9 days of P4 (41.9 ± 6.1 h) and G5 days of P4 (44.0 ± 6.7 h) groups. The mean duration of estrus did not differ ($p > 0.05$) between animals from the various groups (28 ± 15.5 h for G14 days of P4, 30 ± 12.1 h for G9 days of P4, and 26 ± 8.3 h for G5 days of P4). Conception rates are shown in Fig. 3. The G14 days of P4 group had a higher pregnancy rate (83.3%) than sheep in the G5 days of P4 group (47.8%; $p < 0.05$) but not ones in the G9 days of P4 group (60.0%).

## Discussion

In the current study, the effect of norgestomet administration for different lengths of time on estrus synchronization and conception rates in ewes was evaluated. The increased conception rates observed with the 14-day treatment period are contrary to the results from a study by Martins *et al*. [13] using medroxiprogesterone acetate (MAP) for 12 and 6 days. Additionally, our findings conflict with the theory that long-term exposure to progestagen results in the excessive growth and persistence of dominant follicles. According to this hypothesis, oocytes released from these follicles during ovulation are of poor quality [22]. The pregnancy rate of 83.3% obtained in this experiment with long-term (14-day) hormone treatment suggests that the released oocytes retained a satisfactory level of quality and were able to establish pregnancies. Conflicting results from our investigation and those in the literature may be due to differences in the breed of animals or drugs that were used. Another explanation is that compromised fertility may result from long-term protocols involving the use of intravaginal

devices due to the vaginal/cervical environment becoming suboptimal for sperm transport. Our protocol would have overcome this drawback because progestagen was administered through a subcutaneous device implanted in the animal's ear. Furthermore, norgestomet implants placed subcutaneously in the ears of ewes are known to increase serum progesterone levels [20].

The similarity of the 14-day protocol with the physiological events of the luteal phase may provide a reasonable explanation for the relatively high conception rates obtained in our study. It is known that corpus luteum regression in ovine females occurs between 13 and 15 days after ovulation, and that the rapid decline in progesterone is essential for the next onset of estrus and subsequent ovulation [6]. This may explain why the G14 days of P4 group had the highest conception rates since a percentage of ewes in the G9 days of P4 and G6 days of P4 groups may have had an active corpus luteum present in the ovary at the time of implant removal. For those animals, follicular development and ovulation events were surely delayed until progesterone secreted from the corpus luteum decreased following prostaglandin-induced luteolysis. Likewise, a percentage of these same animals would have benefited from delaying FTAI to optimize the conception rate.

For births to occur at the beginning of lambing season, estrus synchronization technology can be used to maximize the number of ewes becoming pregnant at the beginning of the breeding season. The interval between implant removal and estrus onset was significantly shorter in the G14 group (34.2 ± 8.9 h; $p < 0.05$) compared to the G9 days of P4 (41.9 ± 6.1 h) and G5 days of P4 (44.0 ± 6.7 h) groups. This result is similar to findings from studies by Salehi *et al.* [19], and Martemucci and D'Alessandro [12] in which the synchronization of estrus for 14 days via controlled internal drug release or fluorogestone acetate intravaginal sponges, respectively, promoted the onset of estrus within 36 h or 33.1 ± 4.3 h after removing the device.

In the current study, the percentage of ewes in estrus was similar ($p > 0.05$) among the three groups. This observation concurred with results obtained by Salehi *et al*. [19]. In addition, there was no significant difference in the duration of estrus between the groups. Our data indicate the effectiveness of the three protocols to synchronize estrus among the ewes.

In conclusion, treatment with norgestomet for different periods of time resulted in a high percentage of ewes in estrus within the 72-h observation period. Estrus duration among the three groups of ewes was also similar. However significant shortening of the interval between norgestomet implant removal and estrus in the animals treated with progestagen for 14 days ($p < 0.05$) was accompanied by a higher conception rate following FTAI. Future studies are required to evaluate the effect of inducing luteolysis at least 48 h prior to implant removal along with the impact on estrus dynamics and pregnancy following FTAI.

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## Conflict of Interest

There is no conflict of interest.

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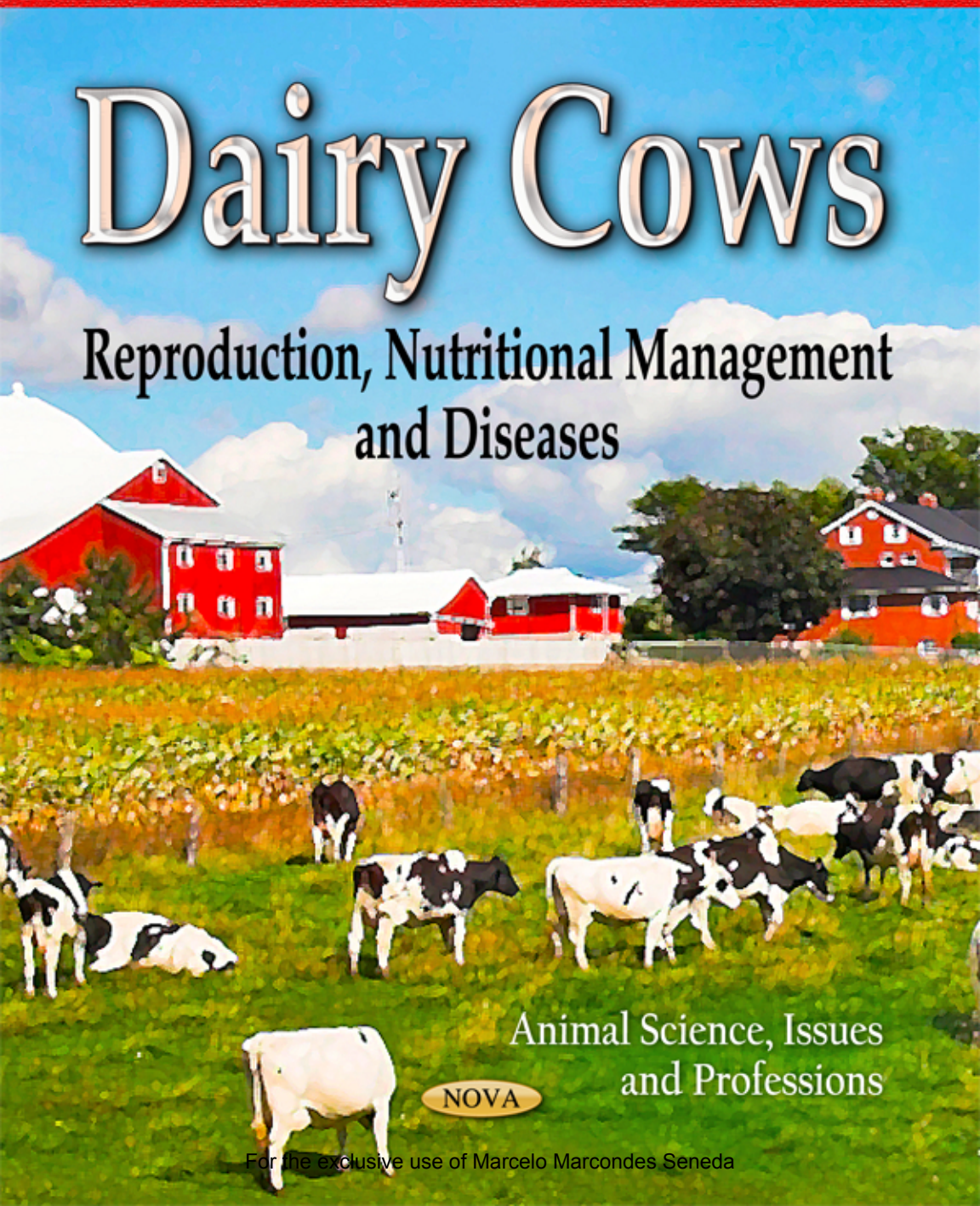
# Dairy Cows

## Reproduction, Nutritional Management and Diseases



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*Chapter 4*

# STRATEGIES TO IMPROVE THE REPRODUCTIVE EFFICIENCY OF DAIRY CATTLE

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## ABSTRACT

The reproductive performance of a lactating herd is a major component of the profitability of a dairy farm. Factors such as negative energy balance, heat stress and failures in heat detection can severely compromise reproductive parameters. To overcome these problems, a variety of strategies can be used. For example, failures in estrus detection can be solved with the use of fixed-time artificial insemination (FTAI). Progesterone implants combined with estradiol administration are very effective in promoting the onset of a new follicular wave. With the use of a luteolytic agent and an inducer of ovulation, AI can be performed at the appropriate time without the need for estrus observation. In countries where the use of such drugs is not allowed, protocols based on GnRH and PGF2α may also offer optimal synchronization of ovulation. In locations where pregnancy rates are compromised by high temperatures, a viable

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alternative to FTAI may be the fixed-time embryo transfer (FTET). Embryos at the morula and blastocyst stage are more resistant to heat stress than gametes and embryos in early stages of development. Thus, embryo transfer (ET) on day 7 of development can ensure satisfactory pregnancy rates throughout the year, even in months and/or regions with higher average temperatures. ET has also been effective in preventing early embryonic mortality and increasing pregnancy rates in repeat breeders. Another strategy that can enhance the reproductive efficiency of dairy herds is the cryopreservation of embryos; embryos are stored and can be used at strategic times, such as in the warmer months of the year. This chapter will discuss technological strategies that can lead to higher breeding efficiency, improved reproductive efficiency and increased profitability of livestock on dairy farms.

## 1. INTRODUCTION

Over the last 50 years, reproductive efficiency has declined greatly in the dairy industry. Currently, parameters such as conception at first service, services per conception and calving interval have shown to be deteriorating. The worsening of these features can be explained by modifications in the management of dairy farms and by negative genetic correlations between milk production and fertility. This decline in reproductive performance is of great concern to milk producers, given the significance of good fertility to the dairy industry. In addition to adequate nutritional management, which is of major importance to achieving positive results, a variety of strategies can be used to ensure better reproductive outcomes. Fortunately, there is currently enough technology to at least partially reverse this setback. One of the most important approaches developed to improve reproductive efficiency is artificial insemination (AI). In the present context, AI is a hugely widespread technology, and most milk producers use it at some level in their herds. However, the failure to cycle and display estrus frequently leads to unsatisfactory outcomes when only AI is used. Therefore, hormonal protocols have been used to induce estrus and synchronize the moment of ovulation, increasing the percentage of pregnant animals in the herd.

In addition to fixed-time artificial insemination (FTAI), embryo transfer (ET) has provided fairly positive pregnancy rates. This strategy is especially useful in regions with high temperatures because not even FTAI protocols can offer acceptable rates in cattle with heat stress. The transfer of both in vivo and in vitro-produced embryos has been widely used to ensure satisfactory

pregnancy rates throughout the year, even in countries with high temperatures in the summer. This chapter aims to present some of the most promising technologies currently available to improve reproductive efficiency in dairy cattle, as well as their pros and cons. Recent data will be reviewed, and the most suitable situations to use each technique will be discussed.

## 2. FIXED-TIME ARTIFICIAL INSEMINATION

The detection of estrus is a determining factor for obtaining good pregnancy rates. In dairy herds, over 50% of the cows in heat are not detected due to a lack of mating behavior displayed by the animals (Van Eerdenburg, 2002). High milk production seems to seriously impact this reproductive factor, as low estrus detection rates are even more pronounced among high-producing animals (Jeong et al., 2012). The failure to detect estrus also represents a significant challenge in programs that use cattle with some proportion of *Bos indicus* blood, as these animals experience an estrus of shorter duration and their estrus frequently begins and ends at night (Bó et al., 2003). In recent years, the use of pharmacological methods to control the estrus cycle (i.e., FTAI protocols) has done away with the need to monitor for estrus. These protocols control the rise of follicular waves and regulate luteal dynamics, promoting synchronization of the moment of ovulation (Nasser et al., 2004). In addition to increasing the number of animals subjected to insemination, FTAI also has brought dairymen great advantages in strategic management of the reproduction of the herd. A wide range of synchronization protocols for FTAI based on a variety of natural and synthetic drugs are available on the market. Some of the most commonly used hormones are prostaglandins, progestagens, estradiol, equine chorionic gonadotropin (eCG), human chorionic gonadotropin (hCG) and gonadotropin-releasing hormone (GnRH).

