Universidade Estadual de Londrina

JOSÉ HENRIQUE FORTES PONTES

ASPECTOS APLICADOS DA PRODUÇÃO *IN VITRO* DE EMBRIÕES *Bos indicus*

LONDRINA
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Tese 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. Dr. Marcelo Marcondes Seneda

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Londrina, 01 de dezembro de 2009.

Dedicatória

A minha esposa Ellen

Todas as virtudes que
sonhei encontrar um dia encontram-se
representadas em você
Obrigado pelo apoio pela
dedicação, pelo carinho e principalmente pelo seu
amor.

Aos meus pais Claudomiro e Izabel e aos meus filhos Gabriel e Henrique

“Quando cheguei ao mundo, não o encontrei despovoado. Assim como meus ancestrais plantaram pra min, eu planto para os meus filhos”.
Talmud

Dedico.

Agradecimentos

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Suas ideias foram cruciais para a realização deste trabalho.

“Sem saber que era impossível, foi lá e fez.”

“O trabalho deve ser um elo de fraternidade.”

Dados curriculares do autor

Médico Veterinário formado pela UNESP – Jaboticabal em 1993. No mesmo ano assumiu a direção da Fazenda Haras São Francisco, gerenciando a produção de aproximadamente 10.000 litros de leite por dia. Após vários anos nessa atividade, realizou seu mestrado junto à *Faculté de Médecine Vétérinaire de L'Université de Montréal*, na cidade de *Saint Hyacinthe*, em Quebec, Canadá. Durante seu mestrado, participou da clonagem do touro *Starbuck*, um dos principais reprodutores da raça Holandesa. Após o mestrado, na mesma Fazenda e Haras São Francisco estruturou a empresa In Vitro Brasil, em Mogi Mirim, SP, atuando no segmento da tecnologia de embriões, com produção *in vitro* e clonagem de bovinos e ovinos.

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Resumo

Os objetivos gerais consistiram em analisar dados de obtenção de oócitos, produção de embriões e taxas de prenhez de doadoras *Bos indicus*, bem como propor novas estratégias para aumentar a eficiência desta biotécnica. Inicialmente, foram analisadas 1.504 aspiração foliculares e procedimentos de produção *in vitro* de embriões, a partir de fêmeas Nelore (n=420). Durante cinco anos, 27.966 oócitos foram obtidos, sendo 22.809 (81,6%) considerados viáveis, os quais resultaram em 7.709 embriões transferidos e 2.579 prenhezes (33,4%). Estes dados mostraram uma média de produção de oócitos inferiores aos relatos e comunicações pessoais anteriores, além de uma grande variação individual na produção de oócitos em fêmeas Nelore. No segundo trabalho, para investigar porque no Brasil tem ocorrido a substituição do método *in vivo* pelo método *in vitro* para obtenção de embriões, comparou-se estes dois métodos nas mesmas doadoras Nelore (n=30), considerando produção de embriões e taxas de prenhez. A média de embriões obtidos por OPU/PIVE (9.4 ± 5.3) foi maior ($P<0.05$) do que na MOET (6.7 ± 3.7). Para vacas Nelore, com um intervalo mínimo de 15 dias entre OPUs, foi possível obter mais embriões e mais prenhezes no método *in vitro* do que *in vivo*. No terceiro trabalho descreve-se um programa em larga escala de produção *in vitro* de embriões a partir de fêmeas leiteiras *Bos taurus* (Holandês), *Bos indicus* (Gir), and *indicus-taurus* (Girolanda) com uso do sêmen sexado. Analisou-se dados de 5.407 OPUs e as taxas de prenhez também foram comparadas. Oócitos viáveis (n = 64.826) foram maturados *in vitro* por 24 h a 38.8°C e 5% CO_2 em ar. Utilizou-se sêmen congelado sexado para fêmea de touros Gir (n = 8) ou Holandês (n = 7). Os embriões resultantes foram cultivados em condições semelhantes de temperatura e atmosfera da maturação, mas embriões em D2, D3, D4 ou D5 tiveram seu estágio final de desenvolvimento realizado em uma estufa portátil. Após protocolo de transferência em tempo fixo, os embriões foram transferidos a fresco, depois de 24 a 72 horas de transporte para cobrir distâncias acima de 2.000 km. O número médio de embriões por OPU/PIVE e taxa de prenhez, foram, respectivamente, 3,2 (n = 12.243/3.378) e 40% para vacas Gir, 2,2 (n = 2.426/1.138) e 36% para Holandês, 3,9 (n = 1.033/267) e 37% para 1/4 Holandês x 3/4 Gir, e 5,5 (n = 1.222 / 224) para 1/2 Holandês-Gir. Demonstrou-se a influência do gado *indicus* na produção de oócitos, comparando-se dois níveis de *indicus*/*taurus*. Além disso, demonstrou-se a eficiência da sexagem de espermatozóides para a produção rápida e em larga escala de produtos *indicus* para a pecuária leiteira. O esquema para o cultivo de embrião durante o transporte por longas distâncias apresentou resultados interessantes, com perspectivas amplas para o comércio internacional de embriões. No último trabalho foi comparado o uso do meio sequencial G1/G2 em relação ao meio SOF, mais os efeitos da alta (20%) ou baixa (5%) tensão de oxigênio durante o cultivo de embriões. A taxa de clivagem foi positivamente ($p <0,05$) influenciada pelo meio G1/G2 (68,2%, n = 521) comparado a SOF (59,0%, n = 455). Não foi observada diferença para o desenvolvimento de blastocisto (30,2 e 28,0% para a SOF e G1/G2, respectivamente). As taxas de gestação revelaram uma diferença marcante para o grupo G1/G2, que resultou em níveis mais altos de prenhez no dia 30 (53%, n = 100) e no dia 60 de gestação (50%) em relação ao grupo SOF (28,2 e 22,8% nos dias 30 e 60, respectivamente, n = 121). Com relação às taxas de prenhez, o índice mais alto ocorreu com baixa tensão de oxigênio, 45,0% n = 248) em relação à alta tensão de oxigênio (34,5%, n = 174). Considerando todos os trabalhos, foi possível apresentar dados específicos da produção de embriões a partir de doadoras *indicus*, além de aspectos inovadores na produção de embriões *in vitro* em larga escala.

Palavras-chave: *Bos indicus, Bos taurus, in vitro*, embriões, sêmen sexado, aspiração folicular, TE

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Abstract

Our general goal at this thesis was to analysis commercial data of oocyte retrieval, *in vitro* embryo production and pregnancy rates from *Bos indicus* donors, as well as to establish new strategies for improving the efficiency of the *in vivo* method for obtaining bovine embryos. First, we analyzed 1,504 ovum pick up / *in vitro* production (OPU/IVP) performed on Nelore (*Bos indicus*) donors (n=420). During five years, 27,966 oocytes were obtained, being 22,809 (81.6%) considered viable resulting in 7,709 transferred embryos and 2,579 pregnancies (33.4%). These data show general means of oocytes quite lower than previous reports/abstracts from Nelore and individual variation on oocyte production seems to be very high in this breed. In the second work we investigate why the preferred means to produce bovine embryos in Brazil has changed from *in vivo* to *in vitro*, comparing these two approaches in the same Nelore cows (n=30) and assessed total embryo production and pregnancy rates. The average number of embryos produced by OPU/IVP (9.4 ± 5.3) was higher ($P<0.05$) than the MOET method (6.7 ± 3.7). We concluded that in Nelore cows, with an interval of 15 d between OPU procedures, it was possible to produce more embryos and pregnancies compared to conventional MOET. In the third work we describe a large-scale commercial program for producing *in vitro* embryos from dairy *Bos taurus* (Holstein), *Bos indicus* (Gir), and *indicus-taurus* (Girolanda) donors using sexed sperm. We analyzed data from 5,407 OPU and pregnancy rates of the cows were also compared. Viable oocytes (n = 64,826) were *in vitro* matured for 24 h at 38.8°C in an atmosphere of 5% CO_2 in air. Frozen-thawed sexed sperm (X-chromosome bearing) from Gir (n = 8) or Holstein (n = 7) sires (2 x 10^6 /dose) were used for fertilization. Oocytes were fertilized by incubating sperm and oocytes for 18–20 hrs, and the resulting embryos were cultured in similar conditions of temperature and atmosphere of IVM, but embryos at Day 2, 3, 4 or 5 finished the developmental stage in a portable incubator. Using a fixed time protocol in the recipients, all embryos were transferred fresh, after 24 to 72 hrs of transportation to cover 2,000 km. The mean number of embryos produced by OPU/IVF and the pregnancy rates were, respectively, 3.2 (n = 12,243/3,378) and 40% for Gir cows, 2.2 (n = 2,426/1,138) and 36% for Holstein cows, 3.9 (n = 1,033/267) and 37% for 1/4 Holstein x 3/4 Gir, and 5.5 (n = 1,222/224) for 1/2 Holstein-Gir. We clearly demonstrated the influence of *indicus* cattle on oocyte yield by comparing two levels of *indicus/taurus* breeds. In addition, we demonstrated the efficiency of sexed sperm for quick and large production of *indicus* dairy female calves. The scheme for culturing embryo during transportation for long distances presented interesting results, with wide perspectives for international business of embryos. In the last work we evaluated the use of the sequential medium G1/G2 compared to SOF, plus the effects of atmosphere oxygen tension during embryo culture. The cleavage rate was positively ($p<0.05$) influenced by G1/G2 medium (68.2%, N=521) compared to SOF (59.0%, N=455). No difference was observed for blastocyst development (30.2 and 28.0% for SOF and G1/G2, respectively). Pregnancy rates revealed a striking difference for G1/G2 group, which resulted in higher levels of pregnancy at day 30 (53%, n =100) and at day 60 of gestation (50%) compared to SOF group (28.2 and 22.8% at day 30 and 60, respectively, n = 121). Regarding pregnancy rates, low oxygen tension during embryo culture was higher (45.0% n = 248) over high oxygen tension (34.5%, n = 174). Taking all together, our data show specific reproductive data of *indicus* donor e novel aspects for *in vitro* embryo production in large-scale.

Keywords: *Bos indicus, Bos taurus, in vitro*, embryo, sexed sperm, OPU, MOET

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Introdução

Há mais de uma década o Brasil tem apresentado um crescimento contínuo no segmento da biotecnologia de embriões. Após um amplo e consolidado conhecimento obtido com a obtenção pelo método *in vivo*, ou superovulação e transferência de embriões (*Multiple ovulation and embryo transfer – MOET*), o país passou também dominar a aspiração folicular, ou *Ovum pick up* (OPU) e a produção *in vitro* de embriões (PIVE).

O domínio do método *in vitro* colocou o Brasil em uma posição de destaque no segmento, despertando o interesse de outros países não só pelas biotécnicas envolvidas, mas também pelo fato do país possuir o maior rebanho do mundo e ser o principal exportador de carne bovina.

Além do aspecto do gado de corte, os trabalhos referentes ao sistema de produção de leite a pasto, com animais do cruzamento de Gir e Holandês – Girolanda – apresentaram-se extremamente promissores, pelo contexto de se obter um animal resistente aos desafios das regiões tropicais, com uma apreciável produção de leite a baixo custo.

O cenário favorável de produção de leite e carne certamente vincula-se ao predomínio de animais *Bos indicus*, ou Zebu, no rebanho nacional. A utilização da biotecnologia de produção de embriões a partir de doadoras *indicus* revelou um aspecto inédito da fisiologia ovariana desses animais: uma quantidade elevada de folículos a serem aspirados, disponibilizando expressivo montante de oócitos, sem qualquer procedimento exógeno.

Verificou-se, assim, uma tríade bastante favorável ao panorama do progresso biotecnológico da produção de embriões: 1) um tipo racial – Zebu – realmente

adequado às condições tropicais, com valorização crescente, tanto no mercado interno, quanto externo; 2) o natural aporte de oócitos das fêmeas *indicus*; e 3) o domínio da biotécnica da PIVE pelo segmento privado.

Em poucos anos, o segmento comercial da obtenção *in vitro* de embriões estabeleceu-se progressivamente, mas algumas lacunas no contexto acadêmico tornaram-se evidentes.

A produção de oócitos a partir de fêmeas zebuínas somente era reportada por relatos de comunicação pessoal ou resumos, sem um levantamento capaz de proporcionar análise mais criteriosa do tema. Outra lacuna referia-se à uma comparação entre os métodos MOET e OPU/PIVE nas mesmas doadoras, para se procurar esclarecer com mais precisão um aspecto único do Brasil: a substituição do método *in vivo* pelo *in vitro*.

Além desses dois aspectos, de contexto mais geral, verificava-se também um grande interesse em se acompanhar os resultados do sêmen sexado e a PIVE em larga escala, particularmente em gado leiteiro, pois gradativamente tornou-se viável a utilização desse recurso no segmento aplicado.

Finalmente, partindo de conhecimentos específicos da modernização dos meios e condições de cultivo, surgia a necessidade de se verificar até que ponto modificações da PIVE se refletiam nos índices gestacionais.

A partir dessas lacunas e questionamentos, procurando reunir, de maneira direta, os interesses do segmento aplicado com os desafios acadêmicos, o presente trabalho foi proposto, considerando-se alguns dos principais trabalhos na área, conforme a revisão a seguir.

Capítulo 1 – Revisão de literatura

1. Cenário geral da produção *in vitro* de embriões no Brasil e no mundo

O Brasil é o primeiro país do mundo em número de embriões produzidos *in vitro*. Durante o ano de 2003, mais de 60.000 embriões foram gerados por esse método e aproximadamente 87.000 embriões pelo método *in vivo* (Thibier, 2004), sendo esta situação mantida em levantamentos mais recentes (Thibier, 2007).

Apesar do país também apresentar expressivo número de embriões obtidos por lavagem uterina, verifica-se uma clara tendência de continuidade ao aumento de embriões produzidos *in vitro*. Em muitos países, a produção *in vitro* de embriões (PIVE) é proposta como a última opção para obtenção de embriões, quando a recuperação pela lavagem uterina é inviável.

Já no Brasil, muitas vezes a PIVE constitui-se a primeira opção para a multiplicação de animais de interesse zootécnico e/ou comercial, e isto certamente tem correlação com o predomínio da raça Nelore no plantel nacional. Para fêmeas Nelore, pode-se admitir uma maior quantidade de embriões por procedimento com a PIVE, quando comparada à colheita e transferência de embriões (Nonato Jr. et al., 2004).

Além da situação referente à raça Nelore, o cenário da PIVE nacional pode ser compreendido por algumas outras particularidades. A grande quantidade de laboratórios privados com domínio da técnica e os altos preços, por vezes especulativos, alcançados por animais Nelore. Embora nos últimos anos a situação de mercado tenha se estabilizado, tais aspectos em muito contribuíram para a consolidação da técnica no Brasil. A partir destes aspectos, profissionais brasileiros estabeleceram em outros países o sucesso obtido aqui, e atualmente há laboratórios comerciais em países da América Central, do Norte, do Sul e mesmo em outros continentes.

Mesmo com toda a tecnologia referente à produção *in vitro* de embriões em larga escala no Brasil, uma grande lacuna refere-se à escassez de informações sobre grandes programas de PIVE em *Bos indicus*, pois a maior parte dos relatos e levantamentos decorre de informações pessoais ou dados de associações, condições que não permitem uma análise mais detalhada dos aspectos técnicos do processo.

2. Produção *in vitro* de embriões Girolanda – impacto na pecuária leiteira em regiões tropicais

Um dos aspectos fundamentais da importância da proteína animal, pela sua magnitude social, é a produção de leite. O Brasil apresenta um potencial extraordinário para este segmento, embora pouco aproveitado. Por muitas décadas, buscou-se realizar em nosso país o mesmo modelo dos países frios, localizados no hemisfério norte, ou seja, a criação de animais de raças européias, confinados em espaços restritos. Embora tal sistema seja possível em algumas regiões específicas, a maior parte do território nacional presta-se a outra forma de produção de leite, envolvendo o manejo de pastagem, a utilização de animais adaptados e a difusão de técnicas para o produtor rural, tanto de pequeno quanto de médio e grande porte (Carvalho et al., 2008).