### 2.1. Drugs Most Frequently Used for Estrus Synchronization

#### *2.1.1. Prostaglandin*

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and its synthetic analogues interrupt luteal activity and are widely used to regulate the estrus cycle of lactating dairy cows. The action of this drug depends on how developed the dominant follicle (DF) is at the time of the administration. If the DF is too mature to ovulate,

only the DF of the next wave will be affected, resulting in a longer time span until the next rut occurs.

However, if a DF able to ovulate is present, the manifestation of estrus occurs approximately 48 h after the treatment with $PGF_{2\alpha}$. In general, approximately 50-60% of cows treated with $PGF_{2\alpha}$ rut within a period of 36 to 130 h after administration (Whittier et al., 1989). Some protocols have been designed based on single or double doses of $PGF_{2\alpha}$ to reduce treatment costs. However, these protocols require estrus detection for a few days and/or the detection of a corpus luteum (CL) that is likely to be responsive to the treatment (CL of day 6 to 17 of the estrus cycle). One effective way to use the luteolytic effect of $PGF_{2\alpha}$ is to combine $PGF_{2\alpha}$ with progesterone in cycling cows. In general, females are treated with progesterone for 7 to 9 days and $PGF_{2\alpha}$ is administered 0 to 2 days before progesterone withdrawal.

### *2.1.2. Progesterone and Estradiol*

The use of progesterone with estradiol controls the rise of follicular waves by promoting atresia of the developing follicles and regulates CL function and ovulation time. When combined with a luteolytic agent and an ovulation inductor, this treatment allows the use of AI at a defined time without estrus observation and with stable pregnancy rates. Estrus observation is not needed and is actually not recommended because FTAI systems frequently induce fertile ovulation without estrus expression (Thatcher et al., 2002). Such hormonal protocols can also increase ovarian activity. Progesterone and its synthetic analogues (progestagens) prevent the manifestation of heat because they mimic the endocrine activity of a CL. Progesterone supplementation is also an effective method for treating anestrus, which is a common problem in dairy production systems.

The removal of the progesterone is followed by estrus, ovulation, and a normal-length luteal phase in a large percentage of treated cows (Lucy et al., 2004). Currently, synthetic progestagens are best supplied in the form of subdermal ear implants; intravaginal implants are mostly used to release progesterone itself. The releasing devices vary in shape, design and the amount of hormone released. Estradiol suppresses the release of FSH, thus inducing the atresia of minor follicles. Progesterone decreases the release of LH, thus restricting the activity of larger follicles. It is therefore expected that all follicles in the ovaries reach atresia. It is also likely that a new follicular wave appears after 3 to 4 days of treatment with estradiol and progesterone (starting from any day of the estrus cycle).

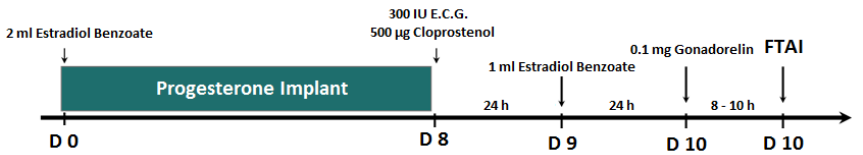


Figure 1. Example of a fixed-time artificial insemination (FTAI) hormonal protocol used for lactating dairy cows. Estradiol benzoate is administered on a random day of the estrus cycle with a progesterone-releasing device; on day 8, the device is removed, together with a cloprostenol (a synthetic analogue of prostaglandin $F_{2\alpha}$) and eCG (equine chorionic gonadotropin) administration; 24 h later, a new dose of estradiol benzoate is administered; gonadorelin (synthetic analogue of GnRH - gonadotropin-releasing hormone) is given 24 h later; following 8 to 10 h, timed-AI is performed.

Additionally, estrogen is essential for the expression of oxytocin receptors in the endometrium which are critical to the release of $PGF_{2\alpha}$ for CL regression. Therefore, estrogen has been used for both the synchronization of follicular waves and the promotion of luteolysis. Several drugs accomplish these purposes. Esters of estradiol, estradiol benzoate (EB) or estradiol cypionate (EC) have the lowest cost and are the most commonly used inductors in traditional protocols. Intervals to wave emergence depend on the dose and the half-life of the ester used. Hormones such as GnRH, LH and hCG may also be used as ovulation inducers, but their use is less common due to their higher costs. With the use of ovulation inductors, FTAI can be performed 52 h after the removal of progesterone, i.e., 8 to 12 h before the planned time of ovulation (Hanlon et al., 1997). An example of a FTAI protocol with the use of a progesterone-releasing device and estradiol is illustrated in Figure 1.

### *2.1.3. GnRH*

GnRH is a hypothalamic hormone that acts by triggering the pituitary release of FSH and LH, thereby inducing ovulation. Despite having a short half-life in plasma (2 to 4 min), a single administration of GnRH results in an enhanced and simultaneous release of both LH and FSH in a log-dose response manner. In FTAI protocols, the ovulatory GnRH dose is typically given 48 h after a luteolytic dose of $PGF_{2\alpha}$. In countries where the use of certain drugs such as estrogen is not allowed, protocols with GnRH and $PGF_{2\alpha}$ are highly convenient for FTAI in dairy cattle. Both hormones were of great importance for the consolidation of the FTAI protocol and the initiation of a series of other protocols. In addition, GnRH can effectively be used to restore the cycling of a female after calving because the ability of the pituitary to respond to GnRH is restored approximately 20 days postpartum.

### *2.1.4. eCG*

Known as a mixed bioactive drug, eCG has been widely used in FTAI protocols. It can be employed both at the end of the treatment and at the emergence of the new wave because eCG is composed of 2/3 FSH and 1/3 LH. Souza et al. (2009) reported improvement in the fertility of dairy cows with low BCS treated with eCG on day 8 of the FTAI protocol. This effect was most likely due to an increase in activity of the ovulatory follicle (and consequently, of the CL) stimulated by this drug. High-producing milk cows have high levels of hepatic metabolism of ovarian steroids; the decrease in circulating progesterone during the luteal phase may therefore suppress early embryonic development. Treatment with eCG prior to ovulation can increase circulating progesterone levels and provide higher pregnancy rates in animals with low BCS.

## 2.2. Association of GnRH and $PGF_{2\alpha}$: Ovsynch

Based on a GnRH and $PGF_{2\alpha}$ combination, the Ovsynch program and its variations are the most commonly used protocols for FTAI on North American dairy farms. The first administration of GnRH occurs on a random day of the estrus cycle and stimulates the ovulation of the current dominant follicle. Ovulation is expected to happen within 2 or 3 days followed by the beginning of a new follicular wave. In order for the luteolytic process to occur, a $PGF_{2\alpha}$ injection is required; such an injection takes place 7 days after the first supply of GnRH. A second dose of GnRH is used 48 h after the $PGF_{2\alpha}$ administration to promote ovulation of the new DF. Ovulation then occurs in approximately 24 h, enabling the use of FTAI within 16 to 20 h (Pursley et al., 1995).

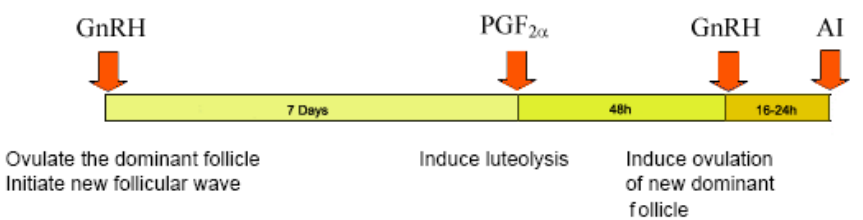


Figure 2. Ovsynch is a fixed-time artificial insemination (FTAI) program that combines GnRH and $PGF_{2\alpha}$. GnRH is administered in a random day of the cycle; 7 days later, $PGF_{2\alpha}$; 48 h later, GnRH again; and AI 16 to 24 h after the last GnRH administration.

The Ovsynch program is represented in Figure 2. It is possible that some females may not respond to the initial application of GnRH, which can occur in phases where there is no dominant follicle to ovulate, such as in the beginning or middle of the estrus cycle, and can disrupt the entire sequence of the protocol.

This is one of the critical problems associated with the Ovsynch program. Another important factor to consider is the number of follicular waves. The higher the number of waves, the less satisfactory the results will be. At the beginning of every follicular wave, there is a range of 4 days during which GnRH has no effect. When there are two follicular waves in the estrus cycle, there are two intervals of 4 days (the beginning of the waves). These periods where no response to GnRH can occur increase when the cycle has three waves, or three 4-day intervals, corresponding to 12 days in which the first application of the protocol will not be effective.

The number of waves is influenced by a number of factors, including the age of the cow. Two-wave cycles are more common in older cows than in heifers (Moreira et al., 2000). Diet can also influence the number of waves, as the type of feed provided can greatly influence the hepatic metabolism of steroid hormones. During digestion, a diet rich in grains requires greater hepatic activity, consequently generating a low serum concentration of steroid hormones such as progesterone. Lower levels of progesterone will lead to an increase in LH levels because less of this gonadotropin will be inhibited by progesterone. With higher levels of LH in the system, the DF present in the first wave of the cycle may remain available for longer, extending its wave duration and delaying the start of the next one. As a result, the third wave will not have time to occur because the hormonal profile at the end of the second wave will match that of the beginning of the third, thus culminating in ovulation.

This type of highly concentrated diet is typically given to high-producing cattle, explaining why these cows tend to have two follicular waves rather than three. This high metabolic clearance rate, leading to a reduction in the levels of circulating estradiol, can also result in a short duration of estrus, which explains why it is difficult to detect signs of estrus in dairy herds (Wiltbank et al., 2006). One approach for obtaining a better reproductive outcome in dairy cattle is to use three consecutive FTAI protocols and later relocate the cow for natural service by bulls. This allows the servicing of cows with several inseminations before transfer and typically results in a 15% greater chance of pregnancy. The resynchronization can be performed with either Ovsynch program protocols or with progesterone implants.

### 2.3. Important Considerations for FTAI In Dairy Herds

The use of FTAI protocols in dairy cattle is intended to decrease the common flaws that occur when relying on estrus observation and to optimize handling. However, the expected pregnancy rates with the use of hormonal protocols are similar to the ones obtained without the use of any drug when simply monitoring cows for signs of estrus. Therefore, the cost-benefit ratio and the pros and cons of the implementation of FTAI must be clear and rationally determined before using this tool. Most protocols of pharmacological control of estrus provide benefits, and major additions to reproductive efficiency can be expected when they are used in herds with adequate conditions.

Some dairy breeds are particularly sensitive to climatic variations. European breeds such as Holsteins and Jerseys suffer great thermal discomfort under conditions of high temperatures and high humidity. Thermal stress is very damaging to FTAI as gametes are sensitive to these conditions. Nevertheless, it is important to remember that the male is responsible for half of the process of fertilization; therefore, it is also necessary to rigorously analyze the quality of the semen.

## 3. EMBRYO TRANSFER IN DAIRY COWS

South America currently leads the world in both in vitro and in vivo bovine embryo production. Such a position is interesting when considering that the predominant racial type in South America is *Bos taurus indicus*, also known as Zebu, which is highly adapted to the tropics. Zebu cows naturally produce more oocytes than taurine breeds, which leads to a higher number of in vitro-produced (IVP) embryos and pregnancies. The larger number of pregnancies justifies, in many occasions, replacing the in vivo-production of embryos with the in vitro technique. This finding contributes to a large-scale commercial application of in vitro fertilization (IVF). By promoting a prior selection of donors and bulls, it is possible to achieve high levels of productivity; this usually results in lower costs, thus enabling the use of IVF on a commercial scale.

Despite the rapid development of the technique since its emergence in the late 1980s, until a few years ago, IVEP was only used as a last resort when traditional techniques failed. However, the high genetic gains provided by

IVEP to the herds and the constant pregnancy rates achieved with this system have contributed to making this system a first-line option.

## 3.1. Embryo Transfer

Embryo transfer (ET) is a term generally used to define more than one biotechnological technique. It involves the production and transfer of embryos generated either in vivo by the technique of Multiple Ovulation and Embryo Transfer (MOET) or in vitro. In both cases, the purpose is to obtain genetically superior embryos and to generate pregnancies in female recipients. These biotechnologies aim to produce a much larger number of offspring than would be possible by natural methods of reproduction throughout the lifetime of a female.