No campo da produção animal, e mais especificamente da reprodução animal, o Brasil apresenta-se como liderança mundial inconteste na produção de embriões bovinos (Thibier, 2007). Tais características apresentam conotação ainda mais importante, quando se considera os tipos raciais predominantes no nosso país, basicamente animais *Bos indicus*, denominados zebuínos, altamente adaptados aos trópicos (Anualpec, 2007). Graças à rusticidade dos animais zebuínos, como a raça Girolanda, por exemplo, torna-se possível a utilização de áreas menos nobres para o

pastoreio, liberando as terras mais férteis para o cultivo agrícola, mantendo assim uma perfeita integração entre a agricultura e a pecuária, independente da extensão territorial da propriedade (Mello et al., 2004). Tal proposta está em acordo com o movimento mundial de favorecer a permanência do homem no campo, tanto pela necessidade de geração de alimentos, quanto para evitar a explosão demográfica dos grandes centros urbanos (Pinstrup-Andersen et al., 2008).

A utilização de animais adaptados aos trópicos representa uma extraordinária área de expansão da atualidade, pois é muito grande a carência de proteína animal em países situados na latitude tropical (Pinstrup-Andersen et al., 2008). Regiões situadas na Índia, África, além de vários países do Continente Americano poderiam ser largamente beneficiados com a aquisição de embriões de raças leiteiras rústicas e produtivas, como a Girolanda.

Para os animais de origem européia, direcionados para produção leiteira, o grande desafio observado no Brasil é o mesmo de outros países com bacias leiteiras situadas em regiões de verão quente e úmido, como a Flórida e a Califórnia: os índices irrisórios de prenhez nos meses de estresse calórico. Algumas propriedades brasileiras optam por medidas extremas, como não inseminar as fêmeas em cio nos meses mais quentes, pois o estresse térmico causa efeitos deletérios nos oócitos, espermatozóides e no desenvolvimento embrionário inicial, acarretando índices insatisfatórios (Hansen 2006). A alternativa mais viável consiste na transferência de embriões em substituição à inseminação artificial, sendo a transferência realizada em tempo fixo ainda mais promissora (Rodrigues et al., 2009).

Dessa forma, a biotecnologia da reprodução dos embriões apresenta-se como a etapa inicial e fundamental para a multiplicação de animais voltados à produção de leite, para ambos os mercados, interno e externo. Considerando-se o avanço do uso do

sêmen sexado, a aplicação desta biotécnica na PIVE representa uma grande perspectiva na rápida multiplicação de fêmeas Girolanda, com perspectivas promissoras na cadeia produtiva do leite para regiões tropicais e subtropicais.

3. Produção *in vivo* e *in vitro* de embriões bovinos

As fêmeas bovinas têm sido alvo de numerosas pesquisas visando o aproveitamento racional dos gametas. Gordon (2003) relata que bezerras ao nascimento possuem mais de 100.000 oócitos em seus ovários, que pelas vias naturais podem gerar 0,01% de produtos viáveis, totalizando por volta de dez descendentes durante a vida reprodutiva. Como propostas para aumentar o aproveitamento dos gametas destacam-se a indução de múltipla ovulação e transferência de embriões (MOET) e a produção *in vitro* de embriões (PIVE); (Bousquet et al., 1999; Merton et al., 2003).

Após a descrição da técnica não invasiva de recuperação de embriões por Greve (1977), a MOET teve um crescimento expressivo em bovinos, sendo o principal método de produção de embriões em escala comercial (Galli et al., 2003; Merton, et al., 2003). Entretanto, a variabilidade das respostas a superovulação foi o principal obstáculo para melhora nos índices na MOET (Mapletoft et al., 2002). Entre 20 a 30% das doadoras tratadas não respondem à terapia superovulatória e aproximadamente 30% das doadoras produzem menos do que 6 embriões viáveis por colheita (Reichenbach, 2003; Galli et al 2003). Mapletoft et al., (2002) atribuíram parte destes resultados à proporção superior a 20% de LH nos extratos hipofisários utilizados para superovulação. Em uma revisão, Sartori (2004) relaciona os achados desfavoráveis a distúrbios no transporte de gametas e efeitos adversos nos CCO, os quais comprometeriam a fecundação e a viabilidade embrionária. Outros fatores relevantes são a disponibilidade de alimentos, escore da

condição corporal e condições de estresse térmico das doadoras durante os tratamentos (Fernandes, 2003).

Atualmente, consegue-se uma média geral em torno 6 embriões viáveis por colheita em doadoras *Bos taurus* e *Bos indicus* (Castro-Neto et al., 2005) Diante das limitações da MOET, a PIVE se apresentou como técnica complementar ou mesmo alternativa para incrementar o uso dos oócitos bovinos (Brackett et al., 1982; Bousquet et al., 1999). Inicialmente, a técnica foi realizada com oócitos provenientes de abatedouro (Galli & Lazzari, 1996). Com o advento das técnicas de aspiração folicular transvaginal guiada pela ultra-sonografia (OPU) foi possível obter oócitos de animais vivos, permitindo assim a expansão desta biotécnica (Pieterse et al., 1988). Merton et al (2003) relataram a possibilidade de fecundação de grupos de oócitos com sêmen de diferentes touros, contribuindo assim o uso da PIVE em escala comercial. A possibilidade de obtenção de embriões de doadoras impossibilitadas de produzir embriões *in vivo* contribuiu para a expansão da PIVE (Seneda et al., 2000). Adicionalmente, resultados animadores foram obtidos em animais saudáveis, em fêmeas pré-púberes e em fêmeas gestantes, possibilitando o encurtamento nas gerações e maior rapidez nos programas de melhoramento genético (Meintijes et al., 1995; Seneda et al., 2001; Snell-oliveira et al., 2003).

Com objetivo de incrementar o potencial de maturação dos oócitos na tentativa de maior exploração do potencial genético das doadoras, vislumbrou-se a possibilidade de submeter as doadoras às duas técnicas de produção de embriões. Bousquet et al. (1999) obtiveram resultados semelhantes de produção embrionária com a PIVE e a MOET, 4,7 e 4,3 embriões viáveis por sessão, respectivamente. Mas na literatura consultada não foi possível identificar trabalhos descrevendo tal associação em fêmeas

Nelore, apesar desta raça ser a predominante do maior rebanho bovino do mundo – o brasileiro.

4. Técnica da produção *in vitro* de embriões bovinos

Os primeiros resultados obtidos de maturação e de fecundação *in vitro* de oócitos bovinos foram de Iritani e Niwa (1977). Cinco anos depois surge o primeiro bezerro produzido pela equipe de Brackett (1982), com oócitos maturados *in vivo*. Somente em 1986, Hanada et al., relataram o primeiro bezerro a partir de oócitos maturados e fertilizados *in vitro*, porém, utilizando o método de cultivo *in vivo.* Finalmente, em 1987 Lu e colaboradores descreveram o sucesso com o primeiro bezerro após maturação, fecundação e cultivo *in vitro*. Desde então, a produção *in vitro* de embriões bovinos tornou-se cada vez mais acessível e eficiente, sendo atualmente considerada uma técnica consolidada (Hansen, 2006).

De maneira geral, a PIVE pode ser dividida em duas principais etapas, quais a obtenção dos oócitos, pela aspiração folicular ou *Ovum pick up* (OPU), e a etapa laboratorial, esta subdividida em maturação, a fecundação e o desenvolvimento ou cultivo embrionário.

4.1 Aspiração folicular ou Ovum pick up (OPU)

Após uma evolução técnica considerável a partir de procedimentos poucos eficientes, como laparotomia, colpotomia, laparoscopia transvaginal ou paralombar (Lambert et al., 1986) a aspiração folicular encontra-se relativamente estabilizada quanto ao uso de equipamentos e aparatos técnicos. Uma dupla bem treinada é capaz de

promover aspiração folicular e seleção de oócitos de aproximadamente 20-25 vacas por dia, número altamente satisfatório e capaz de gerar suficiente demanda para as etapas seguintes da produção *in vitro* de embriões. Algumas situações ainda requerem adaptações, como a aspiração de bezerras pré-púberes. No entanto, a demanda para estes casos é bastante pequena e provavelmente permanecerá restrita a ocorrências particulares.

Uma importante conquista brasileira refere-se ao pioneirismo da utilização do transdutor endo-vaginal humano, ou micro-convexo. Esta probe mostrou-se altamente adequada às condições anatômicas de vacas pequenas e novilhas pré-púberes, além de permitir ótima visualização dos folículos em vaca, pela facilidade de manipulação ovariana, além de apresentar uma imagem com alta resolução e possuir imagem com grande ângulo de abertura.

Inicialmente, a aspiração era realizada apenas com agulhas longas (55 cm), altamente eficientes, pois produzidas especificamente para aspiração folicular (Pieterse et al., 1988), mas atualmente as agulhas hipodérmicas descartáveis representam a principal opção dos profissionais.

Com relação às bombas de vácuo, as da empresa Handle Cook permanecem como as de máxima eficiência. No entanto, dado ao seu elevado custo, as bombas alternativas são cada vez mais comuns. Bombas de aspiração odontológica, de fluidos endotraqueais e de infusão controlada funcionam de maneira satisfatória (Seneda et al., 2005).

A aspiração folicular tem sido realizada, principalmente, em momentos aleatórios do ciclo estral. O principal aspecto para esta situação refere-se à praticidade desta opção para a equipe de aspiração, pois alterações no calendário podem ser feitas facilmente, sem conseqüências para quaisquer segmentos – pecuarista, laboratório de

PIVE, etc. No entanto, a crescente necessidade de maior eficiência e o acréscimo de demanda para situações menos favoráveis (animais com menor produção de oócitos) sinalizam para um incremento na utilização de protocolos pré-aspiração folicular. Naturalmente, o número de folículos disponíveis para a aspiração folicular apresenta considerável variação conforme a dinâmica folicular, sendo o início de onda o momento mais favorável para a recuperação, pelo maior número de folículos e pela melhor eficiência de captação dos oócitos a partir de folículos menores (Seneda et al., 2001).

Apesar de haver relatos em animais zebuínos (Nonato Jr. et al., 2003), é importante ressaltar que a utilização de gonadotrofinas tem acontecido principalmente em vacas de raças européias. Os animais *Bos indicus* apresentam naturalmente maior número de folículos por onda, e talvez por este motivo o FSH tenha tido um impacto menor no crescimento folicular e conseqüente disponibilidade de oócitos para a produção de embriões (Blaschi et al., 2004). Recentemente demonstrou-se tal diferença na resposta gonadotrófica entre *taurus* e *indicus* também em outros aspectos (Monteiro et al., 2009).

Desta forma, para animais zebuínos, os protocolos pré-aspiração têm sido considerados somente situações de produção insatisfatória. Estes protocolos seguem os princípios dos fármacos destinados à inseminação artificial em tempo fixo (IATF), com algumas alterações. A principal alteração refere-se ao término do protocolo, ou seja, o momento da aspiracão folicular. Uma vez que a ovulação não é desejada, e a eficiência de recuperação de oócitos é melhor com a punção de folículos pequenos (Seneda et al., 2001), preconiza-se a aspiração quando os folículos se apresentam com 4 a 6 mm de diâmetro.

Outra particularidade em relação aos protocolos para aspiração refere-se ao implante de progesterona utilizado. Os implantes intra-vaginais causam, em alguns

animais, uma secreção vaginal mucóide. Embora esta secreção geralmente seja asséptica, decorrente de processo irritativo, sua consistência viscosa dificulta o procedimento da aspiração folicular, por causar acúmulo de muco na extremidade do aparato de aspiração, com risco de entupir a agulha.

Em suma, a aspiração folicular encontra-se hoje como técnica estabelecida, de reconhecida eficiência, servindo como instrumento para a próxima etapa do processo de produção *in vitro* de embriões: a maturação oocitária.

4.2. *Maturação in vitro*

A maturação *in vivo* depende da estimulação hormonal do folículo pré-ovulatório por gonadotrofinas como hormônio folículo estimulante (FSH) e hormônio luteinizante (LH).

Após o pico pré-ovulatório de LH, ocorre, então, a retomada da seqüência de eventos que culminam com a maturação nuclear (Kruip et al., 1983). Paralelamente e independentemente, o oócito deve passar por uma maturação citoplasmática, na qual ocorrem importantes alterações necessárias para a fecundação e desenvolvimento embrionário posterior (Thibault et al., 1987).

No procedimento *in vitro*, ocorre o fenômeno conhecido como maturação meiótica espontânea, que leva o oócito a maturação nuclear independente do estimulo gonadotrófico. Ainda que a maturação nuclear seja facilmente identificada, pouco se sabe sobre a maturação citoplasmática nos oócitos maturados *in vitro.* Por isso, nem todos os oócitos maturados *in vitro* tem a capacidade de serem fertilizados e gerar um embrião viável (Gordon, 2003).

Vários experimentos têm sido realizados com o intuito de aprimorar as condições de maturação *in vitro*. Basicamente o meio de maturação mais empregado é o TCM-199, tamponado com bicarbonato e acrescido de soro fetal bovino (SFB), antibiótico, piruvato e hormônios. Alguns fatores de crescimento como o EGF ou IGF-1 têm sido utilizados com a intenção de diminuir a apoptose, promovendo a melhora da qualidade dos oócitos maturados *in vitro* (Ali & Sirard, 2002).

Vários compostos com efeito antioxidante têm sido empregados com o intuito de diminuir a formação de radicais livres que causam sérios danos aos embriões cultivados *in vitro*. De Matos e colaboradores (1996) demonstraram que a suplementação do meio de MIV com cisteamina melhorou o desenvolvimento e a qualidade embrionária. Trabalhando com doadoras *Bos taurus*, Blondin et al., (2002) comprovaram que um protocolo de superovulação aumenta a viabilidade dos oócitos aspirados. Entretanto, tais protocolos não produziram resultados satisfatórios quando aplicados em animais *Bos indicus* (Barros et al.., 2009).

Atualmente, a maturação *in vitro* apresenta altos índices de eficiência, sendo possível obter 80 a 90 % de oócitos em condições de serem submetidos à próxima etapa da PIVE – a fecundação *in vitro* – FIV.

4.3. Fecundação in vitro - FIV

O espermatozóide bovino é uma célula extremamente especializada e, para se tornar capaz de fertilizar o oócito, precisa sofrer uma série de alterações bioquímicas para atingir um estado denominado de capacitação.

Várias proteínas produzidas pelas glândulas seminais ligam-se a superfície do espermatozóide no momento da ejaculação. Quando *in vivo,* essas proteínas são

removidas no momento em que os espermatozóides entram em contato com o líquido existente na tuba uterina. Após a remoção dessas proteínas, o processo de capacitação inicia-se. No processo *in vitro*, dentre os vários métodos desenvolvidos para a capacitação espermática, o uso da heparina tem demonstrado ser o mais eficaz (Parrish et al., 1994). Ainda que outros métodos alternativos existam, a capacitação induzida pela heparina tem produzido melhores resultados sob situações adversas, apesar do mecanismo preciso ainda não ser totalmente estabelecido (Gordon, 2003).

Foi demonstrando que o sucesso nos programas de produção *in vitro* de embriões está intimamente ligado ao uso de touros previamente selecionados e classificados como de alta fertilidade (Xu et al., 2006). Além desse importante aspecto da variação individual, o sêmen a ser usado na PIVE necessita ser separado dos diluentes e dos agentes crioprotetores. O método mais utilizado comercialmente é a centrifugação em gradiente *Percoll*. Entretanto, estudos recentes demonstraram outras técnicas viáveis para separação espermática, mais eficientes e com uso indicado quando o *Percoll* não alcança bons resultados (Lee et al., 2009).

4.3.1. Sêmen sexado na produção in vitro de embriões

Atualmente, o método utilizado para sexagem de espermatozóides é a citometria de fluxo, baseada em uma diferença de 3,8% entre no conteúdo do DNA do espermatozóide bovino X e Y (Johnson et al., 1999). A utilização de sêmen sexado, até poucos anos atrás, parecia ser algo destinado a um longo caminho até ser uma opção ao alcance do pecuarista. No entanto, esta ferramenta já faz parte do leque de opções para incremento da eficiência reprodutiva, e de início verificou-se imensa expectativa de acréscimo na eficiência de obtenção de fêmeas.

Infelizmente, a realidade nos primeiros trabalhos mostrou-se outra. O processo de separação do sêmen acaba reduzindo, drasticamente, a viabilidade dos espermatozóides (Catt et al., 1997; Maxwell e Johnson, 1997) e os primeiros resultados de inseminação artificial mostram desanimadores índices de prenhez (Seidel, 2007). No entanto, outra é a situação quando se aborda a produção *in vitro* de embriões com sêmen sexado (Wheeler et al., 2006). Graças à maior precisão na manipulação dos gametas durante o processo *in vitro*, os índices de produção embrionária mostraram-se animadores, embora ainda com grande variabilidade nas taxas de obtenção de blastocistos (Lu et al., 1999; Wilson et al., 2005, 2006).

Esta variabilidade nos resultados de produção embrionária parece estar vinculada a danos ao DNA espermático causados pela técnica de citometria de fluxo, resultando em embriões com alterações morfológicas nas mitocôndrias, na membrana nuclear e em outras organelas (Palma et al., 2008).

Mesmo com tais limitações, a produção *in vitro* de embriões muito tem contribuído para uma maior difusão do uso do sêmen sexado. De maneira geral, o preço do sêmen sexado ainda é pouco atrativo para uso em larga escala na IA. Entretanto, no processo da PIVE, cada dose de sêmen tem sua utilização maximizada, tornando a opção mais atrativa ao pecuarista, atestando assim a viabilidade do sêmen sexado na PIVE mesmo em escala comercial (Wilson et al., 2006).

A despeito dos primeiros relatos de uma tendência das taxas de produção de blastocistos serem inferiores quando se utiliza o sêmen sexado (Merton et al., 1997), a eficiência nas etapas seguintes – taxa de prenhez e produtos nascidos – recentemente mostrou-se similar aos embriões produzidos com sêmen convencional (Xu et al., 2006). Desta forma, o maior número de fêmeas nascidas poderia compensar uma possível menor eficiência da PIVE com o uso do sêmen sexado.

Como já mencionado, os principais desafios para uma mais ampla disseminação do uso do sêmen sexado na PIVE referem-se às alternâncias de resultados. Poucos laboratórios apresentam estabilidade nos procedimentos e, por isto, os meios para a fecundação *in vitro* e os protocolos de separação dos espermatozóides viáveis mostram-se em processo de evolução. Considerando-se os preços extremamente elevados do sêmen de alguns touros, mais a necessidade de ajustes de individuais dos protocolos, deduz-se que um progresso mais rápido neste segmento irá ocorrer quando houver maior eficiência na sexagem de espermatozóides e menor custo por dose, como já preconizado há quase trinta anos (Van Vleck, 1981).

No presente momento, os principais ajustes têm sido propostos em diferentes concentrações de heparina, diminuição da gota de fecundação e diferentes protocolos de separação de sêmen, como alternativas para aumentar a eficiência do sêmen sexado na PIVE (Blondin et al., 2009; Xu et al., 2009).

Apesar das limitações naturais de uma técnica em fase de desenvolvimento, é inegável o grande avanço proporcionado pelo uso comercial de sêmen sexado à indústria de embriões bovinos, com perspectivas altamente favoráveis especialmente no segmento da pecuária leiteira.

5. *Cultivo ou desenvolvimento embrionário in vitro*

A partir da década de 70 começaram a ser desenvolvidos, com sucesso, os primeiros meios de cultivo para embriões bovinos e ovinos, apesar de restrições importantes, como o bloqueio embrionário que ocorria no estádio de 8 células (Thompson, 2000).

Numa tentativa de superar este bloqueio, foram introduzidos sistemas de cultivo associados a algumas células somáticas, ou seja, os sistemas de co-cultivo. Várias células demonstraram ser capazes de secretar fatores embriotróficos que simulavam o ambiente materno, permitindo aos embriões bovinos estágios mais avançados de desenvolvimento. As células da tuba uterina foram as primeiras a serem utilizadas com sucesso em bovinos e ovinos (Gandolfi et al., 1986). Outras células utilizadas foram: células uterinas, células da granulosa, *Buffalo Rat Liver* (BRL) e células VERO (Gordon, 2003). Os meios utilizados no sistema de co-cultivo apresentaram dois grandes problemas. Primeiro, devido à presença de células, era necessária a adição de soro ao meio, caso contrário as células não se desenvolveriam. Segundo, os meios eram desenvolvidos para atender as necessidades das células, não dos embriões. Substâncias presentes no soro e, também as produzidas pelas células, contêm um número desconhecido de agentes ativos, incluindo alguns fatores de crescimento, com potenciais conseqüências deletérias futuras no desenvolvimento fetal. A presença de soro fetal bovino (SFB) tem sido relacionada com a síndrome do bezerro grande (Thompson, 1995) e com alterações no desenvolvimento do fígado e coração em fetos ovinos (Sinclair et al., 1999) e bovinos (Farin & Farin, 1995). O SFB também tem sido vinculado a outras alterações no metabolismo embrionário, como o rompimento da estrutura mitocondrial, desenvolvimento de grandes vesículas de lipídios, alteração da estrutura embrionária (Lane et al., 2003), modificações na expressão gênica, (Lazzari et al., 2002) e formação prematura de blastocele (Holm et al., 2002).

Outros meios foram subsequentemente desenvolvidos, denominados “meios complexos”, em cuja composição há aminoácidos, vitaminas, precursores de ácido nucléico, minerais e normalmente são suplementados com 5 a 20% de soro. Como já

descrito anteriormente, não são meios que levam em conta a necessidade nutricional dos embriões (Gardner 1999).

Já as "soluções de sais adicionados de substratos energéticos" são meios inicialmente formulados para linhagem de animais de laboratório. Foram baseados em concentrações dos componentes encontrados no tuba uterina ou útero, como por exemplo: 1) SOF (*Sintetic Oviduct Fluid*), desenvolvido por Tervit et al. (1972), baseado em tuba uterina de ovinos; 2) HTF (*Human Tubal Fluid*), desenvolvido por Quin *et al.* (1995), baseado na tuba uterina humana; 3) KSOM, desenvolvido por Lawitts et al. (1992) para ser usado em animais de laboratório. Esses meios são formulados para todo o período de cultivo embrionário e por isso são chamados de estáticos, pois não ocorre mudança de meio ou de formulações durante o cultivo. Por serem meios simples, usualmente necessitam da adição de soro ou albumina sérica bovina (BSA) para atingirem índices aceitáveis de produção embrionária.

Os meios mais próximos das condições fisiológicas são os denominados "meios sequenciais", estabelecidos mais recentemente. Tais meios levam em conta as mudanças nas exigências nutricionais de acordo com as diferentes etapas do desenvolvimento embrionário. Alguns exemplos destes meios são: 1) G1/G2, desenvolvido por Gardner (1994), para uso em bovinos e humanos; 2) P1, associado com *Blastocyst Medium*, desenvolvido por Bertheussen et al. (1997) para uso em humanos. Os meios sequenciais mimetizam com mais exatidão o ambiente do trato reprodutivo feminino, pois *in vivo* o embrião passa por constantes mudanças de ambiente, com distintas concentrações de nutrientes. Além disso, o embrião também muda sua exigência nutricional de acordo com o estádio do desenvolvimento (Gardner & Lane, 1999). Altas concentrações de glicose e aminoácidos essenciais são benéficos para mórulas e blastocistos, e extremamente prejudiciais para a fase entre duas e oito células.

Contrariamente, as baixas concentrações de glicose exigida no início do desenvolvimento são extremamente prejudiciais para mórulas e blastocistos, pois quando estes são cultivados em condições de baixa glicose, produzem menor taxa de desenvolvimento fetal (Gardner & Lane, 1996). Shizari et al., (2009) demonstraram que diferentes meios de cultivo podem alterar a crio-tolerância dos embriões produzidos, mesmo sem afetar a taxa de produção de embriões. Wan et al., (2009) trabalhando com embriões ovinos, também observaram efeito dos meios de cultivo na qualidade dos embriões, tendo encontrado uma maior taxa de apoptose quando os embriões eram cultivados na presença de soro fetal bovino.

A partir de tais considerações, constata-se a maior viabilidade dos meios sequenciais, apesar de uma lacuna na literatura quanto à aplicação desses meios em larga escala e com dados de gestação.

Além da importância dos meios utilizados, o desenvolvimento embrionário também é bastante dependente das demais condições de cultivo, particularmente a atmosfera gasosa.

5.1. Atmosfera Gasosa

Vários trabalhos foram feitos procurando o efeito benéfico da redução de oxigênio no cultivo de embriões mamíferos. Nagao et al., (1994) e Alberio et al., (1998) constataram uma maior taxa de produção e uma melhor qualidade embrionária quando o cultivo foi realizado com 5% de O_2. Posteriormente, foi demonstrado que a concentração de O_2 na tuba uterina de mamíferos situa-se na faixa de 5 a 8% (Gardner, 1999).

O mecanismo mais aceito pelo qual a concentração de oxigênio influencia o desenvolvimento embrionário refere-se à prevenção de formação de radicais livres, os quais causariam a peroxidação dos lipídios contidos nos embriões, resultando em danos celulares tanto em embrioes murinos (Nars-Esfahani et al., 1992) quanto em bovinos (Nagao et al., 2008).

O pH do fluido obtido do trato reprodutivo de mamíferos varia entre 7,1 e 7,3 na fase estrogênica e de 7,5 a 8,0 na fase luteal (Gardner & Lane, 1999). Essas variações estão diretamente relacionadas com a concentração de bicarbonato no fluido útero-tubárico, assim como nos meios de cultivo. Por meio de um sistema ácido carbônico e bicarbonato, quanto maior a concentração de CO_2, maior será a acidez do meio. Para se obter o pH desejado, em torno de 7,4, necessita-se de uma concentração de 2,2 mg/mL de bicarbonato e entre 5,0 e 6,0% de CO_2 (Palma, 2001). A exposição de zigotos de 2 células a um pH elevado reduz significativamente sua capacidade de desenvolver até blastocisto, demonstrando a necessidade e se evitar as flutuações do pH do meio durante a manipulação e cultura.

A interação entre meio de cultivo e atmosfera gasosa se apresenta como um item altamente estratégico no atual patamar da PIVE, principalmente considerando-se perspectivas de modular tais aspectos sob condições de longos períodos de transporte.

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Objetivos

Gerais

- Analisar dados de campo da produção *in vitro* de embriões *Bos indicus*;
- Testar estratégias capazes de aumentar a eficiência da produção de embriões em larga escala

Específicos

- Analisar dados de cinco anos de aspiração folicular, produção *in vitro* de embriões e taxas de prenhez a partir de doadoras Nelore;
- Comparar produção de embriões pelos métodos *in vivo* e *in vitro* e taxas de prenhez a partir das mesmas doadoras Nelore;
- Analisar um programa de larga escala de produção *in vitro* de embriões com sêmen sexado, incluindo transferência de embriões em tempo fixo, a partir de doadoras *Bos taurus* (Holandês), *Bos indicus* (Gir) e *indicus-taurus* (Girolanda), com a finalização do desenvolvimento embrionário durante longos períodos de transporte.
- Verificar os efeitos de diferentes meios de cultivo e condições de atmosfera de oxigênio na produção *in vitro* de embriões Nelore, e nas subsequentes taxas de prenhez.

Capítulo 2

Oocyte yield, *in vitro* embryo production and pregnancy rates from a large scale commercial program of Nelore (*Bos indicus*) donors

Artigo submetido ao periódico *Theriogenology*, em fase de correção, com duas recomendações para publicação.

Oocyte yield, *in vitro* embryo production and pregnancy rates from a large scale commercial program of Nelore (*Bos indicus*) donors

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Abstract

Despite Brazil has been a leader country on embryos produced *in vitro*, most of the available information about that comes from field reports with speculative numbers. For a real understanding about this situation, we collected data during five years from a commercial embryo centre. We analyzed 1,504 ovum pick up / *in vitro* production (OPU/IVP) performed on Nelore (*Bos indicus*) donors (n=420) without hormone stimulation or follicular wave control. All donors were submitted to OPU at least one and no more than 23 times (3.6 on average), without a specific schedule, respecting only a minimum interval of 15 days between procedures. During five years, 27,966 oocytes were obtained, being 22,809 (81.6%) considered viable resulting in 7,709 transferred embryos. All embryos were immediately transferred producing 2,579 pregnancies (33.4%) at Day 30 and 2,307 (29.9%) at Day 60 ($P<0.05$). The average of viable oocytes by OPU session was 15.2, which resulted in an average of 5.1 embryos and 1.5 pregnancies per OPU-IVP procedure. Considering the donors (n=5) with the highest average of oocyte yield, it was obtained 2,869 oocytes and 1,004 viable embryos after 75 OPU-IVP, being the following averages per procedure: 31.6 viable oocytes, 13.4 viable embryos and 5.8 pregnancies. These data show general means of oocytes quite lower than previous reports/abstracts from Nelore and individual variation on oocyte production seems to be very high in this breed.

Keywords: In Vitro fertilization, Ovum pick up, Embryo, Cattle, Nelore, *Bos indicus*

1. Introduction

Brazil has been considered as the first country in the world on embryos obtained by OPU/IVP [1]. Despite the economic aspects involved with the embryo industry, this particular situation on Brazilian herd is not completely understood. As we have recently described [2], some aspects may be considered to explain the expressive embryo production by *in vitro* method in this country. Nelore (*Bos indicus*) is the breed that represents around 80% of Brazilian herd (approximately 200 million of animals), and values for animals of this beef breed has been very high since the end of 1990's. In the same period, the *in vitro* method for embryo production presented a wide expansion by private companies, being this activity naturally beneficiated by a particular aspect of Nelore females: the high production of oocytes.

Bos indicus cows normally present a larger numbers of ovarian follicles when compared to *B. taurus* breeds, with reports in the literature showing averages ranging from 18 to 25 recovered oocytes per OPU session [3-5], but these data are mostly described in abstracts or reports, being difficult to provide a better comprehension of reproductive particularities of Nelore donors.

Comparing females from *Bos indicus* and *Bos taurus* breeds, *B. indicus* donors present more follicular waves [6,7] than *B. taurus* females and also a greater number of follicles < 5 mm per wave [8], being the follicle size an important fact to improve efficiency of oocyte recovery [9]. Other reproductive aspects, like LH surge, seem to be quite peculiar on *B. indicus* females [10] as well as different patterns for hormonal metabolism [11]. However, the physiologic aspects involved with number and size of follicles in Nelore are not established yet.

Comparing the same Nelore donors submitted alternatively to MOET and OPU/IVP, we recently demonstrated a clear advantage for using the *in vitro* method for embryo production, considering the higher number of pregnancies by period of time [2]. However, these data were obtained with only 30 donors and other situation of *in vitro* embryo production in Nelore should be expected by considering a larger number of donors during a long period of time.

The aim of this study was to describe five years of *in vitro* production of Nelore donors, considering oocyte yield, embryo production, pregnancy rates and embryonic mortality. Data were collected from the commercial embryo centre *In vitro* Brasil.

2. Materials and Methods

2.1. Local

The study is a data collect from the embryo center *In vitro* Brasil., located in Mogi Mirim, São Paulo State, from January 2001 to May 2006. All animals were part of the commercial production of *in vitro* embryos from this company.

2.2. Animals

Non-pregnant, healthy, non-lactating cycling Nelore females (n=420) of high genetic value were used as oocyte donors. The average body score condition were 3.1 ± 1.1 (from 2.8 to 4.0; scale 0 to 5). Multiparous cows (n=298) and heifers (n=122) were used, with ages ranging from 20 to 144 months (48 ± 27 mo). All animals were evaluated by transrectal palpation and ultrasonography before each procedure. All

animals were kept on pasture and mineral supplementation. There was no hormone stimulation or follicular wave control. Also, there was not a specific schedule for OPU; it was only performed according to customer's solicitation. A minimum of 15 days was established between follicular aspirations. Each donor was submitted on average to 3.6 procedures of follicular aspiration, being at least one and no more than 23, performing a total of 1,504 OPU sessions.

Crossbreed heifers (16 to 23 mo old) were used as recipients. Criteria for choosing recipients included normal ovarian cycles, appropriate body condition, and to be free of diseases. Selected animals with a detectable CL were given 500 μg cloprostenol im (Ciosin, Schering-Coopers, Cotia, São Paulo, Brazil) to induce estrus. Those with a well-developed CL 6 to 8 d after standing estrus were used as recipients.

2.3. Follicle aspiration

Previously described procedures were used for follicular aspiration [9,12]. Briefly, each visible follicle 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 1/2'' hypodermic needle (Becton Dickinson, Curitiba, Parana, Brazil) connected to a 50 mL conical tube (Corning, Acton, MA, USA) via a silicon tubing (0.8 m; 2 mm id). Aspiration was performed using a vacuum pump (Cook Veterinary Products, Queensland, Australia) with a negative pressure of 10-12 mL of water/min. 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, São Paulo, Brazil) and 10,000 IU/L sodium heparin (Sigma H-3149).