An additional advantage provided by these biotechnologies is a better sanitary control of the reproductive process, especially with regard to diseases of the reproductive tract. In addition to preventing sexually transmitted diseases, the embryos can undergo sanitary procedures before the transfer, further minimizing the chance of transmission of pathogens. Embryo transfer can also be used to obtain descendants from donors that have acquired reproductive disorders or any other non-congenital condition that could prevent the female from producing and carrying healthy embryos. Thus, it is possible to prevent the premature discarding of genetically superior females that have acquired different types of reproductive disorders. Finally, these biotechnologies allow the performance of basic research on the physiology of reproduction and of surveys to assist the development of other biotechnologies such as cloning and transgenesis.

## 3.2. Selection of Donors

To be selected as embryo donors, females must have high levels of milk production. It is also very important to evaluate the reproductive performance of the donors; if their reproductive efficiency is good, the chances of getting the expected results with ET are higher. Donors must be free of infectious diseases such as brucellosis, tuberculosis, leucosis, leptospirosis, IBR and BVD.

Although some of these diseases can be controlled by treating the embryos before the transfer, the efficiency of embryo production in these animals may

be compromised by the infectious process. Donors with stressful conditions such as hoof or udder disorders generate poor results and should therefore be treated or allowed to recover from such situations before being included in ET programs.

## 3.3. Multiple Ovulation and Embryo Transfer (MOET)

This technique is divided into five steps:

- Selection of donors and recipients;
- Superovulation and artificial insemination of the donor;
- Preparation of recipients;
- Embryo collection;
- Evaluation and transfer of embryos.

Superovulation aims to stimulate the growth and ovulation of the highest possible number of follicles in a follicular wave. The administration of hormones with gonadotrophic activity prevents the phenomenon of dominance which would lead to atresia of many minor follicles. One of the biggest drawbacks of the technique is the high individual variability in the response to superovulation among the donors; an aggravating 20 to 30% of donors do not produce any transferable embryo. The choice of the hormonal protocol should be carefully evaluated, taking into consideration the breed, weight and production of donors, the condition of the property and the skill of the employees who will perform the work. An example of hormonal protocol used for superovulation is described in Table 1. In general, the embryo collection is performed seven days after the insemination of the donor. During this period, the embryos are in the uterine lumen and at the morula or blastocyst stage, the most suitable ones for transfer and cryopreservation. The collection method mostly used currently is uterine flushing via the cervix. The viable embryos that are found are categorized and transferred into the recipients via transcervical or surgical implantation. In general, it is possible to perform MOET in animals ranging in age from 10 months to 18 to 20 years. However, very young heifers exhibit highly variable ovarian responses. Furthermore, the size of the pelvis and the size and tortuosity of the cervix should be considered, as they could potentially hinder the necessary procedures and jeopardize the success of the collection. Senescent cows usually present with lower production levels, and production rates tend to decrease as animals age.

**Table 1. Example of hormonal protocol used for the superovulation of bovine females**

| Day | Time | Treatment |
|---|---|---|
| D 0 | a.m. | P4 Device Insertion + E2 |
| D 4 | a.m. | FSH (20% dose) |
| | p.m. | FSH (20% dose) |
| D 5 | a.m. | FSH (15% dose) |
| | p.m. | FSH (15% dose) |
| D 6 | a.m. | FSH (10% dose) + PGF2α |
| | p.m. | FSH (10% dose) + P4 Device Removal |
| D 7 | a.m. | FSH (5% dose) |
| | p.m. | FSH (5% dose) |
| D 8 | a.m. | Estrus observation |
| | p.m. | A.I. (12 h after estrus) |
| D 9 | a.m. | A.I. (24 h after estrus) |
| D 15 | a.m. | Recipients evaluation |
| | p.m. | Embryo collection + embryo transfer |

Doses: estradiol benzoate (E2): 2 mg IM; prostaglandin (PGF2α): 2 mL IM. FSH doses vary according to breed, animal category and manufacturer.

## 3.4. In Vitro Embryo Production (IVEP)

IVEP aims to produce embryos in the laboratory using oocytes from ovaries of slaughtered or living cows. Oocytes and sperm are prepared and matured in vitro for the fertilization and subsequent embryo formation after a period of in vitro culture. The embryos are then transferred to properly prepared female recipients. In terms of genetic merit and sanitary aspects, the same criteria previously mentioned in this chapter should be applied. Specifically, the reproductive condition of females should be evaluated by ultrasonography at the exact time of oocyte retrieval. As a strategy for improving the cost/benefit ratio of IVEP, ideally only donors with a large number of follicles would be subjected to OPU. Although all visible follicles can be aspirated, there is a better retrieval of oocytes when the diameter of the dominant follicles is <4 mm (Seneda et al., 2001). The animals that are

subjected to follicular puncture should be evaluated periodically to control the appearance and severity of the injuries caused by the aspiration procedure. In general, there are few alterations in the vagina and cervix, and those that do occur are mostly transient irritations. Sequelae such as fibrosis and adhesions can occur in cows subjected to several sessions of follicular aspiration if the procedures were not properly performed (Gibbons et al., 1994). It is important to note that follicular aspiration is a technique that has pros and cons. The risk of sequelae exists and should be considered. The procedure must be performed with minimal damage so that the results outweigh the expected disadvantages.

### *3.4.1. Strategic Use of IVP Embryos in Dairy Farming*

The large amount of oocytes and IVP embryos produced by *Bos indicus* females has made IVEP commercially very attractive in recent years. When the first commercial laboratories emerged, the demand was very limited. The interest from breeders was small and the expense of the services almost restricted their use to elite beef cattle producers. However, over the last six or seven years, dairy farmers have begun to make greater use of the technique of follicular aspiration and IVEP with the goal of rapidly multiplying the number of animals with high genetic potential. During this period, IVEP became a commercially viable alternative to improve pregnancy rates and speed up genetic improvement. However, until a few years ago, large-scale IVEP was limited by several drawbacks. One of them was the long distances between the laboratories and the properties where the recipients were held. In Brazil, for example, the large cattle herds are usually located in new areas of livestock production, such as the northern region. Oocyte donors and the laboratories which service them, on the other hand, are concentrated mainly in the south and southeast regions of the country, thousands of miles away from the farms with the recipient animals. The limited ability of IVP embryos to be cryopreserved greatly restricted the contact between production and transfer sites. Consequently, embryos that could not be transferred fresh were often discarded.

Recently, some of these problems have been overcome. In a large-scale IVEP program using exclusively sexed semen from bulls of Holstein and Gir breeds, embryos were transported over distances ranging from 800 to 2000 km to be transferred to the recipients (Pontes et al., 2010). Using portable incubators, the embryos were transported during the early stages of development. They began the development in the laboratory and were then transported for 2 to 5 days at different stages of development (day 2 to day 5; day 0 = day of IVF). At the end of transportation, embryos were re-evaluated

and loaded into straws to be transferred at morula or blastocyst stages. In this outsized project, 20,000 Girolando (Gir-Holstein crossbred animals) embryos were produced with female-sorted sperm. In just over a year, 8,000 Girolando heifers were produced from Holstein, Gir or Girolando donors. The possibility of transporting fresh IVP-embryos contributed to a satisfactory cost effectiveness of the IVEP process, with pregnancy rates of 36 to 40% and an overall average rate of 39%. In Holstein cows that typically show lower production of oocytes, it is also possible to obtain good results with follicular aspiration. The most effective strategy is to perform a pre-selection of females, assessing follicular populations with the use of an ultrasound. By using only the females with higher numbers of follicles, indices can be quite satisfactory. It is interesting to note that non-lactating cows may have high numbers of follicles and oocytes.

### *3.4.2. Sorted Sperm*

In dairy cattle, the possibility of choosing the gender of the calf is extremely advantageous because male calves are not of economic interest. Some properties choose to produce embryos with unsorted sperm and determine the gender of embryos by biopsy and DNA analysis. Although this method is an accurate and sensitive technique, approximately 50% of the embryos are found to be male and discarded. In addition, pregnancy rates are considerably lower with biopsied embryos than with intact embryos (Hasler et al., 2002). The use of sorted sperm makes it possible for almost all of the embryos to be of the desired gender and of the same quality as embryos produced with conventional semen. However, the process of sperm sorting reduces sperm viability and pregnancy rates are not always satisfactory with artificial insemination (AI). For IVEP, on the other hand, semen subjected to this technique can successfully be used because this production system requires fewer viable sperm cells. The rates obtained currently in IVEP with sorted sperm are very encouraging. In the IVEP program described in the previous section of this chapter, the use of sorted sperm allowed a more rational use of embryo recipients. When unsorted sperm is used, twice as many recipients are required to achieve the desired number of products of the expected gender. Nevertheless, there were bull and ejaculate effects, showing the need for a rigorous evaluation and previous selection of the best batches of sperm to obtain more advantageous results (Pontes et al., 2010). Therefore, transportation of embryos over long distances and the use of sorted sperm are crucial ways to improve the efficiency of large-scale IVEP.

## 3.5. Heat Stress

In dairy farms located in regions with humid and hot summers such as Africa, India, South America, and the southern United States, pregnancy rates can be very low during the months of heat stress. Some properties even opt for extreme measures, and do not inseminate females in heat during the warmer months of the year. Exposure of the oocytes to increased body temperatures has been linked to the degeneration of thecal and granulosa cells and to low quality oocytes with lower rates of fertilization and conception (Chebel et al., 2008). Given the greater sensitivity of gametes and embryos to high temperatures in early stages of development, ET on day 7 after fertilization seems to be the most viable option. For this reason, ET has been used efficiently to increase pregnancy rates in dairy cattle in months and/or regions of higher average temperature (Hansen, 2007; Vasconcelos et al., 2011).

## 3.6. Recipients

Selection of recipients must be carefully conducted, as they represent a step of fundamental importance to the success of MOET or IVEP programs. Adequate recipients do not need to have superior genetic qualities, but they must present with an appropriate size (suitable for the breed of the fetus) and have good milk production and proven maternal ability to nurture the neonates properly. The health criteria applied to the recipients should be the same as those that are applied to the oocyte or embryo donors. To ensure the success of IVEP programs and to achieve better pregnancy rates, a large number of animals should be made available to allow a rigorous selection of the most suitable recipients. However, the high cost of maintaining these animals may be prohibitive and compromise the economic viability of the embryo industry. The possibility of transporting fresh embryos in vitro solved the major obstacle of large distances. It was originally cost-prohibitive to use recipients located near the laboratories because the availability of these animals was low. With the transport of IVP embryos over long distances, it became possible to use animals from the largest recipients commercial herds situated far away from the laboratories and establish IVEP on a commercial production scale.

Consequently, the use of large herds of embryo recipients resulted in changes in the pattern of females used. Informally, the best recipients were considered to be crossbred heifers, usually half-blood Nelore or European breeds, such as Simmental and Braunvieh, with some production capacity.

However, due to a limited supply and high demand of these animals, this type of recipient became costly and scarce. Particularly for large ET projects, it became cost-prohibitive to use this type of heifer as embryo recipient. A successful case in Brazil involved the use of recently calved beef cows (Nelore - *Bos indicus*) as embryo recipients. This category of females is the most numerous in South America, providing an adequate supply of recipients at a fair price. Contradicting previously held ideas, these females have provided acceptable pregnancy rates (approximately 40%), and more than 10,000 calves have been born to these cows (In Vitro Brasil Ltda, Mogi Mirim, SP; unpublished data). If animals have adequate nutritional and health statuses, the use of cows may indeed present certain advantages over heifers. In general, cows may have a higher recipient transferred-to-treated rate with FTET because they usually show better response to hormonal protocols and heifers often require a pre-synchronization. Cows usually have also been more exposed to pathogens and thus may have greater disease resistance and better quality colostrum. Finally, the frequency of dystocia tends to be higher in heifers than in cows. Until a few years ago, the importance of recipients was based mainly on their ability to respond to synchronization protocols and to maintain pregnancy. However, with current advances in epigenetics, it became evident that other factors, such as diet quality and maternal behavior, can interfere with gene expression of the calf. Despite the fact that the genetic sequence is established at the first cell division, the expression of genes may be altered due to environmental factors. The follicular ovarian reserve of the fetuses, for instance, can be hugely affected by the nutritional status of the recipient during pregnancy. As a result, a future donor may have compromised reproductive performance if the recipient underwent nutritional deprivation during pregnancy (Ireland et al., 2011). Similarly, it was shown that females with strongly maternal behavior influence DNA methylation and thus interfere with the expression of important genes for environmental adaptation (Weaver et al., 2004).