2.4. *In vitro* embryo production

Embryos were produced as previously described [2]. Briefly, immediately after recovery, the aspirated material was filtered through an EmCon filter with phosphate buffered saline (PBS-Nutricell, Campinas, São Paulo, Brazil) supplemented with 5% FCS. Cumulus oocyte complexes were classified as follows: 1, more than three layers of compact cumulus cells; 2, at least one layer of cumulus cells; 3, denuded; and 4, atretic, with dark cumulus cells and signs of cytoplasmic degeneration [9]. After evaluation, only atretic oocytes were discarded. Prior to *in vitro* maturation (IVM), Cumulus Oocyte Complexes (COC's) were washed three times in TCM-199 hepes (Gibco Life Technologies, Grand Island, NY, USA), supplemented with 10% FCS and 50 μg gentamycin sulfate, and once in bicarbonate TCM-199 (Gibco Life Technologies) supplemented with 10% FCS, 5 μg luteinizing hormone (LH- Ayerst, Rouses Point, NY, USA), 0.5 μg follicle stimulating hormone (FSH- Folltropin, Vetrepharm, Belleville, ON, Canada), 1 μg estradiol (Estradiol 17β- Sigma E-8875), 2.2 μg pyruvate (Sigma P-4562) ,and 50 μg gentamycin/mL of medium. The COC's of each category were separately cultured for 24 h in 100 μL drops of maturation medium under mineral oil (D'Altomare, Santo Amaro, São Paulo, Brazil) at 39 °C and 5% CO_2 in air [13,14]. Only frozen semen from sires of known fertility was used. For IVF, two straws were thawed for 20 s in a 35 °C water bath. Semen was then washed and centrifuged through a 90% - 45% Percoll gradient at 200 x g for 30 min. Sperm was capacitated using heparin (30 μg/mL) and motility was stimulated by the addition of 40μL/mL of PHE

[15]. Concentration was adjusted to 25 x 10^6 live sperm/mL, and each fertilization drop received 4 μL of sperm (final concentration 100 x 10^3 cells per drop) [9]. After maturation, COC's were washed three times in pre-fertilization medium TCM 199 supplemented with 25 mM hepes and 0.3% BSA (Sigma A-9647), and once in TALP fertilization medium supplemented with 10 μg/mL heparin and 160 μL PHE solution [16,17]. After IVF, the oocytes and the *Cumulus* cells were transferred to drops of 100 μL of culture medium of embryos, a modified oviduct synthetic fluid, the SOFaa BSA, containing 8 mg / mL BSA (Sigma, USA) free of fatty acid and 1 mM glutamine, under the same conditions of temperature and gaseous atmosphere of IVF, staying for 5 to 8 days in this medium. The osmolarity was maintained at 270 - 280 mOsmol and the pH was 7.4. The blastocyst rate was obtained from the total of matured oocytes.

Embryos were classified according to IETS criteria [18], and only grades I and II embryos were used. Morula and blastocyst stage embryos from Days 6 to 7.5 post insemination were individually transferred to each recipient.

2.5. Pregnancy evaluation

Between Days 23 to 28 (hereafter designated Day 30) after embryo transfer, the pregnancy status of recipients was determined by ultrasound evaluation. Recipients diagnosed pregnant were re-evaluated by ultrasound 30 to 35 d later (designated Day 60) to confirm pregnancy.

2.6. Statiscal Analysis

The data were normally distributed and were calculated using an analysis of variance (ANOVA), with differences between groups compared by Tukey Test. Tests were performed with $p \leq 0.05$.

3. Results

A total of 27,966 oocytes were recovered from 420 females in 1,504 sessions of follicular aspiration. Considering only viable oocytes (22,809) we obtained a developmental rate of 33.8 % of embryos, which resulted in 2,579 pregnancies at Day 30 and 272 (3.5%) pregnancy losses until Day 60, as showed on Table 1.

Considering stage of embryonic development, most of the pregnancies (58.6%) were obtained from expanded blastocyst and there was no difference on embryonic mortality according to embryonic phase (Table 2).

From the 420 females evaluated, we took the donors (n=5) with the highest total number of oocyte yield. These cows were subjected to 75 sessions of follicular aspiration, of which 2,869 oocytes were obtained, being 2,372 of them considered viable, producing 1004 embryos and 438 pregnancies (Table 3).

4. Discussion

We present here a data collection of an expressive amount of *in vitro* embryo production from Nelore (B. *indicus*) donors. Despite this breed has been closely involved with the fact of Brazil stays on the top of the world for embryos produced *in vitro*, there was little information in the literature about oocyte yield, embryos and pregnancy in large scale. Most of the numbers come from abstracts, reports, or articles

with few animals. We highlight two aspects in this article: i) general average of oocytes and IVF embryos seems to be lower than previously reported; ii) individual variation is very expressive and has contribute to create the speculative impression of a higher average of oocytes.

According to Thibier [3], in a report of data from personal communication cited by Brazilian colleagues, in 2003 Brazil made 11,000 follicular aspiration procedures, retrieving 267,000 oocytes, with an average of 25 oocytes per session of follicular aspiration. In the same year, the world production of *in vitro* embryos reached 106,220 embryos, being 60,000 (56.5%) produced in Brazil. The aspect of high production of oocytes from Brazilian donors has always been considered to justify the amount of *in vitro* embryo from Brazil, considering this country is repeatedly mentioned at Data Retrieval from IETS [3]. However, the speculated average initially reported – 25 oocytes per procedure – was higher than the average of 18 oocytes that we obtained in the present work and the 18.4 we described earlier [19]. Interestingly, our team also related means as high as 25, like 24 [20] and 25 [2] very close to the number reported by Thibier [3]. Taking all together, we believe in a strong individual variation in number of follicles and consequently oocyte recovery in Nelore cows. Despite individual variation has been previously reported for European breeds, with averages of oocyte ranging from 1.9 to 6.1 oocytes/session [21], the magnitude of number of oocytes is clearly more expressive for *B. indicus* donors. This is particularly noted when considering the average of the five donors with the highest oocyte yield (Table 3). Analyzing our data and field reports, it is clear that cows producing more oocytes have been preferentially selected for OPU over the time, contributing to a super estimated average to the breed. Despite this aspect, the average reported in this article - 18.6 - is clearly higher than reports for *B. taurus*, as 4.9 [22] and 9.9 [23].

It is not clear why *B. indicus* donors may produce much more oocytes than *B. taurus* females. There was no difference in the number of preantral (primordial, primary and secondary) follicles in ovaries of fetuses and heifers of females *B. indicus* and *B. taurus*, suggesting that the total number of follicles in the ovaries is not the answer for this difference on oocyte yield (manuscript in preparation). It should be possible to expect a lower rate of follicular atresia for *B. indicus*, so it would provide more follicles to be aspirated. Another hypothesis to be considered is the controversial point of the follicular renewal [24,25], but this new concept needs to be better accepted before being considered [26,27].

It is particularly intriguing how some Nelore donors may produce more than 60 oocytes on average after several OPU procedures [2] and also reports of hundreds of oocytes per OPU session [28]. About this individual variation of oocyte yield in Nelore donors, some genes as GDF9, FGF8, BMP15 and BMP15 receptor have been analyzed. The FGF8 seems to be related with an increasing of 2.26±1.08 oocytes on average and a possible variation of 7.36 ± 1.12 when all genes were considered together [29].

The variation on oocyte yield obviously reflects on the final number of embryos produced. Our general average of embryos /OPU-IVP was 5.1, very close of the five to six transferable embryos obtained from MOET for *B. taurus* breeds [30], and little higher than average of 4.3 per OPU/IVP for Holstein cows [23]. Considering the highest averages from top five donors, we obtained 13.4 embryos OPU /IVP procedure, a similar result from another study with Nelore donors of high performance [2]. For these animals with high performance, there is no doubt about the advantages for *in vitro* method, when compared to MOET. But also considering the lower mean of *in vitro* embryos, e.g., 5.1, it is important to consider we did not use hormonal stimulation and follicular wave control. These aspects, added by the short interval between procedures –

15 days – may explain why OPU/IVP has been preferred in detriment of MOET in the Brazilian herd for *B. indicus* donors. However, again the importance of individual variation seems to be very critical for Nelore, since there are reports of means as high as 9.3 and 10 embryos per uterine flushing [31].

The pregnancy rate obtained at D30 in our study was 33.4 %, very close to 33.5% that we recently described for similar circumstances in the same embryo center [2]. We believe this pregnancy rate represents a real situation for embryos obtained by OPU/IVP in Brazil. Considering that only embryos of quality 1 and 2 were transferred, it is a quite low pregnancy rate, but in Brazil there is a chronic problem with recipients. Because of the high number of embryos that have been generated in the last years and the attractive values of pregnancies for Nelore animals, there is a continuing deficit for recipients. Corpus luteum with poor quality and critical body condition score are common problems, and these situation may certainly be considered to explain this low pregnancy rate. We obtained 10% more pregnancies (43.6) with embryos from the top five donors (Table 3). It is not clear why this situation occurred, since all other donors and recipients were maintained under the same conditions, as well as the laboratory conditions. One hypothesis should be a better quality of the chosen embryos to be transferred, as a natural consequence because of the larger number of embryos produced. It has been demonstrated the importance of the oocyte for the embryo quality [32], and we also hypothesize that these best donors could present oocytes with better quality.

Regarding the development stage of embryos transferred analyzed, the general pregnancy rate were not different ($P< 0.05$). Despite Looney et al. [33] showed a better result of pregnancy rate for hatching blastocyst, our results are consistent with data from Hasler et al. [34] who reported no difference between the embryo development stage

and period of culture (D7 or D8). However, because we could not find similar works with *B. indicus* embryos, we are comparing our results only with data of embryos from European breed donors, and it is important to consider there are differences on morphology and organelles between embryos *B. taurus* and *B. indicus* [35].

All viable embryos were immediately transferred, because an efficient protocol for cryopreservation for *B. indicus* embryos produced *in vitro* is not available yet. It has been reported a higher susceptibility of *B. indicus* embryos for the freezing process, at least for embryos obtained *in vivo* [36] and the same was reported for *in vitro* embryos [2]. Considering the increasing interest of other countries for acquiring *B. indicus* genetics [37], the discovery of an appropriate protocol for cryopreservation of *B. indicus* produced *in vitro* will certainly contribute to not only to the embryo industry in Brazil but for other countries as well.

Considering embryo culture, our present data were obtained from a similar system that we recently described [2], except for the use of SOFaa instead of Menezo B2. There is an important influence of medium on embryonic and fetal development for *in vitro* embryos [38]. However, because most of the pregnancies were sent to different farms of the customers, we could not properly evaluate embryonic and fetal problems, as congenital malformations, dystocia, Large Offspring Syndrome, etc. However, very few reports of alterations were collected, suggesting a regular situation of offspring in general.

Finally, this study demonstrated, from an expressive amount of data, a clear high oocyte yield from Nelore donors, but general averages were lower than previous personal reports and abstracts. Individual variation seems to be very important, and it is probably a reason for discrepancies when studies with small number of animals are

analyzed. We emphasize the importance of a better understanding on this natural capacity of *B. indicus* females to produce oocytes for *in vitro* embryo production.

5. Acknowledgments

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6. References

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Table 1. Means of oocytes, embryos, pregnancies and embryonic death obtained from OPU/IVP procedures (n=1,504) in Nelore donors (n=420).

	%	Mean /OPU-IVP Session
Viable oocytes (viable/total)	81.6 (22,809/27,966)	15.2
Viable embryos (embryos/viable oocytes)	33.8 (7,709/22,809)	5.1
Pregnancies on D30	33.4 (2,579/7,709)	1.7
Pregnancies on D60	29.9 (2,307/7,709)	1.5
Embrionic death (Pregnancy loss/ transferred embryos)	3.5 (272/7,709)	0.2

* Difference between pregnancies on D30 and D60.

Table 2. Proportion of each developmental embryonic stage of transferred embryos on pregnancy at 30 and 60 days. Data obtained from Nelore donors (n=420) in 1,504 OPU/IVP procedures.

Developmental stage	% (developmental stage / transferred embryos)	Pregnancy D30 % (pregnancies / transferred embryos)	Pregnancy D60 % (pregnancies / transferred embryos)
Morula	6.5 (500/7,709)	27.8[a] (139/500)	24.6[a] (123/500)
Early Blastocyst	11.3 (869/7,709)	28.4[a] (247/869)	25.4[a] (221/869)
Blastocyst	23.6 (1,821/7,709)	33.6[a] (612/1,821)	29.8[a] (542/1,821)
Expanded Blastocyst	58.6 (4,519/7,709)	35.0[a] (1,581/4,519)	31.4[a] (1,421/4,519)
Total	100 7,709/7,709	33.4[a] (2,579/7,709)	29.9[a] (2,307/7,709)

In the same row, equal letters do not differ significantly ($P<0.05$).

Capítulo 3

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Comparison of embryo yield and pregnancy rate between *in vivo* and *in vitro* methods in the same Nelore (*Bos indicus*) donor cows

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Abstract

To investigate why the preferred means to produce bovine embryos in Brazil has changed from *in vivo* to *in vitro*, we compared these two approaches in the same Nelore cows (n=30) and assessed total embryo production and pregnancy rates. Without a specific schedule, all cows were subjected to ultrasound-guided ovum pick up (OPU)/*in vitro* production (IVP) and MOET, with intervals ranging from 15 to 45 d between procedures, respectively. To produce *in vivo* embryos, cows were superovulated and embryos were recovered nonsurgically from 1 to 3 times (1.4 ± 0.6), whereas OPU/IVP was repeated from 1 to 5 times (3.2 ± 1.2) in each donor cow during a 12-mo period. Embryos obtained from both methods were transferred to crossbred heifers. On average, 25.6 ± 15.3 immature oocytes were collected per OPU attempt. The average number of embryos produced by OPU/IVP (9.4 ± 5.3) was higher ($P<0.05$) than the MOET method (6.7 ± 3.7). However, pregnancy rates were lower ($P<0.05$) following transfer of IVP (33.5%) versus *in vivo*-derived embryos (41.5%) embryos. Embryonic losses between Days 30 and 60 and fetal sex ratio were similar ($P>0.05$) between *in vivo* and *in vitro*-derived embryos. We concluded that in Nelore cows, with an interval of 15 d between OPU procedures, it was possible to produce more embryos and pregnancies compared to conventional MOET.

Keywords: In vitro fertilization; Ovum pick up; Embryo transfer; Cattle; Nelore

1. Introduction

In a recent report, Brazil was ranked as the second country in the world for the total number of embryos produced by MOET, and is at the top of the list for embryos produced by OPU/IVP [1]. There are several reasons for the growing popularity of the OPU/IVP method for producing bovine embryos in this country. First, the commercial value of Nelore (*Bos indicus*), the most popular breed of cattle in Brazil, has been very high during the past 10 y. Second, there is increasing interest in other tropical countries to acquire Nelore genetics from Brazil [2]. Furthermore, Nelore cows normally have a larger numbers of ovarian follicles compared to *B. taurus* breeds, with averages ranging from 18 to 25 recovered oocytes per OPU session [3-5]. This large population of follicles in this beef breed is present without the use of exogenous hormones or synchronization protocols. Consequently, more than 40 private IVP laboratories are currently operating in Brazil [6].

There are physiological similarities, as well as differences, between *Bos indicus* and *Bos taurus* breeds [7]. For instance, Nelore cows are similar to other *B. indicus* and *B. taurus* breeds when comparing the average embryo production by MOET [8,9]. However, *Bos indicus* breeds tend to have more follicular waves [10,11] and a larger population of small follicles (< 5 mm) compared to *B. taurus* breeds [12]. It was also reported that Nelore cows have smaller dominant follicles and CL, and shorter estrus than *B. taurus* breeds [13,14]. Despite the physiological importance of these differences, the high number of oocytes obtained via OPU seems to be a unique characteristic of Nelore cows.

Little information is available in the literature regarding oocyte recovery, embryo production and particularly pregnancy rates in the Nelore breed. As indicated

by Hansen [15], embryos produced *in vitro* could play a central role in dairy and beef production, but with the exception of Brazil, the use of *in vitro* embryo technologies is still very limited in the cattle industry. A better understanding of all aspects affecting *in vitro* embryo production in the Nelore breed should not only benefit the embryo industry, but also facilitate the application of other reproductive biotechnologies, such as transgenesis and cloning.

The aim of this study was to compare the efficiency of superovulation/embryo recovery versus OPU/IVP, using the same Nelore cows, on embryo production per donor and pregnancy rates over time.

2. Materials and methods

2.1. Animals

Non-pregnant, healthy and cycling Nelore cows (n=30) were used as donors for oocytes and embryos. The donors cows included two heifers (24 mo old) and 28 multiparous cows (41 ± 7 mo old, range, 32 to 58) with 517 ± 13 kg (range, 447 to 631) live weight and average body score condition score of 3.3 ± 0.1 (range, 3.0 to 3.5; scale 0 to 5) [16]. The average postpartum interval was 284 ± 88 (range, 191 to 401 d). All animals were selected on the basis of genetic merit and had regular ovarian activity (based on transrectal palpation and ultrasonography). These cattle were kept on pasture, with mineral supplementation ad libitum. Experiments were conducted over a 12 mo interval, in a commercial embryo production center. All cattle were submitted to both OPU (96 procedures) and embryo recovery (43 procedures). An average of 3.2 ± 1.2 (1 to 5) OPU and 1.4 ± 0.6 (1 to 3) superovulation and embryo recovery procedures were

performed per donor cow. Because the experiments were conducted in a commercial embryo production center, animals were randomly used in OPU/IVP or MOET, without a specific schedule or predetermined sequence. For each donor cow, there was a minimum interval of 15 d between OPU/IVP and also when OPU/IVP was performed before or after MOET. The minimum interval between MOET was 45 d.

Crossbreed heifers (15 to 19 mo old) were used as recipients. Potential recipients were selected for body condition, normal cyclicity, and health status. Those with a detectable CL were given 500 μg cloprostenol im (Ciosin, Schering-Coopers, Cotia, São Paulo, Brazil) to induce estrus. Those with a well-developed CL 6 to 8 d after standing estrus were used as recipients.

2.2. Donor preparation

Before each procedure, feces were removed from the rectum and the perineal area was cleaned with tap water and 70% ethanol. Prior to embryo collection or OPU, each cow received pidural anesthesia, using 7 mL of 2 % lidocaine (Anestésico L, Pearson, São Paulo, São Paulo, Brazil) to decrease peristalsis and discomfort.

2.3. Superovulation

All 30 donor cows received the same treatment, which consisted of an intravaginal progesterone implant (CIDR, Pfizer, Hamilton, New Zealand) and 2 mg estradiol benzoate im (Estrogin, Farmavet, São Paulo, São Paulo, Brazil) on Day 0. Between Days 4 and 7, FSH (Pluset, Serono, Rome, Italy) was administered twice daily in decreasing doses of 133, 100, 65, and 35 IU (total dose, 333 IU). In the afternoon of

Day 6, donors were given 500 μg cloprostenol im (Ciosin, Coopers, São Paulo, São Paulo Brazil) and the progesterone implants were removed 12 h later (morning of Day 7), with AI 36 and 48 h after progesterone implant removal.

2.4. Embryo recovery and transfer

Uterine flushing was performed 7 d after AI and embryos were collected using a two-way Foley catheter passed through the cervix. The catheter's tip was placed in the uterine body, caudally to the external bifurcation of the uterus, and both horns were flushed simultaneously. The uterus was flushed three or four times using 1 L total volume of Dulbecco's Phosphate Buffered Saline (DPBS, Nutricell, Campinas, São Paulo, Brazil). Embryos were collected on a filter, counted and evaluated according to IETS criteria [17]. Embryos graded as 1, 2 and 3 were defined as viable. All viable embryos were individually transferred non-surgically to synchronous recipient heifers.

2.5. Follicle aspiration

Animals were used independent of their estrous cycle stage. Previously described procedures were used for follicular aspiration [18]. Briefly, each visible follicle 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 1/2'' hypodermic needle (Becton Dickinson, Curitiba, Parana, Brazil) connected to a 50 mL conical tube (Corning, Acton, MA, USA) via a silicon tubing (0.8 m; 2 mm id). Aspiration was performed

using a vacuum pump (Cook Veterinary Products, Queensland, Australia) with a negative pressure of 10-12 mL of water/min. 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, São Paulo, Brazil) and 10,000 IU/L sodium heparin (Sigma H-3149).

2.6. *In vitro* embryo production

Immediately after recovery, the aspirated material was filtered through an EmCon filter with phosphate buffered saline (PBS-Nutricell, Campinas, São Paulo, Brazil) supplemented with 5% FCS. Cumulus oocyte complexes were classified as follows: 1, more than three layers of compact cumulus cells; 2, at least one layer of cumulus cells; 3, denuded; and 4, atretic, with dark cumulus cells and signs of cytoplasmic degeneration [18]. After evaluation, only atretic oocytes were discarded. Prior to *in vitro* maturation (IVM), Cumulus Oocyte Complexes (COC's) were washed three times in TCM-199 hepes (Gibco Life Technologies, Grand Island, NY, USA), supplemented with 10% FCS and 50 μg gentamycin sulfate, and once in bicarbonate TCM-199 (Gibco Life Technologies) supplemented with 10% FCS, 5 μg luteinizing hormone (LH- Ayerst, Rouses Point, NY, USA), 0.5 μg follicle stimulating hormone (FSH- Folltropin, Vetrepharm, Belleville, ON, Canada), 1 μg estradiol (Estradiol 17β-Sigma E-8875), 2.2 μg pyruvate (Sigma P-4562) ,and 50 μg gentamycin/mL of medium. The COC's of each category were separately cultured for 24 h in 100 μL drops of maturation medium under mineral oil (D'Altomare, Santo Amaro, São Paulo, Brazil) at 39 °C and 5% CO_2 in air [19, 20]. Only frozen semen from sires of known fertility

was used, and the same sire was used for both *in vivo* and *in vitro* embryo production in each donor. For IVF, two straws were thawed for 20 s in a 35 °C water bath. Semen was then washed and centrifuged through a 90% - 45% Percoll gradient at 200 x g for 30 min. Sperm was capacitated using heparin (30 μg/mL) and motility was stimulated by the addition of 40μL/mL of PHE [21]. Concentration was adjusted to 25 x 10^6 live sperm/mL, and each fertilization drop received 4 μL of sperm (final concentration 100 x 10^3 cells per drop) [18]. After maturation, COC's were washed three times in pre-fertilization medium TCM 199 supplemented with 25 mM hepes and 0.3% BSA (Sigma A-9647), and once in TALP fertilization medium supplemented with 10 μg/mL heparin and 160 μL PHE solution [22,23]. Presumptive zygotes had their cumulus cells stripped off (by pipetting) 20 h after insemination and were washed three times in pre-fertilization medium and once in Menezo's B2 medium (Pharmascience, Paris, France), supplemented with 10% FCS. Embryos were cultured in 50 μL drops of medium at 38.5 °C and 5% CO_2 in air for 48 to 72 h, when 50 μL of fresh development medium was added [24].

Embryos were classified according to IETS criteria [17], and only grades I and II embryos were used. Morula and blastocyst stage embryos from Days 6 to 7.5 post insemination were individually transferred to each recipient.

2.7. Pregnancy evaluation

Between Days 23 to 28 (hereafter designated Day 30) after embryo transfer, the pregnancy status of recipients was determined by ultrasound evaluation. Recipients diagnosed pregnant were re-evaluated by ultrasound 30 to 35 d later (designated Day 60) to confirm both pregnancy and fetal sex.

2.8. Statistical analysis

Statistical analysis was performed using the software Minitab 14 [25]. Means of procedures and embryos were compared by ANOVA, with differences analyzed by Tukey's test. Pregnancy rates were evaluated by Chi square. For all analyses, $P \leq 0.05$ was considered significant.

3. Results

A total of 2,463 oocytes were collected in 96 OPU sessions performed in the 30 donor cows. On average, 25.6 ± 15.3 oocytes were obtained per procedure and 89.3% (2200/2463) were considered viable.

Oocyte production from 10 randomly selected donor cows is shown (Table 1). In some donors, the average number of oocytes produced per OPU was consistently high (> 30; A and H) or low (<14; J), whereas others (E) had substantial variation (from 5 to 40 oocytes) between OPUs. That the schedule between OPU/IVP and MOET was not predetermined and the intervals to obtain the oocytes were not controlled, these data must be cautiously interpreted.

Individual data of embryo production and pregnancy rates for both methods are shown (Table 2). Some cows (e.g., Donor III) with the usual average of embryos for the *in vivo* method (i.e, 6.5 per flushing) produced on average approximately four times more embryos (24.5) with the OPU/IVP method. Conversely, Donor VI produced an average of 5.3 embryos per uterine flushing and only 3.8 embryos per OPU/IVP session.

From 2200 oocytes submitted to IVF procedures 910 embryos (41.4%) were produced and transferred. Non-surgical collections of 43 superovulated donors yielded 376 embryo/ova and 289 viable embryos. This group of 30 Nelore cows produced an average of 9.4 ± 5.3 embryos per IVP session and 6.7 ± 3.7 embryos using the conventional embryo collection method ($P<0.05$). Mean number of procedures per animal were 3.2±1.2 and 1.4±0.6, for *in vitro* and *in vivo* systems, respectively.

Pregnancy rates were different between *in vivo* and IVP systems at Day 30 after embryo transfer (45.6 vs 37.4%, respectively) and at Day 60 (41.5 and 33.5%). For these systems, embryonic losses between Days 30 and 60 were 8.9 and 10.5% ($P=0.12$). Embryos produced by IVP resulted in 52.8% male and 47.5% female calves, whereas *in vivo*-produced embryos resulted in exactly 50% male and female calves (Table 3).

4. Discussion

To our knowledge, this is the first study comparing OPU/IVP and MOET in the same Nelore cows. These data should be of interest to the embryo production industry because they indicate that over the same interval, more embryos can be produced using *in vitro* compared to *in vivo* methods. Our findings were consistent with Brazil as the leading country in the world for number of embryos produced by OPU/IVP [1].

This study also confirmed the high oocyte production from Nelore cows, a unique aspect of this *Bos indicus* breed. The number of oocytes produced per session (25.6) seemed much higher than results reported by Hasler et al. [25] and Bousquet el al., [26] in *Bos taurus* breeds (average collection of 4.9 and 9.9 oocytes per OPU session, respectively). Furthermore, our previous studies with Holstein cows resulted in 4.1 oocytes collected per OPU session [18], which represented approximately 16% of

the number obtained herein with Nelore cows. The average in the present work (25 oocytes/session) was similar to another report from our team [27].

Zebu cows have more follicles per wave in comparison to European breeds [28] but it is not clear if Nelore females have a larger follicular population, or just more recruited follicles per wave. Another possible aspect to be considered regarding the higher number of oocytes in Nelore is the predominance of three follicular waves per cycle, with reports of four waves [10,11], which generally exceeded the two or three waves described in Holstein cows [29,30]. Considering a better efficiency in oocyte collection when aspirating small follicles [18], a higher number of follicular waves results in a greater probability of finding small follicles in animals with three versus two follicular waves. In agreement with this are previous studies reporting the existence of a larger population of follicles <5 mm in diameter in *Bos indicus* compared to *Bos taurus* heifers [12]. We speculate that this contributes to the high number of oocytes recovered from Nelore cows.

The total number of follicles in Nelore ovaries remains to be better established. Apparently, the number of primordial and primary follicles in Nelore ovaries is similar to European breeds, but there are only a few reports describing this aspect [31]. Perhaps Nelore females have a larger number of germinal cells at the fetal stage or even a longer period of mitosis during the formation of oogonia. The controversial hypothesis of follicular renewal [32,33] seems plausible, and was previously mentioned in the IVF context [26]. However, this new concept needs to be better established and receive greater acceptance before being considered [34,35]. That some Nelore cows produced more than 200 oocytes in one OPU procedure without receiving any kind of hormonal stimulation is difficult to explain. For instance, one OPU session performed in a Nelore cow by our team resulted in 251 oocytes (Seneda MM, unpublished data), with similar

findings reported by several practitioners. Our current studies investigating pre-antral follicular population in Nelore females of different ages are expected to provide additional insights to explain these striking findings.

Consistent individual variation in oocyte production seemed to occur in Nelore cows . Indeed, such individual variation is frequently reported by practitioners and in field studies, there were specific donor cows producing a remarkably large number of oocytes, whereas other cattle, especially old cows, had very poor oocyte production [5]. That cows producing more oocytes are preferentially selected for OPU could also contribute to the superior rates of oocyte yield in Nelore cows.

In the present study, by performing only to five OPU per donor, it was not possible to evaluate if the large oocyte production per donor cow was consistently maintained over a prolonged interval. Attempts to obtain this information from other embryo production centers in Brazil were unsuccessful. However, based on only anecdotal information, it seems rare to find a donor cow that maintains excellent oocyte production for a prolonged interval under an OPU/IVF program. Due to contractual issues in the commercial embryo production centers, these high production cows are frequently used for few (i.e. one or two) OPU/IVP sessions, because the expected number of pregnancies is quickly achieved.

Regarding the MOET method, an average of five to six transferable embryos is well accepted for several European breeds [9,36]. In the present work, we obtained 6.7 viable embryos per recovery, which was very close to the average of five to six previously reported with Nelore cows in Brazil [8,37]. Under Brazilian conditions, a small variation was observed in other Zebu breeds, with 7.3 for Brahman, 4.1 for Gir, and 5.7 for Guzera [8]. We inferred that there was less variation with the MOET compared to the *in vitro* production method in Nelore cows, because similar means have

been described when comparing Nelore with other European and Indian breeds on classical ET programs.

A comparison of OPU/IVP and MOET methods was previously conducted in Holstein cows, with averages of 4.3 and 4.7 embryos, respectively [27]. In the present study, some cattle had good potential for OPU/IVP because they produced very high averages of oocytes and consequently the number of embryos and pregnancies were also high. Conversely, cows that produced lower averages of oocytes were less suitable for OPU/VIP; nevertheless they can still produce acceptable means of embryos by MOET. Perhaps it is possible to select Nelore donor cows that would better respond to *in vivo* or *in vitro* embryo production methods.

The pregnancy rate obtained in our study with IVP embryos (33.5%) seemed relatively lower compared than the rate reported in Holstein cows [26]. That only quality 1 and 2 embryos were transferred, we inferred that the recipients were a major cause of poor fertility. Due to the high value of pregnancies from top genetic quality Nelore cows, there has been increased demand and excessive value of cross-breed heifers to be used as embryo recipients [2]. Also, donors producing high number of embryos in one OPU/IVP procedure have imposed some difficulties for acquiring good recipient females at the appropriate time. This has probably influenced not only the pregnancy rate, but also the embryonic mortality rate.

As described previously [36], there are several differences between embryos generated *in vivo* and *in vitro*. In Holstein embryos, there was a higher degree of apoptosis at the blastocyst stage when they were obtained by the *in vitro* compared to the *in vivo* method [38]. Comparisons of embryos obtained by both methods from donor cows of European breeds showed higher rates of embryonic death in embryos produced *in vitro* [39]. Based on the current findings, there was an advantage of the *in vivo* over

the *in vitro* method in terms of embryo quality, based on morphology and pregnancy rates (Table 3).

A big challenge for the embryo industry in Brazil is cryopreservation of Nelore embryos. Apparently there is a higher susceptibility of *B. indicus* embryos for freezing, as described for embryos obtained *in vivo* [40,41]. Pregnancy rates from *in vitro* Nelore embryos were usually very low and/or highly variable (personal communication with several practitioners). Consequently, only fresh embryos were used in the current study. Due to the difficulty in accessing suitable recipients, there is a large impetus to find an efficient method for freezing *B. indicus* embryos, but so far it is not well established. Although IVP was generally regarded as a mature technology [6], the difficult to successfully cryopreserve IVP Nelore embryos requires further investigation.

The synthetic oviduct fluid (SOF) culture medium has been largely used in many IVP systems worldwide [6]. However, we used Menezo B2 medium because it has provided better results on embryo production in previous experiments performed in the embryo production center (Pontes J.H., unpublished data). A very important consideration regarding embryo culture medium is evaluation of fetal and postnatal development (after embryo transfer). Unfortuately, in the present study, it was not possible to obtain all the information to evaluate those parameters, because pregnant recipients were sent to various farms. The occurrence of Large Offspring Syndrome [42], or other problems such as abortions, dystocia or congenital malformations, were reported by very few farmers. Furthermore, despite the tendency of an altered sex ratio for animals generated by the *in vitro* method [36] our production system resulted in the proportion 1:1 for males and females.

The efficiency of embryo technologies depends on the costs to produce a live calf [36]. That this studies was conducted under commercial conditions, some

economical issues are discussed. The cost of each embryo produced by OPU/IVP was approximately 1.5 fold higher compared to the MOET method, excluding the semen cost. Despite this difference, the OPU/IVP method offered other advantages that must be considered. For instance, embryos generated by the *in vitro* method have preferentially been produced using the most expensive semen. In such situations, a single dose was used to fertilize all the oocytes collected from more than 10 cows. It is also important to consider that the OPU/IVF method did not require any hormonal treatment or follicular stimulation. Finally, the OPU/IVP method can result in more pregnancies over a period of time. The use of the *in vivo* approach with embryo collections at 45-d intervals would produce approximately three pregnancies per month. Therefore, if compared over 1-y period, the OPU/IVP approach would result in many more pregnancies than the MOET method. Adding to this the high market value of Nelore breed in Brazil it is easy to understand why the time has been considered as a key factor for choosing the method of embryo production.