## 3.7. Fixed-Time Embryo Transfer (FTET)

Similarly to timed artificial insemination (TAI), FTET eliminates the need for heat detection, resulting in great advantages for the strategic management of the breeding of the herd. To solve problems related to heat detection and to increase the proportion of suitable recipients to receive embryos, several

hormonal protocols have been developed. An example of FTET protocol is described in Figure 3.

## 3.8. Advantages of Using Embryos Instead of AI

Unlike AI, the use of embryos results in relatively constant pregnancy rates throughout the year, including during periods of high temperatures. ET allows the enhancement of genetic gain. With the use of AI, the number of descendants of sires genetically evaluated for desirable characteristics can be multiplied.

Embryo transfer, in turn, also increases the number of descendants of females of high genetic merit, causing an even more significant impact on the improvement of a herd. Therefore, greater genetic gain and a higher genetic leap are achieved in each generation with ET than with the use of AI alone. Embryo transfer can also increase the reproductive efficiency of repeat breeder cows. These animals are defined as subfertile females that do not have any anatomical or infectious abnormalities yet require three or more services to become pregnant. ET increases conception rates of these animals to levels close to those of normally fertile breeder cows, suggesting that reproductive failures are associated with low oocyte quality and early embryonic developmental defects. ET can therefore be an effective method of preventing early embryonic mortality and increasing pregnancy rates.

Because of these advantages, some dairy farms have completely replaced AI with ET, achieving better pregnancy rates and accelerating genetic gain between generations. In these situations, recipients and donors are dairy cows from the same herd; the cows with the best genetic potential are used as oocyte or embryo donors and the remainders are used as recipients. Thus, efficient genetic selection is achieved from animals from the same herd.

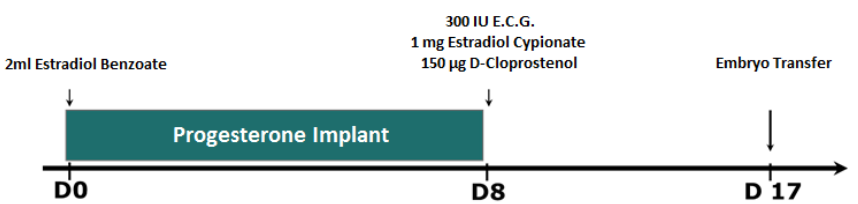


Figure 3. Example of a hormonal protocol for fixed-time embryo transfer (FTET).

## 3.9. Advantages of Using In Vitro- Instead of In Vivo-Produced Embryos

When performing the in vivo production of embryos, it is necessary to superovulate the donor with the administration of hormones. With OPU/IVEP, oocytes can be obtained without the use of drugs and the sequential production of embryos can be performed without exogenous hormone stimulation. The in vitro technique also allows the use of a smaller interval between follicular aspirations in the donor. In general, a minimum interval of 15 to 30 days is used before the females are subjected to a new session of follicular aspiration. There is no established limit for the number of aspirations, and reports of up to 20 procedures performed sequentially without harm to the donor exist. Moreover, superovulation requires intervals of 40 to 60 days and can only be used approximately 3 to 4 times before a gap of several months is recommended. Perhaps one of the major advantages of IVEP is its efficient use of sexed semen. The sorting process often compromises the fertilization efficiency of a portion of the spermatozoa, making the process of in vivo fertilization more challenging. In the in vitro process, the sperm are subject to less demanding conditions, providing better results than AI and in vivo embryo production. Production of embryos by MOET does not allow the use of pregnant animals. In IVEP, it is possible to produce embryos by aspirating the ovary of pregnant animals as long as the aspiration can be performed without excessive pulling. Another advantage of IVEP is that it enables higher use of a fixed dose of semen: a single dose can be used to fertilize oocytes of 10 or more females.

## 3.10. Critical Aspects of the Use Of IVP Embryos:

The OPU/IVEP technique has more steps than the in vivo production technique. Thus, highly trained professionals are needed throughout the process to obtain satisfactory results. The amount of embryos produced can be decisive in the commercial viability of the technique. Because OPU and laboratory staff are associated with fixed costs, higher numbers of attempted pregnancies can result in lower overall costs. Both cryopreservation and rewarming processes are more critical in IVEP than in other techniques. Because they are structurally different from in vivo-generated embryos, IVP embryos cannot be subjected to conventional freezing without suffering severe damage. Currently, the most efficient method to cryopreserve IVP embryos is

vitrification. In this technique, no method of direct transfer exists yet, and vitrified embryos must go through a specific process of rewarming before being loaded and transferred. The genetic impact on a herd is higher with IVF than with other techniques. If the donors are selected properly, IVF can confer a significant improvement on each generation. On the other hand, any mistake in the selection of females can have a major negative impact on the herd. Hence, rigorous and objective criteria must be used in the selection of oocyte donors.

## 3.11. Cryopreservation and Strategic Use of the Embryo

To be cryopreserved, embryos generated in vivo are loaded into straws, in a similar manner to fresh embryos, with an additional cryoprotectant added to the medium. The most commonly used cryoprotectant is ethylene glycol, as it protects the blastomeres from damage caused by the low temperatures. Once they are filled, the straws are put in the freezing machine, which allows a controlled decrease of the temperature. When the temperature reaches approximately - 7 °C, a "seeding" procedure, which aims to induce homogeneous crystallization of the contents of the straw, should be performed. After this procedure has been performed, the machine goes back to decreasing the temperature at a rate of approximately 0.3 to 0.5 °C per minute. This speed is calculated to prevent the formation of ice crystals within the embryo (if freezing occurs too rapidly) or the induction of cell damage due to the rapid dehydration of the embryo and hyper-saline concentration inside the cell (if freezing occurs too slowly). When the final temperature is reached (-32 to -35 °C), the straws are immersed in liquid nitrogen and stored in nitrogen tanks.

Unlike in vivo-generated embryos, IVP embryos are more susceptible to the damage caused by cryopreservation and rarely survive the freezing process. The greater sensitivity of IVP embryos to cooling appears to be directly related to higher lipid accumulation caused by the in vitro culture (Abe et al., 2002). These lipids can undergo irreversible changes and severely compromise embryonic development. A new method of increasing the cryotolerance of these embryos that has proven effective is chemical delipidation.

Forskolin, a substance derived from the Indian plant *Coleus forskohlii*, has been successfully used to promote intracellular lipolysis in embryonic cells (Men et al., 2006). When applied at strategic periods of the in vitro culture, this substance is able to increase the embryonic cryotolerance to levels that

provide good pregnancy rates, enabling the commercial use of the technique. The pregnancy rates obtained by the laboratory In Vitro Brasil with vitrified embryos previously exposed to forskolin are described in Table 2 (unpublished data). With the advances in cryopreservation techniques of IVP embryos, it is possible to have a stock of female-sexed embryos for strategic use during the months of greatest heat.

**Table 2. Pregnancy rates of IVP Bos indicus embryos treated with forskolin for 48 h in culture and submitted to vitrification**

| Breed | Transferred embryos (n) | Pregnancies (n) | Pregnancy rate (%) |
|---|---|---|---|
| *taurus-indicus* | 87 | 37 | 42.5[a] |
| Gir | 701 | 314 | 44.8[a] |
| Guzera | 680 | 274 | 40.3[a] |
| Nelore | 440 | 200 | 45.5[a] |
| Total | 1,908 | 825 | 43.2 |

[a] Within a column, rates with a common superscript are similar (P > 0.05).

In addition to enabling better pregnancy rates than AI, IVP embryos have the added advantage of generating pregnancies of the desired gender. The use of IVP embryos in dairy farming has been the most promising feature of recent years, second only to the advances brought about by the pharmacological control of the estrous cycle and ovulation. Highly efficient technologies are already available, and large dairy farms have sought strategies to improve their herds using technology from biotech companies.

## CONCLUSION

The failure to detect estrus in an accurate manner is one of the most common reasons for the failure of reproductive programs on dairy farms. Heat stress also represents a significant challenge, causing low pregnancy rates and therefore high economic losses. The technologies of FTAI and embryo transfer have directly benefited dairy farms of all sizes (micro, small, medium and large). However, the importance of factors such as nutrition and management must be considered before implementing these strategies because these factors directly influence reproductive efficiency. Any variation in these factors can

affect the result of a FTAI protocol or rates of embryo production. Thus, a proper analysis of the technique to be used combined with adequate conditions for the herd can provide optimal results.

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# Large-scale programs for recipients of *in vitro*-produced embryos

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## Abstract

*In vitro* embryo production (IVEP) has become an accessible option for meat and milk producers, and Brazil is now the leader of IVEP worldwide. Recipient females represent one of the most significant costs in embryo transfer (ET) programs. Thus, hormonal protocols may increase the proportion of suitable recipients to receive embryos and improve the efficiency of IVEP programs. Besides improving the amount of available recipients, it is important to select high quality animals. Due to the great demand, the type of females that were considered ideal for ET has become scarce, especially for large-scale programs. Therefore, new approaches have successfully emerged, as the use of Nelore cows recently calved as embryo recipients. For being the most numerous category in Brazil, these animals can be acquired for fair prices. Embryo production from slaughterhouse ovaries also represents an innovative strategy for large-scale IVEP. With the use of sorted sperm and large amount of pregnancies, is has become an interesting alternative compared to AI. In this article, recent advances in embryo IVP are discussed, as well as some of the most used hormonal protocols for estrus synchronization of recipients in large-scale IVEP programs.

**Keywords**: *Bos taurus indicus*, estrus induction/ synchronization, FTET, IVP.

## Introduction

*In vitro* embryo production (IVEP) has been a powerful tool for increase and dissemination of high quality genetic animals. IVEP has become more accessible in recent decades, and can currently be considered as a consolidated technique (Thibier, 2006).

Brazil occupies the first place in the world ranking of cattle IVEP (Thibier, 2006), and this leadership is certainly related to the predominance of Nelore cattle (*Bos taurus indicus*) in the national herd. Nelore has higher number of ovarian follicles and more oocytes recovered per ovum pick up (OPU) session when compared with european breeds (Pontes *et al*., 2009).

To ensure the success of IVEP programs, a crucial aspect concerns the availability of recipients. To achieve the best pregnancy rates, it would be ideal to have a large number of animals, to allow rigorous selection of the most suitable ones. However, the greater the number of recipients the higher the cost for maintaining these animals. High costs may be prohibitive and compromise the economic viability of the embryo industry. Thus, the efficiency of recipient preparation protocols becomes a factor of extreme importance for successfully obtaining products through the application of embryo transfer technology.

In dairy herds, more than 50% of cows in heat are not detected, by lack of mating behavior (Van Eerdenburg, 2002). This problem is more pronounced when high producing dairy cows are used as recipients, since high rates of estradiol metabolism make signs of estrus even less intense (Rodrigues *et al*., 2010). The failure to detect estrus also represents a great challenge in programs that use cattle with some proportion of *Bos indicus* blood due to the increased occurrence of short duration estrus and the high incidence of estrus at night (Bó *et al*., 2003).

In order to solve problems related to heat detection and to increase the proportion of suitable recipients to receive embryos, several hormonal protocols were developed, allowing embryo transfer (ET) without the need of estrus observation. These protocols enable the transfer of embryos in a fixed time (FTET), by using hormones that control follicular and luteal dynamics and promote synchronization of the moment of ovulation (Bó *et al*., 2002; Nasser *et al*., 2004).

This article describes the importance of large-scale recipient programming for IVEP, discussing some of the commonly used FTET protocols in programs involving large numbers of recipients.

## Advances in large-scale IVEP

Until a few years ago, large-scale IVEP was limited by several drawbacks. One of them was the long distances between laboratories and recipient properties. Large herds of cattle are usually located in new areas of livestock production, such as the North of Brazil. Those areas are located thousands of miles away from oocyte donors and IVEP laboratories, which are concentrated mainly in South and Southeast regions. The limitations for cryopreservation of *indicus* IVP embryos greatly restricted the connection between production and transfer of IVP embryos. Thus, the disposal of embryos not transferred was a common practice.

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Another problem associated with the scarcity of recipients was the large number of calves of unwanted gender. When unsexed sperm is used, twice as many recipients are required to achieve the desired number of products of the expected gender.

Therefore, transportation of embryos over long distances and the use of sexed sperm are important procedures for a more rational use of recipients, improving the efficiency of large-scale embryo production (Pontes *et al*., 2010).