In conclusion, our findings confirmed that Nelore cows produced high numbers of oocytes per OPU session, which seemed to be unique to this breed. The high oocyte production in this breed is an important component to explain how OPU/IVP became the preferred option for embryo production in Brazil. However, other factors, including cattle marketing and predominance of the Nelore breed, have also accounted for the expansion in the use of this technology for embryo production in Brazil.

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Table 1. Example of individual production of oocytes in Nelore donor cows. Data represent the number of oocytes obtained in 4 or 5 OPU procedures from 10 donors (A - J), randomly selected from a group of 30 animals. Intervals between follicular aspirations were at least 15 d.

	Donors (A – J)									
	Oocyte production (n)									
OPU	**A**	**B**	**C**	**D**	**E**	**F**	**G**	**H**	**I**	**J**
1	46	37	11	22	20	13	19	61	44	14
2	48	23	35	60	5	15	18	75	40	13
3	33	24	13	68	40	21	15	65	23	15
4	39	25	13	46	17	21	34	46	28	13
5	30	19	8	-	32	-	-	-	-	-

Table 2. Variation in embryo production among 6 Nelore cows (I – VI), comparing *in vitro* (OPU / IVF) versus *in vivo* (MOET) procedures.

	Donors (I – VI)					
	I	II	III	IV	V	VI
Total no. OPU IVF	5	5	4	4	5	5
Mean no. oocytes/collection	36.6	25.6	49	29.7	22.8	16
Mean no. viable oocytes/collection	32.2	23.4	45.2	26	19.6	14.4
Mean no. embryos/OPU IVF	15.6	10.4	24.1	10.3	6.8	3.8
Mean no. pregnancies/OPU IVF	4.8	2.8	9.25	4.3	2.2	1
Total no. MOET	2	3	2	2	2	3
Mean no. embryos/collection	10	4.3	6.5	2	12.5	5.3
Mean no. pregnant/collection	5.5	2	1	1.5	6.5	1.3

Table 3 – Pregnancy rates at Days 30 and 60, embryonic losses, and sex ratios from embryos obtained by *in vivo* or *in vitro* procedures from 30 Nelore cows

Method	Total no. transferred embryos	Pregnancies on Day 30 No. (%)	Pregnancies on Day 60 No. (%)	Embryonic loss No. (%)	Fetal gender diagnosed Male No. (%)	Female No. (%)
In vitro	910	341 (37.4)[a]	305 (33.52)[a]	36 (10.5)	159 (52.5)	144 (47.5)
In vivo	289	132 (45.6)[b]	120 (41.52)[b]	12 (9.0)	60 (50)	60 (50)

[ab]Within a column, means without a common superscript differ (P<0.05)

Capítulo 4

Large - scale *in vitro* embryo production and pregnancy rates from *Bos taurus*, *Bos indicus*, and *indicus-taurus* dairy cows using sexed sperm

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Large-scale *in vitro* embryo production and pregnancy rates from *Bos taurus*, *Bos indicus*, and *indicus-taurus* dairy cows using sexed sperm

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Abstract

We describe here a large-scale commercial program for producing *in vitro* embryos from dairy *Bos taurus*, *Bos indicus,* and *indicus-taurus* donors using sexed sperm. We analyzed data from 5,407 OPU and compared the number of recoverable (n = 90,086), viable (n = 64,826) oocytes and the number of embryos produced *in vitro* (IVP) from Gir (*Bos indicus*), Holstein (*Bos taurus*), 1/4 Holstein x 3/4 Gir, and 1/2 Holstein-Gir crossbreed cows. The pregnancy rates of the cows were also compared. Oocytes were obtained from each donor cow during a 12-mo period using OPU and the procedure was repeated 4 to 7 times (5.7 ± 2.4 times) per donor. On average, 16.7 ± 6.2 oocytes were obtained per OPU procedure and 72.0% were considered viable. The cumulus-oocyte complexes (n = 90,086) obtained were classified according to the presence of cumulus cells and the oocyte cytoplasm aspect (homogeneous or heterogeneous/fragmented). Viable oocytes (n = 64,826) were *in vitro* matured for 24 h at 38.8°C in an atmosphere of 5% CO_2 in air. Frozen-thawed sexed sperm (X-chromosome bearing) from Gir (n = 8) or Holstein (n = 7) sires (2 x 10^6 /dose) were used for fertilization. Oocytes were fertilized by incubating sperm and oocytes for 18–20 hrs, and the resulting embryos were cultured in similar conditions of temperature and atmosphere of IVM, but embryos at Day 2, 3, 4 or 5 finished the developmental stage in a portable incubator. All embryos were transferred fresh, after 24 to 72 hrs of transportation to cover 2,000 km. The mean number of total oocytes per OPU procedure was 17.1 ± 4.4 for Gir cows (n = 617), 11.4 ± 3.9 for Holstein cows (n = 180), 20.4 ± 5.8 for 1/4 Holstein x 3/4 Gir (n = 44), and 31.4 ± 5.6 for 1/2 Holstein-Gir crossbreed females (n = 37, $P < 0.01$). The mean number of viable oocytes per OPU procedure was 12.1 ± 3.8 for Gir cows, 8.0 ± 2.6 for Holstein

cows, 16.8 ± 5.0 for 1/4 Holstein x 3/4 Gir, and 24.3 ± 4.7 for 1/2 Holstein-Gir crossbreed donors ($P < 0.01$). The mean number of embryos produced by OPU/IVF and the pregnancy rates were, respectively, 3.2 (n = 12,243/3,378) and 40% for Gir cows, 2.2 (n = 2,426/1,138) and 36% for Holstein cows, 3.9 (n = 1,033/267) and 37% for 1/4 Holstein x 3/4 Gir, and 5.5 (n = 1,222/224) for 1/2 Holstein-Gir. We clearly demonstrated the influence of *indicus* cattle on oocyte yield by comparing two levels of *indicus/taurus* breeds. In addition, we demonstrated the efficiency of sexed sperm for quick and large production of *indicus* dairy female calves. The scheme for culturing embryo during transportation for long distances presented interesting results, with wide perspectives for international business of embryos.

Keywords: Oocytes, Embryos, Ovum pick up, IVF, cattle, *Bos indicus*.

1. Introduction

The *in vitro* embryo industry has been constantly improving during the last decade, and the numbers of embryos *in vivo* and *in vitro* are closer now than in the past [1]. Compared to other countries, there is a unique situation of IVP in Brazil, with a strong tendency for replacing MOET by OPU / IVP, as we have recently described [2]. One of most important reasons to explain this situation is the natural larger production of follicles / oocytes from *Bos indicus* (or Zebu) compared to *Bos taurus* [2], and, as of yet, there is no biological explanation of this intriguing difference. Most of the available information of OPU / IVP in Brazil, however, was obtained only from Nelore, a Zebu beef breed that represents around 80% of the Brazilian herd (approximately 200 million animals).

Despite the importance of Nelore as a beef breed very adapted for tropical areas, there is also a growing interest for Zebu dairy breeds, such as Gir, considering their adaptability for producing large amounts of milk under challenging conditions, including hot weather, parasites, and poor pastures. These interesting characteristics have been maintained in Gir-Holstein animals [3], usually named Girolanda, a popular breed for dairy cattle in Central and South America and potentially other tropical areas.

Recently, the *in vitro* method for embryo production has been considered for Girolanda donors because of the growing efficiency of sexed sperm on IVF [4], which has allowed the production of a large number of females for the milk industry in a short period of time.

The objective of the present study was to analyze oocyte yield using OPU and *in vitro* embryo production with sexed sperm and to analyze pregnancy rates after long-term embryo transportation from *Bos taurus* (Holstein), *Bos indicus* (Gir), and *indicus*-

taurus (Holstein x Gir) breeds. All results were obtained from In Vitro Brasil, a large commercial IVF production center.

2. Materials and Methods

2.1. Animals

Non-pregnant, healthy, and cycling Gir (*Bos indicus* n = 617), Holstein (*Bos taurus* n = 180), 1/4 Holstein x 3/4 Gir (n = 44), and 1/2 Holstein-Gir (n = 37) cows were used as oocyte donors. The mean body condition score was 3.5 ± 0.5 (scale, 1 to 5) [5], and the mean age was 5 ± 2.3 years (range, 3 to 7 years). The median postpartum interval was 296 days. All donors were selected based on genetic merit and had regular ovarian activity (based on transrectal palpation and ultrasonography). All cows were used for OPU (5,407 procedures), with a mean of 6.2 ± 2.4 (range, 4 to 7) OPUs performed per donor cow. Since the oocytes were collected at a commercial embryo production center, the animals were randomly used in OPU/IVP without a specific schedule or predetermined sequence, only considering a minimum interval of 15 days between procedures. None of the females were submitted to hormonal treatment before OPU/IVP. Embryo production was conducted over a 12-month interval in a commercial embryo production center, with laboratories located in Mogi-Mirim, Sao Paulo, and Goiania, Goias, both in Brazil.

Crossbreed heifers (15 to 19 mos old) and cows (24 to 48 mos old) were used as recipients. These animals were located in eight farms at North Area of Brazil, in the States of Para and Mato Grosso, with a distance of over 2,000 km from the IVP

laboratories. Potential recipients were selected for body condition, normal cyclicity, and health status.

2.2. Donor Preparation

Before each procedure, feces were removed from the rectum and the perineal area was cleaned with tap water and 70% ethanol. Prior to OPU, each cow received epidural anesthesia using 7 mL of 2% lidocaine (Anestésico L, Pearson, São Paulo, São Paulo, Brazil) to decrease peristalsis and discomfort.

2.3. Follicle Aspiration

Previously described procedures were used for follicular aspiration [6]. Briefly, each visible follicle 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 1/2'' hypodermic needle (Becton Dickinson, Curitiba, Parana, Brazil) connected to a 50-mL conical tube (Corning, Acton, MA, USA) via silicon tubing (0.8 m; 2 mm id). Aspiration was performed using a vacuum pump (Cook Veterinary Products, Queensland, Australia) with a negative pressure of 10–12 mL of water/min. 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, São Paulo, Brazil), and 10,000 IU/L sodium heparin (Sigma H-3149).

2.4. *In vitro* Embryo Production

Immediately after recovery, the aspirated material from the follicles was washed and filtered through an Emcon Embryo filter (Immuno Systems Inc., Spring Valley, WI, USA) with a phosphate buffer solution (PBS-Nutricell, Campinas, São Paulo, Brazil). The cumulus oocyte complex was classified according to the presence of cumulus cells and the oocyte quality using the following criteria: good, more than three layers of cumulus cells; regular, at least one layer; denuded, partly covered with cumulus cells or without cumulus cells; atretic, dark cumulus oophorus and signs of cytoplasmic degeneration [6]. After evaluation, atretic oocytes were recorded and discarded. The regular and good oocytes were classified as viable oocytes.

Prior to *in vitro* maturation (IVM), cumulus oocyte complexes (COCs) were washed three times in TCM-199 HEPES (Gibco Life Technologies, Grand Island, NY, USA) supplemented with 10% FCS and 50 μg gentamycin sulfate and were washed once in bicarbonate TCM-199 (Gibco Life Technologies) supplemented with 10% FCS, 5 μg luteinizing hormone (LH- Ayerst, Rouses Point, NY, USA), 0.5 μg follicle stimulating hormone (FSH- Folltropin, Vetrepharm, Belleville, ON, Canada), 1 μg estradiol (Estradiol 17β- Sigma E-8875), 2.2 μg pyruvate (Sigma P-4562), and 50 μg gentamycin/mL of medium. The COCs of each category were separately cultured for 24 hrs in 100-μL drops of maturation medium under mineral oil (D'Altomare, Santo Amaro, São Paulo, Brazil) at 39°C and 5% CO_2 in air [19, 20]. Frozen-thawed sexed sperm (2 x 10^6 /dose) from Gir (n = 8) and Holstein (n = 7) sires of known fertility were used. Holstein sexed sperm were used for fertilization of oocytes from Gir, 1/4 Holstein x 3/4 Gir, and 1/2 Holstein-Gir cows, and Gir sexed sperm were used for Holstein

females. For IVF, straws were thawed for 20 s in a 35°C water bath. Sperm were washed by centrifugation at 200 x g for 30 min through a 90%–45% Percoll gradient. Sperm were capacitated using heparin (30 μg/mL) and motility was stimulated by the addition of 40 μL/mL of PHE [7]. After visual estimate of motility, sperm concentration was adjusted to 25 x 10^6 live sperm /mL and each fertilization drop received 4 μL of sperm (final concentration 1 x 10^5 sperm per drop) [6]. After maturation, COCs were washed three times in pre-fertilization medium TCM 199 supplemented with 25 mM HEPES (Gibco Life Technologies, Grand Island, NY, USA) and 0.3% BSA (Sigma A-9647), and were washed one time in TALP fertilization medium supplemented with 10 μg/mL heparin and 160 μL PHE solution [7,8].

Presumptive zygotes had their cumulus cells removed (by pipetting) 20 hrs after the addition of sperm and were transferred to drops of 100 μL of culture medium of embryos, a modified oviduct synthetic fluid, the SOFaa BSA, containing 8 mg / mL BSA (Sigma, USA) free of fatty acid and 1 mM glutamine, under the same conditions of temperature and gaseous atmosphere of IVF, remaining in this medium for 5 to 8 days. The osmolarity was maintained at 270–280 mOsmol and the pH was 7.4. The embryo rate was obtained from the total of aspirated oocytes. Considering all steps involved with transportation, embryos at different developmental stages were considered to be transferred, e.g., embryos from Days 2 to 5, with Day 0 being the day of IVF. Due to the long distance from the laboratories to the recipients, the final stage of embryo development was carried out during the transportation period, as described below.

2.5. Protocol for Embryo Transfer

A timed fixed embryo transfer protocol was used. Each recipient received an intravaginal progesterone implant (CIDR, Pfizer, Hamilton, New Zealand) and 2 mg of estradiol benzoate (Estrogin, Farmavet, São Paulo, Brazil) on Day 0. Progesterone implants were removed on Day 8, when animals were also injected with 300 IU of eCG (Novormon, Syntex, Buenos Aires, Argentina), 150 μg of d-cloprostenol (Preloban, Intervet, São Paulo, Brazil) and 1 mg of estradiol cypionate (E.C.P., Pfizer, Guarulhos, Brazil). No detection of estrus was performed; Day 10 was considered as the day of estrus. Embryos were transferred on Day 17. Before embryo transfer, each recipient was submitted to ovary examination by ultrasound (Aloka SSD 500®, 5 MHz linear transducer, Tokyo, Japan) to confirm the presence and size of CL. Only recipients showing a CL ≥ 13 mm received an embryo.

2.6. Embryo Transportation

Embryos were produced in the States of Sao Paulo (Southeast of Brazil) and Goias (Center of Brazil) and were transferred into recipients located in eight farms at the North area of Brazil. Due to the long distances (over than 2,000 km), embryos were carried by airplanes, transported into microtubes containing 400 μl of the same embryo culture medium described above, under 300 μl of mineral oil. Temperature and atmosphere were similar that those of the in vitro maturation. Each tube received an average of 40 embryos. During the transportation period, 24 to 72 hrs, from the laboratory until the very moment of transfer, all tubes were maintained inside a specific incubator for embryo transport (Ceafepe Tecnologia Veterinaria, Sorocaba, Sao Paulo, Brazil). Prior to the transfer, each embryo was inserted into a 0.5-ml pallet to be transferred non-surgically at the uterine horn. The developmental stage of embryos was

not recorded at the moment of embryo transfer, but the vast majority had reached morula or blastocyst stage during transportation period.

2.7. Pregnancy Evaluation

Between Days 23 to 28 (hereafter designated Day 30) after embryo transfer, the pregnancy status of recipients was determined by ultrasound evaluation. Recipients diagnosed pregnant were re-evaluated by ultrasound 30 to 35 days later (designated Day 60) to confirm pregnancy.

2.7. Statistical Analysis

Statistical analysis was performed using the software Bioestat 5.0 [9]. The oocyte yield collected per donor cow and the number of embryos produced per donor cow were normally distributed and were analyzed by ANOVA. Comparisons between breeds was done by applying the Tukey test. The number of viable oocytes per donor cow was not normally distributed and therefore was analyzed using the Kruskal-Wallis test. The comparison of viable oocytes per donor cow between breeds was conducted with the Dunn test.