*Transportation of embryos produced with sexed sperm over long distances*

Pontes *et al*. (2010) conducted a large-scale IVEP program using exclusively sexed sperm from bulls of Holstein and Gir breeds. A total of 5,047 OPU sessions were conducted in Gir, Girolanda and Holstein females, and the embryos were transported over distances from 800 to 2000 km to be transferred to the recipients. Embryo production rates in relation to total oocytes were similar for all types of donors, *Bos indicus, Bos taurus* and *Bos indicus taurus* (17.4 to 18.9%), as well as pregnancy rates (36 to 40%, overall average of 39%).

Due to the large distance between the laboratory and the farms where the recipients were housed, transportation of embryos during the early stages of embryonic development was proposed. Embryos at different developmental stages (day 2 to day 5; day 0 = day of IVF) were subjected to transportation. To establish the moment to begin the transport, a calculation considering the distance and time needed for the trip was made. The end of the transportation had to coincide with day 7 of culture, and embryos should be at the morula or blastocyst stage. Thus, the latest stages of embryo development occurred during transport. The strategy was to place the embryos in groups of 35-40, in microtubes containing 400 µl of culture medium under 300 µl of mineral oil, in culture conditions similar to the laboratory (39°C and 5% $CO_2$ in air), but in a portable incubator (Ceafepe Veterinary Technology, Sorocaba, or WTA, Cravinhos, SP, Brazil). In situations of longer transportation time, the replacement of the medium and the gaseous atmosphere was carried out by the veterinarian who conducted the portable incubator. At the end of transportation, embryos were re-evaluated and loaded into straws for transferring.

In the program described above, the use of sexed sperm allowed a more rational use of the recipients, because the objective was to obtain female offsprings. Fertilization of oocytes with sexed sperm enables an accuracy of 85 to 95%, being the quality of the embryos similar to those produced with conventional sperm (Peippo *et al*., 2009, 2010). There were effects of bull and ejaculate, showing the need for a rigorous evaluation and previous selection of the best batches of sperm to obtain more advantageous results (Pontes *et al*., 2010).

**Ideal type of recipient**

Selection of high quality recipients is a challenging procedure, especially in countries like Brazil, where there is an excessive number of embryos in relation to the ability of direct transferring them to recipients. Good nutritional and health status, as well as adequate management, are important factors to ensure the success of IVEP programs.

The use of virgin heifers as recipients is usually indicated as the best option for achieving higher pregnancy rates. There used to be an informal concept that the best recipients would be crossbred heifers, usually half-blood Nelore and European breeds with some dairy ability, such as Simmental and Braunvieh. However, due to a limited supply and a high demand, this type of recipients became costly and scarce. Particularly for large ET projects, it became prohibitive to use this type of heifers as embryo recipients.

A successful alternative recently proposed (Pontes *et al*., 2009) refers to the use of Nelore cows recently calved as embryos recipients. This female category is the most numerous in the country, allowing an adequate supply with a fair price. Contradicting preconceived ideas, this animal category has allowed pregnancy rates around 40%, with more than 10,000 calves already born (In Vitro Brasil Ltda, Mogi Mirim, SP; unpublished data). The option of using cows with suckling calves should consider nutrition and health conditions, so that estrous cycles can restart soon after calving, in time to respond to synchronization protocols (Jones and Lamb, 2008).

If an adequate nutritional and health status has been established, the use of cows may effectively present certain advantages compared to heifers. In general, the use of cows may have a higher recipients transferred-to-treated rate on FTET, since they usually show a better response to the hormonal protocol and heifers often require a pre-synchronization. Another interesting aspect is that cows have been exposed more to pathogens. Thus, they may have greater disease resistance and better quality colostrum. Finally, the frequency of dystocia tends to be higher in heifers than in cows.

*Importance of recipients on calf gene expression*

Until recently, the importance of recipients has been based mainly on their ability to respond to synchronization protocols and to maintain pregnancy. However, with the recent advances in epigenetic area, it became evident that diet quality and maternal behavior can interfere on gene expression of the calf. Despite the fact that the genetic sequence is established since the first cell division, the expression of genes may be altered due to environmental factors. For example,

during pregnancy, nutritional status of the recipient may interfere in the follicular ovarian reserve of the fetuses (Ireland *et al*., 2011). Then, a future donor may have its reproductive performance compromised if during its fetal formation the recipient has undergone nutritional deprivation.

Another important aspect of epigenetic events refers to maternal ability of the recipient. It was shown that females with high maternal ability influence DNA methylation of their products, interfering in the expression of important genes for environmental adaptation (Weaver *et al*., 2004).

## Protocols for estrus synchronization

*Prostaglandin F2α*

The simplest treatment for estrus synchronization of recipients is based on a single administration of PGF2α, or on two injections 11 to 14 days apart. Although those procedures are relatively effective and inexpensive, treatment with PGF2α depends on the efficiency of heat detection, as well as the responsiveness of the corpora lutea of animals subjected to protocol.

The corpus luteum (CL) is responsive to PGF2α only from day 5 to 16 of the estrous cycle. Also, by the time of the application each recipient is in a particular stage of the follicular wave, resulting in considerable asynchrony on the onset of estrus (Siqueira *et al*., 2009). For large scale programs, the limitations of estrus synchronization may impair the efficiency of the technique. Therefore, in those cases it would be more suitable to synchronize ovulation and to transfer the embryos in a fixed time (FTET).

*FTET*

In large programs of IVEP, where most of the embryos are transferred fresh, the availability of a significant number of recipients is needed. To reach this goal, protocols of FTET have been increasingly used.

In a recent study with repeat breeders dairy cows, Rodrigues *et al*. (2010) compared a single application of PGF2α to a FTET protocol using progesterone, eCG, estradiol benzoate and estradiol cypionate. Higher recipient transferred-to-treated rate (75.0 *vs*. 34.5%) and pregnancy at 60 days (29.3 *vs*. 16.2%) were observed in FTET group. In the same study, it was found that the FTET protocol was effective regardless of the presence of a CL at the beginning of the treatment. Thus, using the FTET protocol increased the amount of recipients suitable for ET and allowed the use of cows without a CL at the beginning of the protocol, with the same efficiency as those with a CL.

*Estradiol benzoate x cypionate*

In spite of having different pharmacokinetic properties, estradiol benzoate (EB) and estradiol cypionate (EC) are considered effective in inducing ovulation in *Bos taurus* and *Bos indicus* cattle. By having a shorter half-life, EB promotes an LH surge of greater amplitude and shorter duration than EC, resulting in greater synchrony of LH surges (Sales *et al*., 2012). Due to its low solubility in water, EB is released more slowly and promotes a longer lasting LH surge (Vynckier *et al*., 1990).

Protocols of FTET that use EB as ovulation inducer are widely used. One model consists of an injection of EB (2 mg) and insertion of a progestin-releasing implant on a random day of the estrous cycle, which is considered as day 0. On day 5, eCG is administered (400 IU), and on day 8 the implant is removed and an injection of PGF2α is given (0.5 mg). On day 9, another dose of EB is administered (1 mg). Day 10 is considered the day of estrus, and FTET is carried out on day 17.

In protocols that use EC to induce ovulation, it is administered on day 8, along with the removal of the progestin implant. Day 10 is considered the day of estrus and FTET is performed on day 17 (Rodrigues *et al*., 2010). Execution of the EB protocol requires handling animals four times, while replacing EB by EC allows the elimination of one handling, reducing labor costs time. Sales *et al*. (2012) evaluated the effect of both drugs for induction of ovulation on lactating *Bos indicus* cows submitted to FTAI. They concluded that, despite the greater synchronization of LH surge in animals that received EB, synchronization of ovulation was similar between the two groups, with no effect on fertility of the animals exposed to either protocol.

*Use of eCG*

Administration of eCG on day 5 of synchronization protocols increases pregnancy rates and proportion of recipients selected to ET (Bó *et al*., 2002; Nasser *et al*., 2004). For its ability to increase the size of the ovulatory follicle and to induce single and multiple ovulations, this drug provides a higher concentration of progesterone during the subsequent luteal phase (Baruselli *et al*., 2010).

Studies were performed in order to evaluate the effect of delaying eCG administration from day 5 to day 8 of the original protocol, thus avoiding the handling of recipients on the 5th day (Nasser *et al*., 2004, Reis *et al*., 2004). Treatment on day 5 results in higher number of CL and higher plasma progesterone concentration, with recipients treated on day 5 presenting higher pregnancy rates than those treated on day 8. One possible explanation for the better performance of recipients that received eCG on day 5 would be that the high concentration of progesterone from physiological CL together with progestin-releasing implant can affect LH pulsatility and follicular growth (Baruselli *et al*., 2010).

Thus, Ferreira *et al*. (2006) evaluated the effect

of delaying the administration of eCG from day 5 to day 8, but replacing vaginal progestin implant for an auricular implant, containing less hormone. Results showed that, when ear implants were used, it was possible to reduce the number of animal handlings without compromising the efficiency of the synchronization protocol (Table 1). The protocol proposed by Ferreira *et al*. (2006) consists of insertion of a progesterone device and administration of EB on day 0; administration of PGF2α, eCG, CE and device removal of progesterone on day 8, and FTET on day 17. There is also the option of using intravaginal progesterone-releasing device, with the condition that a dose of PGF2α is administered on day 0 at the time of progesterone device insertion. For requiring only three handlings, the new protocol is easily implemented, even if a large number of recipients must be synchronized at the same time.

In order to further facilitate recipient management in large-scale programs, different durations of the progesterone device have been tested. Synchronization protocol is initiated at the same day in the entire group of females (day 0), with the possibility of removing the device in 7, 8 or 9 days. It is possible to start the program in all animals and remove the implant from one third of the lot, starting from day 7 (In Vitro Brasil Ltda, Mogi Mirim - SP, unpublished data). The ovulation inducer (EC), the luteolytic agent and eCG are applied at the same day of the progesterone device removal. Thereby, the group that has the implant removed on day 7 receives embryos on day 16, and so on, making it viable to adjust the synchronization between embryonic developmental stage and recipient uterus. In a program conducted in 2011, 357 Nelore cows with suckling calves were subjected to FTET with P4 removal on days 7, 8 or 9 of the protocol. There was no difference in transferred-to-treated rate or in pregnancy rates among animals that remained with the implant for 7, 8 or 9 days (Table 1).

Table 1. Transferred-to-treated rate and pregnancy rates obtained in synchronization protocols using cows as recipients for IVEP programs.

| Protocol | Breed of recipients | Prior evaluation of CL | Utilization rate % | Pregnancy rate % | Source |
|---|---|---|---|---|---|
| P4 and BE + eCG, PGF2α and CE | Holstein | without CL | 61.2 | 38.2 | Rodrigues *et al*. (2010) |
| P4 and BE + eCG, PGF2α and CE | Holstein | with CL | 75.0 | 42.9 | Rodrigues *et al*. (2010) |
| P4 and BE + eCG, (D5) + PGF2α and BE | *taurus x indicus* | with CL | 76.1 | 31.0 | Siqueira *et al*. (2009) |
| P4 and BE + eCG, PGF2α and CE | *taurus x indicus* | with CL | 96.0 | 59.0 | Ferreira *et al*. (2006) |
| P4 and BE + eCG, PGF2α and CE (D7) | Nelore | No evaluation | 88.0 | 43.0 | In Vitro Brasil |
| P4 and BE + eCG, PGF2α and CE (D8) | Nelore | No evaluation | 88.0 | 42.0 | In Vitro Brasil |
| P4 and BE + eCG, PGF2α and CE (D9) | Nelore | No evaluation | 88.0 | 44.0 | In Vitro Brasil |
| P4 and BE + PGF2α (D7) + eCG, and CE (D9) | Nelore | No evaluation | 61.8 | 42.1 | In Vitro Brasil |
| P4 and BE + $PGF_{2\alpha}$ (D7) + eCG, and CE (D8) | Nelore | No evaluation | 67.9 | 37.2 | In Vitro Brasil |
| | *taurus x indicus* | No evaluation | 73.8 | 45.4 | In Vitro Brasil |

**Replacement of AI by ET and of FTAI by FTET**

In general, embryos are more resistant than gametes, particularly when heat stress is considered. As a result, for dairy cattle, the use of embryos to obtain pregnancies may be more advantageous, especially in months and/or regions with greater average temperatures.

Some dairy farms have completely replaced IA by ET, achieving better pregnancy rates and accelerating genetic gain between generations. In those situations, recipients and donors are dairy cows from the same herd, the ones with best genetic potential are used as embryo donors and the remainder as embryos recipients.