3. Results

A total of 90,086 oocytes were collected in 5,407 OPU sessions. We performed 3,778 ultrasound-guided follicular aspiration of ovaries of Gir cows, 1,138 of Holstein cows, 267 of 3/4 Gir x 1/4 Holstein cows, and 224 of 1/2 Holstein-Gir crossbreed cows.

On average, 16.7 ± 6.3 oocytes were obtained per procedure and 72.0% (64,826/90,086 oocytes) were considered viable, generating an average of 12.0 ± 4.4 viable oocytes per procedure.

For Gir cows (*Bos indicus*), the mean number of oocytes recovered (17.1 ± 4.5) and classified viable (12.1 ± 3.9) per procedure was greater ($P < 0.01$) than for Holstein cows. Holstein cows had the lowest number of recoverable (11.4 ± 3.9) and viable (8.0 ± 2.7) oocytes per procedure ($P < 0.01$, Table 1). The oocyte yield from *taurus* X *indicus* donor cows was greater ($P < 0.01$) than from *Bos indicus* donors. For 3/4 Gir x 1/4 Holstein cows, the mean number of recoverable (20.4 ± 5.8) and viable (16.8 ± 5.0) oocytes per procedure was lower ($P < 0.01$) than for 1/2 Holstein-Gir crossbreed females, which had the highest number of recoverable (31.4 ± 5.6) and viable (24.3 ± 4.7) oocytes per procedure compared with other breeds ($P < 0.01$, Table 1).

Individual results of embryo production are shown (Table 2), representing 64,826 oocytes used for IVF procedures producing 16,924 embryos (26.1%) that were transferred into recipients. The mean number of embryos produced per IVP session from 1/2 *taurus* x *indicus* donor cows was greater ($P < 0.01$) than from *Bos indicus* cows. For 3/4 Gir x 1/4 Holstein cows, the mean number of embryos (3.9, n = 1,033/267) produced per donor cow was similar to 1/2 Holstein-Gir (5.5, n = 1,222/224) crossbreed females per procedure ($P > 0.05$, Fig. 2). Mean number of procedures per donor was 2.9 ± 1.4.

Percent of cleaved oocytes, embryos per oocyte, and embryos per cleaved oocyte obtained with sexed sperm from Gir and Holstein sires are shown (Fig. 1).

Considering embryos from all donors, we obtained a 39% pregnancy rate. Because of the large proportion of this embryo transfer program, we could not recover

the data of all transferred embryos, but we are showing pregnancy data for 60% (10,049) of the embryos (Table2).

4. Discussion

To our knowledge, this is the largest program of *in vitro* embryo production with dairy *indicus* cattle using sexed sperm. We present commercial data of more than 5,000 OPU / IVP procedures, with interesting aspects of embryo production and pregnancy rates involving Holstein, Gir, and Holstein-Gir donors. We also describe an interesting strategy for transferring embryos at long distances from the laboratory to the recipients. This information can be used to strengthen and expand OPU/IVF programs that utilize donors of both Zebu and *B taurus* breeds.

The number of recoverable oocytes from Holstein cows (11.4 ± 3.9) was greater than that reported previously [10,11]. In these studies, approximately 4 oocytes were collected per OPU session. However, the number of recovered oocytes from Holstein donors in the present study was similar to the results reported by Bousquet et al. [11], who reported a mean of 9.5 (n = 4,145/437) oocytes collected per OPU session. It is possible that the higher number of oocytes collected in our study (11.4 ± 3.9) is representative, due to the numbers of procedures performed (n = 1,138). We believe that two factors can explain our success and results: our use of donors that were reproductively sound and the work scheme of our OPU technicians. Veterinarians from the embryo center usually perform 15 to 20 follicle aspirations per day, every workday, allowing them to gain expertise in this procedure.

The number of recovered oocytes per OPU session from Gir cows (17.1 ± 4.5) was higher to that reported by Viana et al. [12]. These authors collected an average of

11.6 oocytes per OPU session from Gir cows. Our results are similar to those reported by the same authors [13] in a different study, collecting an average 18.8 oocytes per procedure. Based on the number of OPU procedures performed in the present study (n = 3,778) we believe that a collection of 17 to 18 oocytes is more representative for this breed. We could not find references in the literature of oocyte yield from Holstein-Gir donors. For these animals, we obtained means of oocytes ranging from 20 (¾ Holstein-Gir, n = 267) to 31 (½ Holstein-Gir, n = 224). It seems that the blood shock promoted an increase on number of follicles / oocytes, considering that crossbreed animals presented higher averages than pure breeds.

Considering only Zebu cows, the variation in oocyte yield seems to be related to the gene sequence, at least for the Nelore breed [14]. These authors performed studies with genetic sequencing according the oocyte yield. The genes *GDF9*, *FGF8*, *BMP15*, and *BMP15* receptor were analyzed. Considering only the *FGF8* effect, these authors reported an increase of 2.3 ± 1.1 oocytes on average and a possible variation of 7.4 ± 1.1 oocytes when all genes were considered together. Despite this promising study, there are several factors yet to be addressed pertaining to oocyte yield in Zebu cattle. High oocyte yield seems to be a unique aspect of *Bos indicus* breeds. Although the total number of follicles in *Bos indicus* ovaries remains to be determined, the number of primordial and primary follicles in *Bos indicus* ovaries seems to be similar to *Bos taurus* breeds [15].

Four hypotheses can be considered to explain the high oocyte yield from *Bos indicus* donors. It is possible that *Bos indicus* females have a greater number of germinal cells at the fetal stage or maybe a longer period of mitosis during the formation of oogonia. Distinctive mechanisms of follicular atresia can also explain the high oocyte yield from *Bos indicus* females. The controversial hypothesis of follicular

renewal [16,17] seems plausible for Zebu cows. However, this new concept needs to be scientifically confirmed before being considered [18,19]. We are currently investigating the pre-antral follicular population in Zebu females to explain this concept.

Donors used for oocyte collection were not treated with hormones. As described earlier by our team, the number of oocytes obtained from Nelore females by ultrasound-guided follicular aspiration did not increase with ovarian superestimulation [20]. This is one intriguing aspect not well understood for *Bos indicus* breeds. Another unexplained factor is a tendency to produce fewer embryos when performing MOET [21]. As with the other aspects mentioned above, the question of hormone stimulation on Zebu breeds remains to be explained.

It is obvious that the variation of oocyte yield reflects the embryo production (Table 1). For Holstein cows, our average of 2.1 embryos / OPU-IVP was lower when compared with the average of 4.3 described by Bousquet et al. [11]. These authors had more efficient *in vitro* embryo production, considering that their oocyte yield per OPU, 9.5, was lower than ours, which was 11.4. This variation can be explained in two ways. First, we worked with double the number of donors, and a higher variation in oocyte quality and embryo production would therefore be expected. The second aspect involves the logistics of our large embryo program, i.e., the distance from the laboratory to the recipients and the use of embryos in different developmental stages. It is a bigger challenge to produce embryos under these conditions compared to having all donors set in the embryo center. This hypothesis seems to be true, because we recently described [2] 9.4 embryos per OPU/IVP when performing OPU from donors located in the embryo center.

For Gir and Gir-Holstein donors, we could not find other works describing *in vitro* embryo production with pregnancy rates. Considering the high number of

procedures performed, we believe the averages of embryos presented in this work to be representative for these types of donors under similar conditions of OPU/IVP. We highlighted the very close rate of embryos / oocytes (Table 1) for all type of donors, which suggests that the *in vitro* system of embryo production works similarly, no matter if oocytes came from *indicus, taurus,* or *indicus-taurus* donors.

The successful use of sexed sperm is efficient for producing a large number of females for a dairy herd. Our team has used sexed sperm to produce Zebu embryos, but this work was our first attempt to generate a large amount of *in vitro* embryos from Holstein and Gir sires. Other teams [4] have already described the efficiency of sex-sorted sperm to produce *in vitro* embryos in Holstein, with higher averages of embryo development, but these authors used X-sorted sperm from a selected Holstein bull, while our results came from 15 sires. It is very clear that there is large individual variation between bulls, as showed in Figure 1 and this aspect has also been previously reported [4]. We have no precise comparisons, but our laboratory team has also reported important variation between ejaculates from the same bull. After internal tests, it was possible to establish best samples, according to the ejaculates from the same bull. Since the major goal of this program was to produce pregnancies, we could not obtain precise information about fetal sex and/or calf sex. However, personal information from practitioners gave us an efficiency rate higher than 95% for females, with very few reports of abortion and other abnormalities.

Our general pregnancy rate was close to 40%, higher than the 33.5% recently described by our team [2] and very close to other reports with sexed sperm (40 and 41%) [4]. However, we emphasize the larger number of embryos transferred in our work, as well the peculiar logistics for transporting embryos for long distances. We probably achieved this good mark because of the protocol for timed fixed embryo

transfer. Due to the constant lack of good recipients available, the use of time-fixed protocols has been considerably improved in very recent years in Brazil [22]. In addition, embryonic development occurred during transportation for the last 1 to 3 days, with conditions not as stable as an incubator in the laboratory. Regarding the transportation of embryos at early stages of embryonic development, we could not find similar reports in the literature. This strategy was proposed at the beginning of the project, considering the long distance from laboratory to recipients. At least under the presented conditions, our results indicate that it is possible to transport embryos at an early stage of development, with actable pregnancy rates.

We clearly demonstrated the influence of *indicus* cattle on oocyte yield by comparing two levels of *indicus/taurus* breeds. In addition, we demonstrated the efficiency of sexed sperm for quick and large production of dairy female calves. The scheme for culturing embryo during transportation for long distances presented interesting results, with wide perspectives for the international business of embryo use.

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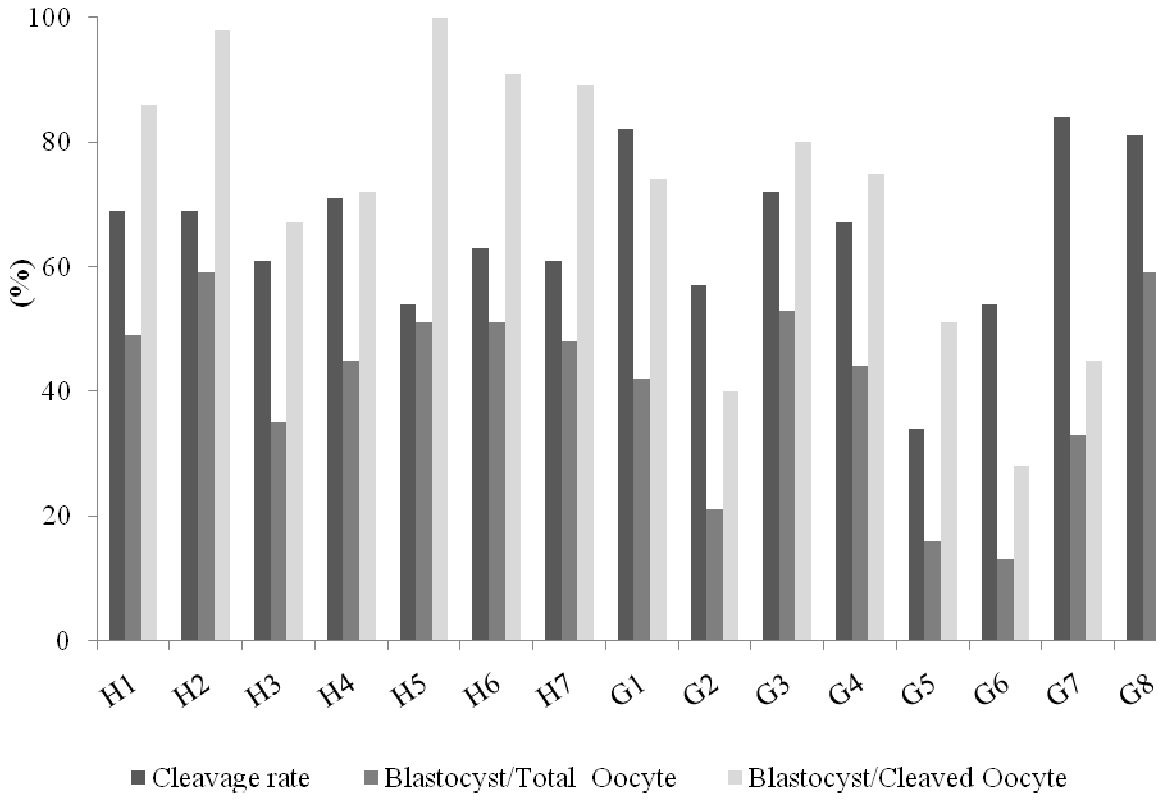

Figure 1. Mean percentages of cleaved oocytes, percentages of hatched blastocysts per oocyte, and percentages of hatched blastocysts per cleaved oocyte obtained with sexed semen from different Gir (G) or Holstein (H) sires.

Table 1. Number of collected and viable oocytes per donor (± SEM), and percentage of embryos on total oocytes per OPU – VIP obtained from Gir, Holstein, and Holstein-Gir crossbreed donor cows.

Type of donor	Mean ± SEM of total oocytes/OPU (n)	Mean ± SEM of viable oocytes/OPU (n)	Embryos/ Total oocytes % (n)
Gir	17.1 ± 4.5 [a] (64,617/3,778)	12.1 ± 3.9 [a] (45,838/3,778)	18.9 [a] (12,243/64,617)
Holstein	11.4 ± 3.9 [b] (12,977/1,138)	8.0 ± 2.7 [b] (9,082/1,138)	18.7 [a] (2,426/12,977)
¾ Holstein ¼ Gir	20.4 ± 5.8 [c] (5,457/267)	16.8 ± 5.0 [c] (4,472/267)	18.9 [a] (1,033/5,457)
½ Holstein ½ Gir	31.4 ± 5.6 [d] (7,035/224)	24.3 ± 4.7 [d] (5,434/224)	17.4 [a] (1,222/7,035)
Total	16.7 ± 6.3 (90,086/5,407)	12.0 ± 4.4 (64,826/5,407)	18.8 (16,924/90,086)

Means ± SEM with different in the same column superscripts differ; $P<0.01$.

Table 2. Data of embryos and pregnancy obtained from Gir (*B indicus*), Holstein (*B taurus*) and *indicus – taurus* donors submitted to OPU – IVP.

Type of donor	Mean of embryos / OPU-IVP (n)	Mean of pregnancy / OPU-IVP * (n)	Pregnancy Rate * (n)
Gir	3.2 [a] (12,243/3,778)	1.2 [a] (3,113/2,2670)	40% (3,113/7,763)
Holstein	2.1[b] (2,426/1,138)	0.7 [b] (604/822)	36% (604/1,698)
¾ Holstein ¼ Gir	3.9 [ac] (1,033/267)	1.3 [ac] (137/103)	37% (137/368)
½ Holstein ½ Gir	5.5 [c] (1,222/224)	1.7 [c] (82/47)	37% (82/220)
Total	3.1 (16,924/5,407)	1.1 (3,936/3,3642)	39% (3,936/10,049)

* Pregnancy data of 10,049 embryos from the total of 16,925 transferred. Data of 6,876 transferred embryos could not be recovered.

In the same column, different letters are significantly different ($P < 0.05$).

Capítulo 5

Effect of culture medium and oxygen tension on bovine embryo development and pregnancy rates

Artigo em elaboração, a ser submetido ao periódico *Theriogenology*

Effect of culture medium and oxygen tension on bovine embryo development and pregnancy rates

Abstract

In commercial bovine embryo *in vitro* production (IVP) settings, the optimization of embryo development and pregnancy rates are fundamental issues. This work aims to present the results of a strategy aimed to increase the efficiency of commercial bovine embryo IVP systems based on embryo pre and postimplantation (i.e. after embryo transfer – ET) development. The effects of embryo *in vitro* culture conditions regarding two variables of great interest for commercial large-scale bovine embryo IVP were evaluated: (i) use of the sequential medium G1/G2 compared to SOF medium, and (ii) the atmosphere oxygen tension in the incubator during IVC. Experiments comparing SOF medium and G1/G2 medium were performed to evaluate their efficiency. The cleavage rate was positively ($p<0.05$) influenced by G1/G2 medium (68.2%, N=521) compared to SOF (59.0%, N=455). No difference was observed for blastocyst development (30.2 and 28.0% for SOF and G1/G2, respectively). Pregnancy rates obtained after ET of embryos produced in the two groups: (i)G1/G2, (ii)SOF revealed a striking difference forG1/G2 group, which resulted in higher levels of pregnancy at day 30 (53%, number of ET=100) and at day 60 of gestation (50%) compared to SOF group (28.2 and 22.8% at day 30 and 60, respectively, number of ET = 121).Regarding the effect of oxygen tension, no difference on cleavage (58.5 and 56.0%) and on blastocyst development (30.0 and 32.1%) was observed for high and low oxygen tension, respectively. Nonetheless, regarding pregnancy rates at day 60 after ET, low oxygen tension in the incubator during IVC was significant advantageous ($p<0.05$; 45.0%, number of ET=248) over high oxygen tension (34.5%, number of ET=174). Based on

pregnancy outcome it was possible to conclude that even though no difference occurred in preimplantation embryo development, low oxygen tension during embryo IVC increased pregnancy rates. Also the use of G1/G2 medium allows higher pregnancy rates ($p<0.05$) over SOF medium . Since there are so many interactions during the bovine embryo IVP process, we show that the strategy of carefully introducing bovine embryo culture conditions optimization, transferring the embryos to recipient cows and analyzing the outcome allows conclusive results for increasing embryo IVP commercial efficiency.