In beef herds, a new perspective is the embryo production using oocytes obtained from slaughterhouse and Y-sorted sperm. With large amounts of pregnancies, a competitive price can be achieved for the embryo, turning it into a commercially attractive alternative compared to semen.

## Conclusions

Despite the wide spread use of ET in the last years, the use of this technology has been restricted by failure on heat detection, in both beef and dairy herds. In both cases, the acquisition and maintenance of the recipient in the herd represent a major cost in IVEP programs. Expenses with food, estrus synchronization and ET rise in each unsuccessful transfer. Therefore, protocols that increase the number of pregnant recipients by synchronization treatment represent the best solution for economically viable programs.

Likewise, several studies were conducted to reduce the number of times that recipients are handled during synchronization protocols. The results obtained to date are considered to be very satisfactory, since the currently available protocols are considered practical and easily applied.

The FTET is an interesting alternative that could result in the achievement of good pregnancy rates all year round, even in repeat-breeder cows and milk-producing cows in the warmer months of the year. Finally, advances in the use of sexed sperm and embryo transport over long distances have enabled the effectiveness of large-scale IVEP programs, benefiting beef and milk livestock.

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# PRODUCCIÓN IN VITRO DE EMBRIONES DE RAZAS LECHERAS A GRAN ESCALA

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**Introducción**

Los datos presentados por la Sociedad Internacional de Transferencia de Embriones a finales de 2008 muestran una mayor participación de Brasil en relación con la producción y transferencia de embriones bovinos en el mundo en 2007, lo que representó casi un tercio de la producción mundial (32,8%). Con respecto a los embriones producidos in vivo total en el mundo, el país estaba por detrás del EUA, Canadá y Japón también. En el mercado de embriones producidos in vitro (PIV), por el contrario, el país ha consolidado un líder absoluto, lo que representa más del 85% del mundo (Thibier, 2008).

En el período 2002 a 2006, tendió a estabilizarse la FIV. La reducción de la tasa de crecimiento que se esperaba, debido a la reducción natural de la demanda y también debido a las limitaciones de la técnica, tales como dificultad para la criopreservación de embriones. A medida que la congelación de embriones es todavía limitado en la PIV (7,4% del total), el crecimiento en la transferencia de embriones (TE) puede reflejar una mayor demanda de embriones criopreservados, ya sea con fines de optimizar el uso de los receptores o fines de comercio (Viana, 2009).

A pesar de las limitaciones de la técnica, Brasil ha mostrado un fuerte impulso para reemplazar TE por *ovum pick up* (OPU) / PIV (Pontes *et al*., 2009). Esto se debe a una peculiaridad del ganado cebú (*Bos indicus*), dado a las vacas que suelen tener más folículos ováricos y más ovocitos recuperados por OPU en comparación con el ganado *Bos taurus* (PONTES *et al*., 2009). No hay ninguna explicación aparente para esta diferencia biológica intrigante. Sin embargo, la información disponible en la mayoría de OPU / PIV en Brasil se obtuvo de Nelore, responsable por aproximadamente el 80% del rebaño brasileño (acerca de 200 x $10^6$ animales) (Pontes *et al*., 2011).

Las razas cebuínas siguen representando la mayoría de los embriones producidos en Brasil, con 94,0% del total. De 2007 a 2009, sin embargo, hay un constante incremento en la participación de las razas lecheras en el mercado de los embriones, especialmente el cebú, como Gir (68,0% del total) y Girolando (22,4%), la única raza compuesta con una importante participación tanto en el TE y en la PIV (Viana, 2009). El interés por las razas cebuínas lecheras se debe a su capacidad de adaptación para producir grandes cantidades de leche bajo condiciones de estrés tales como altas temperaturas, parásitos y pastos pobres. El método de la PIV ha sido considerado para las donantes Girolando debido al aumento de la eficiencia del uso de semen sexado para la fertilización in vitro (Xu *et al*., 2006), lo que facilita la producción de un gran número de hembras para la industria láctea.

La técnica de la OPU tiene la ventaja de ser sencilla, adecuado y permitir la recogida de ovocitos repetidas veces para procedimientos in vitro, incluyendo con aumento del número de folículos después de varias semanas de aspiraciones folicular (Stubbings & Walton, 1995). Otro aspecto positivo de la OPU/PIV es la posibilidad de producción de embriones en hembras preñadas. Esto es posible porque los ovarios mantienen su actividad durante el preñez, haciendo posible la recuperación de ovocitos. Si la técnica de la OPU es bien realizada, no hay riesgo de preñez y puede realizarse como aspiración folicular hasta el tercer mes de preñez, o hasta que el período en que el veterinario puede manipular los ovarios sin necesidad de un fuerte tirón. La aspiración folicular se presenta también como una alternativa a las hembras con limitaciones reproductivas, pero su aplicación es más ampliamente utilizada para una hembra con salude, que puede producir hasta cuatro veces más embriones que en el TE (Kruip *et al*., 1994), pero con mayor costo por embrión (Rodrigues & García, 2000). Además, cada grupo de ovocitos se pueden inseminar con un toro diferente, lo que aumenta el número de posibles combinaciones genéticas (Merton *et al*., 2003).

Es especialmente sin explicación cómo algunos animales se han producido cientos de ovocitos en un solo procedimiento OPU, sin ningún tipo de estímulo. Nuestro grupo de trabajo obtuvo 251 ovocitos por una vez (Seneda, M.M., datos no publicados) y

similares resultados se han relacionado por varios profesionales.Al parecer, existe una variación individual en la producción de ovocitos de donantes en *Bos indicus* (Watanabe *et al*., 1999). Teniendo en cuenta sólo la transferencia de embriones, es bien aceptada una media en torno a cinco a seis embriones transferibles por varias razas europeas (Castro Neto *et al*., 2005; Hansen & Block, 2004), lo mismo que nuestro grupo de trabajo tiene obtenido para Nelore. En condiciones de Brasil, una pequeña variación se observó en otras razas cebú, como Brahman con 7,3 embriones, 4,1 para Gir y el 5,7 por Guzera (Peixoto *et al*., 2006). Teniendo en cuenta todos los datos, llegamos a la conclusión de Nelore donantes presentar una distinción característica sólo en la producción de embriones in vitro.

Hay animales con un buen potencial para la OPU/PIV, debido a que presente de forma natural muy altos promedios de ovocitos y posteriormente buen número de los embriones y los preñeces. Por otra parte, algunos animales con menores promedios de ovocitos no debe ser indicado para la OPU / PIV, pero que pueden producir medio aceptable de embriones de MOET (Pontes *et al*., 2009).

**La producción de embriones**

La PIV se utiliza para acelerar la producción de animales genéticamente superiores. Esta biotécnica implica las etapas de maturación (MIV) y fertilización (FIV) de ovocitos, así como el cultivo (CIV) de zigotos y embriones. Los ovocitos bovinos se pueden obtener de in vitro e in vivo por la punción folicular. El potencial de maduración, fecundación y desarrollo embrionario de los ovocitos se puede estimar por la aparición de células COC, con la siguiente clasificación. Grado I (GI) - Cumulus compacto, que contiene más de tres capas de células, ooplasma con granulaciones finas y homogénea, llenando el interior de la zona y de felpa de color marrón; Grado II (GII) - Cumulus compacto alrededor de los ovocitos o que rodea completamente el ovocito, con menos de tres capas de células, ooplasma con granulaciones distribuidos heterogeneamente y puede ser más concentrado en el centro y más claro en la periferia o condensada en un solo lugar se ve una mancha oscura; Grado III (GIII) - Ooplasma contratado, con espacio entre la membrana celular y zona pelúcida, llenando el espacio perivitelino irregular; Degenerado - vacuolizado o fragmentados; Desnudos - plenamente descubierta por los cúmulos de células o parte cubierta por ellos; Atrésico - oscuro cumulus oophorus o la presencia de signos de degeneración citoplásmica (59). La maduración del ovocito implica las modificaciones nucleares y citoplásmicas y está vinculada con cambios estructurales y bioquímicos que hacen la gameta femenina capaz de fertilización y posterior desarrollo embrionario. La mayoría de resultados indican que la adición de gonadotropinas en el medio de maduración de ovocitos bovinos aumenta la capacidad de fecundación del ovocito y mejora el posterior desarrollo embrionario. Se propone la adición de suero al medio de maduración como un requisito para lograr la perfecta expansión del cumulus y la maduración del ovocito. Después de la maduración de ovocitos, los espermatozoides viables obtenidos con gradiente de Percol o lavado espermático son colocados para la capacitación espermática y fertilización. Durante la pre-implantación de embriones ocurre la activación del genoma embrionario, la totalización y compactación de blastómeros, la diferenciación del trofoblasto y embrioblasto, la formación y expansión de blastocele. Estos eventos pueden verse afectados por factores intrínsecos y extrínsecos, como los iones inorgánicos, tampones, composición de gas de la atmósfera, aminoácidos, pH, factores de crecimiento, la luz, vitaminas y macromoléculas (Gonçalves *et al*., 2001).

**Producción in vitro de embriones a gran escala**

Hasta recientemente, la producción in vitro de embriones de razas lecheras a gran escala se limitaba por algunos obstáculos. El primero es el transporte de los embriones a larga distancia. Teniendo en cuenta que, especialmente en países como Brasil, las granjas con alta disponibilidad de receptores están generalmente a miles de kilómetros de las propiedades dónde están las donantes de ovocitos. Además, como todavía no existRecientemente, algunos de estos obstáculos se han superado y ganado lechero se han beneficiado de la producción eficiente de grandes cantidades de embriones de hembras, que pueden ser transportados con éxito a largas distancias, lo que resulta en buenas tasas de embarazo (Pontes *et al*., 2010). A continuación se presentan algunas de las estrategias utilizadas por nuestro equipo y otros investigadores que han contribuido a facilitar la producción in vitro de embriones de razas lecheras de gran escala.

**Transporte a largas distancias**

En un trabajo reciente, nuestro grupo, en asociación con una empresa brasileña de producción de embriones, evaluó un programa comercial de la producción in vitro de embriones producidos exclusivamente con el uso de semen sexado de los toros Holstein y Gir (Pontes *et al*., 2010). Se evaluaron 5047 OPU celebrada en hembras Gir, Girolando y Holstein. Los embriones fueron transportados a largas distancias para ser transferidos a los destinatarios (Tabla 1). Las tasas de embriones / ovocitos totales fueron similares para todos los tipos de donantes; *indicus*, *taurus*, o *indicus-taurus* (17,4 a 18,9) y también las tasas de embarazo (36 a 40 %).

Con respecto al transporte de los embriones en las primeras etapas del desarrollo embrionario, no hemos podido encontrar informes similares en la literatura. Esta estrategia fue propuesta en el inicio del proyecto, debido a la gran distancia desde el laboratorio a los destinatarios (hasta 2 000 km). Los probables cigotos tuvieron las células del cumulus extraídas y fueron transferidos a gotas de 100 µL de medio de cultivo bajo aceite mineral a 39 °C y 5% de $CO_2$ en el aire, permaneciendo en estas condiciones hasta el momento de la transferencia para la receptora. Embriones en diferentes etapas de desarrollo (Días 2-5; Día 0 = día de la FIV) fueron seleccionados para ser transportados a las granjas donde las receptoras se alojan. Debido a las largas distancias desde el laboratorio a las receptoras, las últimas etapas de cultivo de embriones se llevaron a cabo durante el transporte. A pesar de la duración variable de transporte desde el laboratorio a la granja, todos los embriones fueron transferidos en el Día 7. Los embriones fueron cargados en aviones, transportados en grupos de 40 en microtubos que contienen 400 µL de medio de cultivo, en 300 µL de aceite mineral, la temperatura y la atmósfera similar a la inicial de cultivo en la incubadora. Durante el transporte (24 a 72 h) del laboratorio a la hora de la transferencia, todos los tubos se mantuvieron en una incubadora para el transporte de los embriones (Tecnología Ceafepe Veterinaria, Sorocaba, SP, Brasil). Antes de la transferencia, cada embrión fue insertado en una paleta de 0,5 mL y transferido de manera no-quirúrgica en el cuerno uterino ipsilateral al cuerpo lúteo. No se registró la etapa de desarrollo del embrión en el momento de la transferencia, pero la gran mayoría se encontraba en la fase de mórula o blastocisto. Para aumentar las tasas de éxito de embarazo, se utilizó un protocolo de transferencia de embriones en tiempo fijo (Rodrigues *et al*., 2010).

**Tabla 1.** Número de ovocitos recolectados y viables per donante (promedio ± desvío estándar), número de embriones y preñez obtenidos a partir de donantes Gir (*B. indicus*), Holstein (*B. taurus*) y Girolando (*indicus-taurus*) sometidas a OPU-PIV con semen sexado.

| e | Ovocitos total/ OPU (n) | Ovocitos viables/ OPU (n) | Embriones/ OPU-PIV (n) | Preñez / OPU-PIV (n) |
|---|---|---|---|---|
| **Gir** | 17,1 ± 4,5 [a] (64617/3778) | 12,1 ± 3,9 [a] (45838/3778) | 3,2 [a] (12243/3778) | 1,2 [a] (3113/2670) |
| **Holstein** | 11,4 ± 3,9 [b] (12977/1138) | 8,0 ± 2,7 [b] (9082/1138) | 2,1 [b] (2426/1138) | 0,7 [b] (604/822) |
| **¾ Gir ¼ Holstein** | 20,4 ± 5,8 [c] (5457/267) | 16,8 ± 5,0 [c] (4472/267) | 3,9 [ac] (1033/267) | 1,3 [ac] (137/103) |
| **½ Gir ½ Hosltein** | 31,4 ± 5,6 [d] (7035/224) | 24,3 ± 4,7 [d] (5434/224) | 5,5 [c] (1222/224) | 1,7 [c] (82/47) |
| **Total** | 16,7 ± 6,3 (90086/5407) | 12,0 ± 4,4 (64826/5407) | 3,1 (16924/5407) | 1,1 (3936/3642) |

Promedio ± DS con letras distintas en la misma columna difieren; P< 0,05.
(Pontes *et al*., 2010)

**El uso de semen sexado**

Durante muchos años los ganaderos han buscado una forma de predeterminar el sexo de los terneros producidos, tanto por la necesidad de vaquillas de reemplazo de sus propios rebaños y el valor comercial de los terneros, que es considerablemente mayor para las hembras que para los machos (Wheeler *et al*., 2003). La necesidad de producir embriones con el sexo definido se hace aún mayor cuando la producción se lleva a cabo in vitro, ya que algunos estudios describen un aumento en el porcentaje de machos en relación a los embriones generados in vivo (Rizos *et al*., 2008). Camargo *et al*. (2010) reportaron que la producción in vitro de embriones Gir aumentó el porcentaje de machos en 76,9 % en comparación con la tasa esperada de 1:1. Una alta proporción de los machos hace del sistema de la PIV poco práctico para la producción de leche, provocando la reducción de la eficiencia y aumento de los costos (Camargo *et al*., 2010).

En algunos países, la determinación del sexo de los embriones mediante biopsia y análisis de ADN se practica rutinariamente. Sin embargo, a pesar de ser una técnica sencilla y muy precisa, no es económicamente óptima, teniendo en cuenta que aproximadamente el 50% de los embriones son desechados porque no son del sexo deseado (Peippo *et al*., 2009). Además, las tasas de embarazo son más bajas en embriones biopsiados en comparación con los embriones intactos (Hasler *et al*., 2002).

El uso de semen sexado para la PIV ofrece ventajas sobre la determinación del sexo de los embriones por biopsia. La fecundación de los ovocitos con semen sexado para hembra permite que casi todos los embriones sean del sexo deseado, evitando la eliminación de los mismos. Además, porque no hay necesidad de una biopsia, los embriones producidos a partir del de semen sexado tienen la misma calidad de embriones generados con el semen convencional (Peippo *et al*., 2009; 2010).

El método más utilizado para la producción de semen sexado es la citometría de flujo, que separa los espermatozoides X y Y por el contenido de ADN. Este método es bastante exacto, con 85 a 95% de los espermatozoides mostrando el cromosoma deseado (Seidel & Garner, 2003). A pesar de los espermatozoides que han sido presentados a esta técnica tener una menor movilidad, pueden ser utilizados con éxito para la PIV, teniendo en cuenta que en este sistema de producción un menor número de espermatozoides viables es suficiente para llevar a cabo la fertilización (Peippo *et al*., 2010).

Hay una cierta variación entre los animales en su susceptibilidad al proceso de determinación del sexo de espermatozoides, por lo que la técnica parece poco práctica para algunos toros, en particular (Palmer *et al*., 2008; Peippo *et al*., 2009.). Además, algunos estudios reportan una tasa de blastocisto inferior por el semen sexado en comparación con el convencional (Blondin *et al*., 2009). Sin embargo, en el trabajo realizado por nuestro grupo, se obtuvieron tasas aceptables de embriones / ovocitos totales y de embarazo, incluso con el uso de 15 diferentes toros (Pontes *et al*., 2010).

**Criopreservación de embriones PIV**

Existen básicamente dos técnicas que se utilizan para criopresevación de embriones. La primera es conocida como la congelación convencional, y requiere equipos especiales que promueven una disminución lenta y controlada de la temperatura. Este enfriamiento controlado permite el intercambio entre fluidos intra y extracelular, minimizando el daño osmótico y la deformación de las células (Vajta & Kuwayama, 2006). Esta técnica es ampliamente utilizada para los embriones generados in vivo, y a estos embriones proporciona tasas de embarazo similares a los obtenidos después de la transferencia de embriones frescos (Hasler, 2001). La segunda técnica, la vitrificación, previene la formación de cristales de hielo intracelular a través de la rápida velocidad de enfriamiento y una alta concentración de crioprotectores, causando una reducción de los efectos nocivos de la refrigeración (Saragusty & Arava, 2011). Esta metodología tiene ventajas tales como la simplicidad, rapidez y bajo costo del procedimiento.

Embriones PIV son extremadamente sensibles a la congelación convencional, y aparentemente hay una mayor susceptibilidad de embriones *B. indicus* producidos in vitro, con resultados que tienden a ser muy bajos y / o altamente variables (Visintin *et al*., 2002; Zanenga, 1993). Los embriones PIV son diferentes de los embriones generados in vivo en muchos aspectos. Las principales diferencias son la mayor cantidad de lípidos en el citoplasma (Crosier *et al*., 2000; FAIR *et al*., 2001), la compactación incompleta de los blastómeros (Van Soom *et al*., 1997), reducción de la densidad de las mitocondrias maduras (Crosier *et al*., 2000) y más frágil zona pelúcida (Duby *et al*., 1997). A pesar de todas las diferencias estructurales entre los embriones PIV y los generados in vivo, hay evidencia de que la alta susceptibilidad de los embriones PIV a los daños causados por el proceso de criopreservación se debe principalmente a la gran cantidad de gránulos de lípidos en el citoplasma (Abe *et al*., 2002; Pryor *et al*, 2011).

La vitrificación es considerada el método más adecuado para la crioconservación de embriones PIV. En las últimas décadas, fueron creados nuevos contenedores para el almacenamiento de los embriones durante la criopreservación, con el fin de bajar el volumen y permitir una mayor velocidad de enfriamiento (Saragusty & Arav, 2011). También se crearon técnicas que permiten la transferencia directa de embriones después del recalentamiento (Akiyama *et al*., 2010). Sin embargo, los resultados permanecen estancados en niveles que no permiten la viabilidad comercial de esta técnica, y bajas tasas de embarazo después de la transferencia de embriones criopreservados impiden el uso eficiente de los embriones sobrantes. A pesar de la PIV ha sido considerado como una tecnología madura en general (Van Wagtendonk-De Leeuw, 2005), el aspecto de congelación en embriones, principalmente del ganado cebú presenta una enorme brecha por cubrir.

**Perspectivas**

El nuevo protocolo descrito para transporte de los embriones durante el período inicial de desarrollo ha demostrado ser posible obtener tasas aceptables de embarazo después del transporte de embriones a larga distancia. Los resultados son prometedores, con un gran potencial para las solicitudes de comercio nacional y internacional de embriones bovinos. Además, los últimos resultados descritos en la literatura con el uso de semen sexado han demostrado ser posible su uso exitoso en la PIV de las razas lecheras. Se espera que el uso de semen sexado afecte a la estructura de la industria lechera mediante la creación de una mayor oferta de novillas de reemplazo, que tendrán su costo reducido. El precio del semen sexado tiende a disminuir considerablemente con el tiempo, reduciendo así el costo de producción de leche. Las principales limitaciones para el uso de

semen sexado se han corregido, y la eficiencia de la determinación del sexo del procesador debe aumentar gradualmente durante los próximos años (Wheeler *et al*., 2006), asegurando la expansión del uso de semen sexado en la industria lechera. Aunque no hay una metodología adecuada para la criopreservación de embriones PIV,, esta técnica parece ser más prometedor para la producción a gran escala de hembras lecheras. Una vez que superado este obstáculo, el proceso de producción de embriones de razas lecheras se verá facilitado y más eficiente, contribuyendo aún más al engrandecimento de la ganadería nacional.

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## Fatores que interferem na população folicular e produção de oócitos em bovinos

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Graças à maior precisão na manipulação dos gametas durante o processo in vitro, os índices de produção embrionária mostram-se cada vez mais promissores. Desde a última década, o Brasil se apresenta como líder mundial da tecnologia de produção in vitro de embriões, beneficiando diretamente os produtores de leite e carne de diferentes escalas (micro, pequenos, médios e grandes) por meio do incremento do melhoramento genético proporcionado pelo uso do embrião para gerar prenhez. Pelo último levantamento da IETS (2012), o Brasil produziu quase 270.000 embriões in vitro, uma porcentagem próxima de 80 % da produção mundial.

Para uma melhor compreensão dos eventos relativos à produção in vitro de embriões, a fisiologia ovariana tem sido intensamente estudada e o desenvolvimento folicular tem sido manipulado na esperança de se produzir o maior número possível de oócitos competentes. As informações obtidas até o momento têm proporcionado métodos bastante eficientes para a obtenção de grande número de embriões viáveis.

Vários fatores podem interferir na população folicular e consequente obtenção de oócitos, primeira etapa da produção in vitro de embriões. A seguir apresentaremos os principais aspectos sobre este tópico.

A contagem de folículos antrais (CFA, ≥3 mm de diâmetro) apresenta grande variação entre animais, podendo ser menor do que 5 em alguns animais e maior do que 50 em outros, considerando-se os dois ovários.

A variação individual da quantidade de folículos se reflete em grande variabilidade entre doadoras na produção de embriões, tanto nos procedimentos in vivo como in vitro. Em programas de superovulação, apenas 30% das vacas produzem 70% dos embriões, e 25% das vacas tratadas não produzem embrião algum. Da mesma forma, algumas fêmeas são altamente eficientes na PIVE, e outras produzem resultados bastante insatisfatórios.

Enquanto a população folicular é altamente variável entre indivíduos, existe uma grande repetibilidade do número de folículos antrais em um mesmo indivíduo. Esta contagem de folículos antrais tende a ser constante nas diferentes ondas foliculares de um mesmo animal, de forma que uma única avaliação ultrassonográfica, se realizada no início da onda, pode ser bastante útil identificar fêmeas com baixo, intermediário ou alto número de folículos ovarianos. Em vacas Nelore, a variação individual na produção de oócitos também demonstrou estar associada à produção de embriões e às taxas de prenhez. Doadoras que produzem grande número de oócitos também produzem mais embriões e mais prenhezes do que as doadoras que produzem poucos oócitos (Pontes et al., 2011).

Tratando-se da variação individual, um aspecto interessante diz respeito à raça. As fêmeas da raça Nelore, zebuínas e suas cruzas, normalmente têm maior número de folículos recrutados por onda em comparação com as raças europeias (Seneda et al., 2002). Esta característica certamente favoreceu o rápido crescimento da PIVE no Brasil, uma vez que os animais zebuínos representam aproximadamente 80% do rebanho brasileiro.

No entanto, apesar do potencial similar entre os indivíduos da mesma raça, parâmetros como recrutamento e desenvolvimento folicular podem variar amplamente, mesmo entre pares de gêmeos monozigóticos (Machado et al., 2003). Não está completamente esclarecida a justificativa para esta maior produção oocitária em fêmeas indicus, visto que a população folicular pré-antral entre taurus e indicus tem sido reportada como numericamente semelhante.

A quantidade de folículos antrais e a consequente produção de oócitos também sofrem alterações conforme o peso, a idade e as variações fisiológicas de cada animal. Animais senis produzem oócitos menos competentes, com menor quantidade de camadas de células do cumulus oophorus, que comumente resultam em baixa eficiência na produção embrionária. De forma semelhante, animais muito jovens podem ter os folículos aspirados com uso de equipamento adequado, mas frequentemente os resultados obtidos com a produção de embriões não são satisfatórios devido à baixa competência de desenvolvimento destes oócitos.

A condição corporal das fêmeas também exerce efeito sobre a qualidade dos oócitos, de forma que doadoras que sofreram privação alimentar produzem oócitos com menor capacidade de desenvolvimento até o estágio de blastocisto. O efeito da subnutrição na produção embrionária pode ser revertido, no entanto as fêmeas podem demorar mais de oito semanas para apresentar oócitos com capacidade satisfatória de desenvolvimento embrionário.

Alguns trabalhos afirmam que a competência de desenvolvimento dos oócitos subordinados pode estar comprometida durante a fase de dominância. Todavia, resultados de campo não mostram diferença significativa na qualidade oocitária durante as diferentes fases do ciclo estral, e atualmente a aspiração folicular tem sido realizada em momentos aleatórios da dinâmica folicular. Por outro lado, a taxa de recuperação é maior quando se aspiram folículos pequenos, que podem ser encontrados em maior número no início de cada onda folicular.

De forma semelhante, a fase reprodutiva pode exercer efeito na produção de oócitos e embriões. Em fêmeas leiteiras, as não gestantes frequentemente apresentam mais oócitos melhor produção embrionária do que as gestantes. Considerando-se as fêmeas lactantes, as gestantes proporcionam melhores resultados do que as lactantes vazias na produção de embriões após sessões de OPU/PIV.

A obtenção de quantidade apropriada e boa qualidade de oócitos para PIVE estão diretamente relacionadas a uma metodologia adequada. A resolução do ultrassom e o treinamento da equipe são fundamentais para a obtenção de bons resultados. Um veterinário experiente é capaz de aspirar 8 a 12 vacas por hora, com alto rendimento de obtenção de oócitos. O menor tempo também é benéfico para as etapas laboratoriais, pois um intervalo mais curto entre a primeira e a última vaca aspirada permitirá maior padronização dos procedimentos. Além do menor tempo, a habilidade se reflete em causar mínimos danos ao estroma ovariano, permitindo maior longevidade reprodutiva à doadora.

O intervalo entre sessões de OPU influencia tanto na qualidade como na quantidade de oócitos. A OPU deve ser efetuada com intervalo mínimo de 15 dias, preferencialmente 30, permitindo assim que a doadora retorne ao ciclo naturalmente e de forma a não afetar o funcionamento ovariano.

Os protocolos pré-aspiração estão praticamente restritos à vacas taurus e/ou animais com baixa produção de oócitos. A administração de FSH exógeno pode ser eficaz em aumentar o percentual de folículos médios e grandes, mas nem sempre os oócitos são proporcionalmente competentes. A combinação do FSH com LH parece ser mais eficiente em aumentar o número de oócitos de boa qualidade recuperados em sessões de OPU (Blondim et al., 2002), embora o uso de LH proporcione expansão do cumulus, tornando muito mais

difícil o procedimento de busca e seleção dos oócitos após a aspiração folicular. Os protocolos hormonais, portanto, são utilizados principalmente em raças europeias, já que as fêmeas zebuínas possuem, naturalmente, maior número de folículos recrutados por onda.

Pesquisas recentes mostram que o ambiente uterino pode exercer grande influência na população folicular da reserva ovariana dos fetos, podendo prejudicar a produção oocitária da fêmea adulta. Bezerras nascidas de fêmeas que sofreram restrição alimentar durante o primeiro trimestre de gestação apresentaram redução de 60% na CFA quando comparadas às fêmeas nascidas de mães alimentadas com dieta controle (Evans et al., 2010). Desta forma, o potencial reprodutivo de uma doadora, quanto à população folicular, já deve ser considerado desde a condição corporal da fêmea responsável pela gestação, seja a própria mãe ou a receptora.

O aumento da compreensão dos padrões de desenvolvimento folicular nas últimas décadas tem permitido a manipulação da dinâmica ovariana e a obtenção de bons índices relacionados à produção de embriões. Entretanto, existem animais com bom potencial para a OPU/PIVE, que apresentam naturalmente grande quantidade de oócitos e consequentemente bom número de embriões e de prenhezes. Por outro lado, alguns animais possuem menor disponibilidade de oócitos e podem não ser indicados para a OPU/PIVE, mas podem produzir números aceitáveis de embriões por MOET.

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**CONDOMÍNIO EMPRESARIAL**

# Fatores que interferem na população folicular e produção de oócitos em bovinos

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Graças à maior precisão na manipulação dos gametas durante o processo *in vitro*, os índices de produção embrionária mostram-se cada vez mais promissores. Desde a última década, o Brasil se apresenta como líder mundial da tecnologia de produção *in vitro* de embriões, beneficiando diretamente os produtores de leite e carne de diferentes escalas (micro, pequenos, médios e grandes) por meio do incremento do melhoramento genético proporcionado pelo uso do embrião para gerar prenhez. Pelo último levantamento da IETS (2012), o Brasil produziu quase 270.000 embriões *in vitro,* uma porcentagem próxima de 80 % da produção mundial.

Para uma melhor compreensão dos eventos relativos à produção *in vitro* de embriões, a fisiologia ovariana tem sido intensamente estudada e o desenvolvimento folicular tem sido manipulado na esperança de se produzir o maior número possível de oócitos competentes. As informações obtidas até o momento têm proporcionado métodos bastante eficientes para a obtenção de grande número de embriões viáveis.

Vários fatores podem interferir na população folicular e consequente obtenção de oócitos, primeira etapa da produção *in vitro* de embriões. A seguir apresentaremos os principais aspectos sobre este tópico.

A contagem de folículos antrais (CFA, ≥3 mm de diâmetro) apresenta grande variação entre animais, podendo ser menor do que 5 em alguns animais e maior do que 50 em outros, considerando-se os dois ovários.

A variação individual da quantidade de folículos se reflete em grande variabilidade entre doadoras na produção de embriões, tanto nos procedimentos *in vivo* como *in vitro*. Em programas de superovulação, apenas 30% das vacas produzem 70% dos embriões, e 25% das vacas tratadas não produzem embrião algum. Da mesma forma, algumas fêmeas são altamente eficientes na PIVE, e outras produzem resultados bastante insatisfatórios.

Enquanto a população folicular é altamente variável entre indivíduos, existe uma grande repetibilidade do número de folículos antrais em um mesmo indivíduo. Esta contagem de folículos antrais tende a ser constante nas diferentes ondas foliculares de um mesmo animal, de forma que uma única avaliação ultrassonográfica, se realizada no início da onda, pode ser bastante útil identificar fêmeas com baixo, intermediário ou alto número de folículos ovarianos. Em vacas Nelore, a variação individual na produção de oócitos também demonstrou estar associada à produção de embriões e às taxas de prenhez. Doadoras que produzem grande número de oócitos também produzem mais embriões e mais prenhezes do que as doadoras que produzem poucos oócitos (Pontes et al., 2011).

Tratando-se da variação individual, um aspecto interessante diz respeito à raça. As fêmeas da raça Nelore, zebuínas e suas cruzas, normalmente têm maior número de folículos recrutados por onda em comparação com as raças europeias (Seneda et al., 2002). Esta característica certamente favoreceu o rápido crescimento da PIVE no Brasil, uma vez que os animais zebuínos representam aproximadamente 80% do rebanho brasileiro. No entanto, apesar do potencial similar entre os indivíduos da mesma raça, parâmetros como recrutamento e desenvolvimento folicular podem variar amplamente,

mesmo entre pares de gêmeos monozigóticos (Machado et al., 2003). Não está completamente esclarecida a justificativa para esta maior produção oocitária em fêmeas *indicus*, visto que a população folicular pré-antral entre *taurus* e *indicus* tem sido reportada como numericamente semelhante.

A quantidade de folículos antrais e a consequente produção de oócitos também sofrem alterações conforme o peso, a idade e as variações fisiológicas de cada animal. Animais senis produzem oócitos menos competentes, com menor quantidade de camadas de células do *cumulus oophorus*, que comumente resultam em baixa eficiência na produção embrionária. De forma semelhante, animais muito jovens podem ter os folículos aspirados com uso de equipamento adequado, mas frequentemente os resultados obtidos com a produção de embriões não são satisfatórios devido à baixa competência de desenvolvimento destes oócitos.

A condição corporal das fêmeas também exerce efeito sobre a qualidade dos oócitos, de forma que doadoras que sofreram privação alimentar produzem oócitos com menor capacidade de desenvolvimento até o estágio de blastocisto. O efeito da subnutrição na produção embrionária pode ser revertido, no entanto as fêmeas podem demorar mais de oito semanas para apresentar oócitos com capacidade satisfatória de desenvolvimento embrionário.

Alguns trabalhos afirmam que a competência de desenvolvimento dos oócitos subordinados pode estar comprometida durante a fase de dominância. Todavia, resultados de campo não mostram diferença significativa na qualidade oocitária durante as diferentes fases do ciclo estral, e atualmente a aspiração folicular tem sido realizada em momentos aleatórios da dinâmica folicular. Por outro lado, a taxa de recuperação é maior quando se aspiram folículos pequenos, que podem ser encontrados em maior número no início de cada onda folicular.

De forma semelhante, a fase reprodutiva pode exercer efeito na produção de oócitos e embriões. Em fêmeas leiteiras, as não gestantes frequentemente apresentam mais oócitos melhor produção embrionária do que as gestantes. Considerando-se as fêmeas lactantes, as gestantes proporcionam melhores resultados do que as lactantes vazias na produção de embriões após sessões de OPU/PIV.

A obtenção de quantidade apropriada e boa qualidade de oócitos para PIVE estão diretamente relacionadas a uma metodologia adequada. A resolução do ultrassom e o treinamento da equipe são fundamentais para a obtenção de bons resultados. Um veterinário experiente é capaz de aspirar 8 a 12 vacas por hora, com alto rendimento de obtenção de oócitos. O menor tempo também é benéfico para as etapas laboratoriais, pois um intervalo mais curto entre a primeira e a última vaca aspirada permitirá maior padronização dos procedimentos. Além do menor tempo, a habilidade se reflete em causar mínimos danos ao estroma ovariano, permitindo maior longevidade reprodutiva à doadora.

O intervalo entre sessões de OPU influencia tanto na qualidade como na quantidade de oócitos. A OPU deve ser efetuada com intervalo mínimo de 15 dias, preferencialmente 30, permitindo assim que a doadora retorne ao ciclo naturalmente e de forma a não afetar o funcionamento ovariano.

Os protocolos pré-aspiração estão praticamente restritos à vacas *taurus* e/ou animais com baixa produção de oócitos. A administração de FSH exógeno pode ser eficaz em aumentar o percentual de folículos médios e grandes, mas nem sempre os oócitos são proporcionalmente competentes. A combinação do FSH com LH parece ser

mais eficiente em aumentar o número de oócitos de boa qualidade recuperados em sessões de OPU (Blondim et al., 2002), embora o uso de LH proporcione expansão do *cumulus*, tornando muito mais difícil o procedimento de busca e seleção dos oócitos após a aspiração folicular. Os protocolos hormonais, portanto, são utilizados principalmente em raças europeias, já que as fêmeas zebuínas possuem, naturalmente, maior número de folículos recrutados por onda.

Pesquisas recentes mostram que o ambiente uterino pode exercer grande influência na população folicular da reserva ovariana dos fetos, podendo prejudicar a produção oocitária da fêmea adulta. Bezerras nascidas de fêmeas que sofreram restrição alimentar durante o primeiro trimestre de gestação apresentaram redução de 60% na CFA quando comparadas às fêmeas nascidas de mães alimentadas com dieta controle (Evans et al., 2010). Desta forma, o potencial reprodutivo de uma doadora, quanto à população folicular, já deve ser considerado desde a condição corporal da fêmea responsável pela gestação, seja a própria mãe ou a receptora.

O aumento da compreensão dos padrões de desenvolvimento folicular nas últimas décadas tem permitido a manipulação da dinâmica ovariana e a obtenção de bons índices relacionados à produção de embriões. Entretanto, existem animais com bom potencial para a OPU/PIVE, que apresentam naturalmente grande quantidade de oócitos e consequentemente bom número de embriões e de prenhezes. Por outro lado, alguns animais possuem menor disponibilidade de oócitos e podem não ser indicados para a OPU/PIVE, mas podem produzir números aceitáveis de embriões por MOET.

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