Keywords: pregnancy rate, embryo quality, in vitro culture, developmental competence

Introduction

Commercial bovine embryo *in vitro* production (IVP) has been shown to be an efficient tool for spreading genetic highly valuable animals, especially concerning the Nelore cattle breed (1). Also, this biotechnology is considered to be fundamental for the success of animal cloning and transgenesis (2).

Bovine embryo *in vitro* culture (IVC) conditions, especially post-fertilization culture environment, are critical to blastocyst quality (3, 4). Among IVC conditions, the culture medium chemical composition, the oxygen tension during embryo IVC.

Various culture media for bovine embryo IVP, such as synthetic oviduct fluid (SOF), tissue culture medium 199 (TCM 199), Charles Rosenkranz-1 (CR1), CDM (complete defined medium) and modified simplex optimized medium (KSOM) can used for commercial bovine embryo IVP (5). Nonetheless, the use of sequential culture media, which take into consideration metabolic and nutritional changes during embryo *in vitro*

development, is considered to be a promising approach. An example of a successful sequential medium is the G1/G2 medium (6). Besides avoiding the used of fetal calf serum (FCS), G1/G2 aims to decreasing embryo metabolic stress during IVC (7). The composition of this medium includes metal chelants such as ethylenediamine tetraacetic acid (EDTA) and recommends the use of low oxygen tension (i.e. 5% O2) for embryo IVC. Low oxygen tension in the incubator during embryo IVC avoids oxidative stress, since it mimics the oviduct physiological normal environment. Oviduct oxygen tension is around 8% in hamsters, rabbits, and monkeys and its use for bovine embryo IVP shown to be beneficial (8, 9).

The objective of this work is therefore to use the strategy of carefully introducing and evaluating three variables of high interest for commercial and large-scale bovine embryo IVP, which are (i) the use of sequential medium G1/G2 compared to SOF medium, and (ii) the atmosphere oxygen tension in the incubator during IVC. As shown by our data, robust conclusions can be drawn when large numbers of ET (more than 400 in each of the experiments) are performed. Embryo *in vitro* development rates are not always mirrored by embryo pregnancy rates, confirming the need of employing further *in vitro* evaluations and developing new methods to evaluate embryo competence for further development.

Material and Methods

Chemical reagents and samples

Unless mentioned otherwise, chemicals and growth media were purchased from Sigma-Aldrich (St. Louis, MO, USA). For *in vitro* maturation (IVM) and *in vitro* fertilization

(IVF) incubator conditions were 38.5°C with 5% CO_2 in air and maximum humidity. For embryo IVC, incubator conditions were 38.5°C with 5% CO_2 in air (In experiment 1 for SOF group and In experiment 2 for high oxygen tension group), or 5% CO_2, 5% CO_2 and 90% N_2 (In experiment 1 for G1/G2 group and in experiment 2 for the low oxygen tension group).

Experimental procedures

Oocytes were obtained by OPU (14) of Nelore cows independent of their estrous cycle stage. Briefly, visible follicles were aspirated using a real-time B-mode ultrasound scanner (Scanner 200 Vet, Pie Medical, Maastricht, The Netherlands), equipped with a 7.5 MHz convex array transducer fitted into the intravaginal device (Pie Medical), and a stainless steel guide. Follicular puncture was performed using a disposable 19 gauge 1/200 hypodermic needle (Becton Dickinson, Curitiba, Parana, Brazil) connected to a 50 mL conical tube (Corning, Acton, MA, USA) via a silicon tubing (0.8 m; 2 mm id). Aspiration was performed using a vacuum pump (Cook Veterinary Products, Queensland, Australia) with a negative pressure of 10–12 mL of water/ min. The collection medium used consisted of TCM 199 (Gibco Life Technologies, Grand Island, NY, USA) supplemented with 25 mM HEPES, 5% fetal calf serum (FCS), 50 µL/mL gentamycin sulfate (Schering- Plough, Sao Paulo, Sao Paulo, Brazil) and 10,000 IU/ L sodium heparin.

Immediately after recovery, the aspirated material was filtered through an EmCon filter with phosphate buffered saline (PBS-Nutricell, Campinas, Sao Paulo, Brazil) supplemented with 5% FCS. Prior to IVM, Cumulus oocyte complexes (COCs) were washed three times in TCM-199 HEPES-buffered (Gibco Life Technologies, Grand

Island, NY, USA), supplemented with 10% FCS and gentamycin sulfate. Once in IVM medium, which consisted of bicarbonate-buffered TCM-199 supplemented with 10% FCS, 0.20 mmol/L sodium pyruvate, 83.4mg/mL amicacin, 1 ng/mL FSH (Pluset, Laboratorios Calier do Brasil Ltda, Brazil), 50 mg/mL LH (Vetecor, Laboratorios Calier do Brasil Ltda, Brazil), and 1 mg/mL estradiol, the COCs of each animal were separately placed in 1.5mL cryogenic vias (Nalgene, Rochester, New York, USA) containing 400μL of IVM medium and 300μL mineral oil, and transported to the laboratory at 38.5^{o}C in an portable incubator (CEAFEPE, Sorocaba, SP, Brazil). In the laboratory, oocytes were transferred to droplets of 100μL IVM medium under mineral oil and placed in the incubator.

After 24 h IVM, oocytes were *in vitro* fertilized in TALP-IVF (Tyrod's albumin lactate and pyruvate – *in vitro* fertilization) medium supplemented with 0.6% (w/v) bovine serum albumin (BSA), 30 mg/mL heparin, 18 mmol/L, penicillamine, 10mmol/L, hypotaurine and 1.8 mmol epinephrine. Frozen sex-sorted or non sex-sorted semen was thawed for 20 s in water at 35.8 ^{o}C. Semen was then washed and centrifuged through a 90% - 45% Percoll gradient at 200 x g for 10 min. Sperm was capacitated using heparin (30 mg/mL) and PHE. Concentration was adjusted to 25X 10^{6} live sperm/mL. After 20-24h of oocytes and semen co-incubation, presumptive zygotes had their cumulus cells stripped off by gentle pipetting. Embryos were cultured in groups of up to 25 in 100 μL drops of medium. Two different embryo IVC media were evaluated: (i) SOF (15)supplemented with 2.5% FCS and 0.6% BSA and (ii) G1/G2 supplemented with 0.6% BSA). For embryos cultured in SOF medium, after 72 h of IVC, 50 μL of fresh development medium was added. For embryos culture in G1/G2 medium, after 72h of IVC, embryos were transferred from G1 to G2 medium droplets. Cleavage rate was

evaluated after 72h of embryo IVC. At day 7 of IVC, embryos were counted and classified according to IETS criteria (16).

Based on the commercial bovine embryo *in vitro* production experience, we selected two factors influencing on the routine IVF(culture medium and oxygen tension) and designed two experiments in order to evaluate preimplantation development, and also pregnancy outcome after ET of these embryos.

In the first experiment, a total of 1608 OPU-derived oocytes from 45 different donor cows were divided simultaneous s for *in vitro* culture in SOF in a high oxygen tension(805 oocytes) or in the G1/G2 sequential medium in a low oxygen tension (803 oocytes).

The second experiment was designed to test the effect of the oxygen tension (high oxygen tension – 20% O_2; or low oxygen tension – 5% O_2) on our routine embryo *in vitro* culture, in which SOF is used as the culture medium after IVF. A total of 2467 OPU-derived oocytes from 18 repetitions were divided for *in vitro* culture in SOF mediun in a low oxygen (1213 oocytes) and high oxygen tension(1254 oocytes) .

Embryo Transfer and pregnancy evaluation

Blastocysts were transferred nonsurgically into the uterus of previously synchronized recipient cows. Only grade I and II embryos were individually transferred to recipient cows at the stage of morula and blastocyst from Days 6 to 7.5 post insemination. On days 30 and 60 after ET, the pregnancy status of recipients was determined by ultrasound evaluation.

Statistical analysis

In the experiment 1, data was submitted to ANOVA and interaction has been evaluated. Means were compared by T-student test. Data included 45 repetitions for embryo development (cleavage and blastocyst rate) and pregnancy assessment. The frequency of embryonic losses from 30 to 60 days of pregnancy was tested by Fisher's exact test. The level of 5% of significance was used for all experiments.

Data of experiment 2 (effect of oxygen tension on embryo *in vitro* production and pregnancy rate 60 days after ET) was submitted to ANOVA and means comparison by T-student test. Data regarding embryo development (cleavage and blastocyst rate) consisted of 18 repetitions, and data regarding pregnancy rate consisted of 10 repetitions.

Results

Experiment 1 – Effect culture medium (G1/G2 versus SOF) on bovine embryo in vitro production and pregnancy establishment at day 60 after E.T.

A total of 1608 oocytes fertilized were divided for culture in SOF(805 oocytes) or G1/G2 sequential medium(803 oocytes). According to the experimental design, donor cows had the oocytes fertilized had the oocytes divided randomly for culture in SOF and G1/G2 media. In Table 1 it is possible to observe a positive effect in the cleavage rate [73.85% (593/803)and 58.63% (472/805) for G1/G2 medium and SOF medium respectively.figure 2a], but this difference was not reflected in the blastocyst rate [31.68% (255/805) and 29.02% (233/803) for G1/G2 medium and SOF medium respectively.

Table 1 - Mean percentage and standard deviation of cleavage and blastocyst production of bovine *in vitro* cultured in G1/G2 and SOF medium.

	No.	**Nc.**	**Cleav rate (±SD)**	**Nb.**	**Blast rate (±SD)**
G1/G2	803	593	73.85% [a'] (20.3)	233	29.02% (14.6)
SOF	805	472	58.63% [b'] (19.5)	255	31.68% (15.0)
Total	1608	1065	-	488	-

[a,b] Different superscripts within columns indicate difference between culture media ($P<0.05$). No= number of oocytes; Nc = number of cleaved embryos; Cleav rate = cleavage rate; Nb = number of blastocysts; Blast rate = blastocyst rate

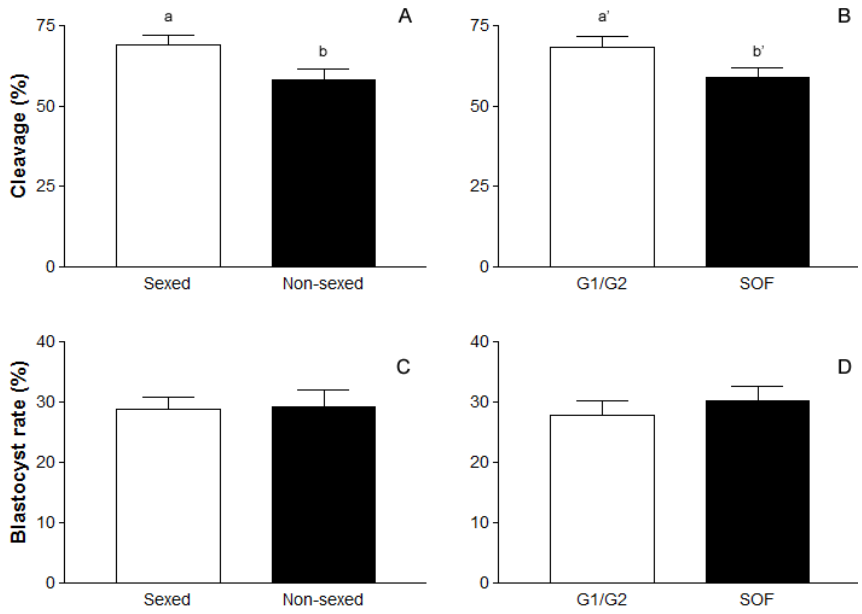

Figure 01 – Cleavage and blastocyst production of bovine embryos produced *in vitro ,* cultured in G1/G2 or SOF media (A and B, respectively). Different letters (a, b) and (a', b') above bars indicate statistical difference ($p<0.05$) between culture media. Bars depict means and whiskers depict SEM.

Regarding pregnancy rates (Table 2 and Figure 2a-b), when oocytes were cultured in G1/G2 sequential medium , pregnancy levels were positively improved both at day 30 after ET [40.38% (86/213)] and at day 60 [37.56% (80/213)] compared to the SOF group, which were [28.9% (71/251) and 24.3% (61/251) at 30 and 60 days after ET, respectively].

Table 2 - Mean percentage and standard deviation of 30- and 60-day pregnancies derived from bovine embryos *in vitro* cultured in G1/G2 and SOF medium.

	Media	**N. ET**	**N. 30d preg**	**30d preg rate (±SD)**	**N. 60d preg**	**60d preg rate (±SD)**
	G1/G2	213	86	40.38 (26.7)[b]	80	37.56(21.6)[b]
	SOF	251	71	28.29 (33.6)[a]	61	24.3 (34.3)[a]
Total	**-**	464	157	-	141	-

[a,b] Different superscripts within columns indicate difference ($P<0.05$). N. ET = number of embryos transferred; N. 30d preg = number of 30-day pregnancies; 30d preg rate= pregnancy rate at 30 days; N. 60d preg = number of 60-day pregnancies; 60d preg rate= pregnancy rate at 60 days.

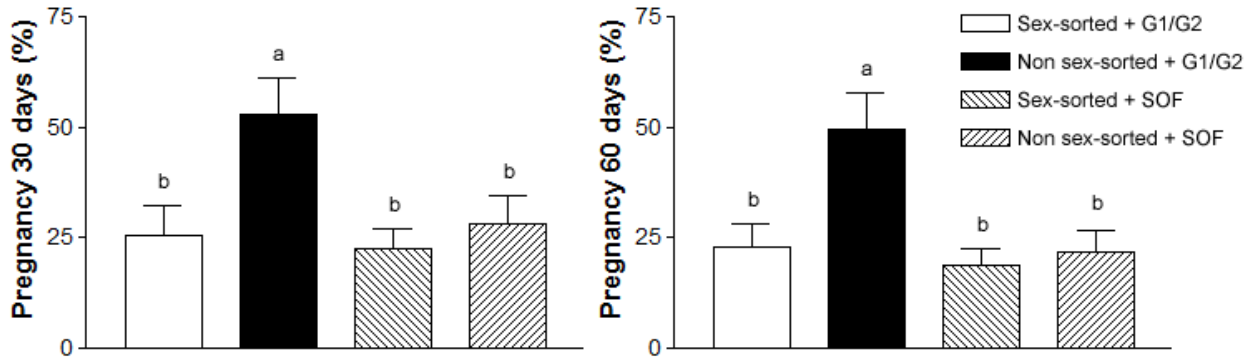

Figure 02 – Pregnancy rates at 30 and 60 days derived from bovine embryos produced by *in vitro* cultured in G1/G2 or SOF media. Different letters (a, b) above bars indicate statistical difference ($p<0.05$). Bars depict means and whiskers depict SEM.

Pregnancy losses from day 30 to day 60 ranged from 7.0 to 14.3% among the groups, which were not significantly different (Table 3).

Table 3 – Embryonic mortality in the period ranging from 30 to 60 days in pregnancies derived from embryos i*n vitro* cultured in G1/G2 and SOF medium.

	No. losses/No. pregnancies	**Embryonic mortality**
G1/G2	6/86	6.98 %
SOF	10/71	15.49 %
Total	16/157	

Experiment 2: Effect of oxygen tension [high oxygen tension (20% O_2) versus low oxygen tension (20% O_2)] on bovine embryo in vitro production and pregnancy establishment.

A total number of 2467 oocytes were used in the Experiment 2. Results (Table 4) show no effect ($p>0.05$) of oxygen tension on embryo cleavage between high O_2 group [57.3% (719/1254);Figure 4a] and low O_2 group [57.1% (639/1213)]. Also, no difference was observed in the blastocyst rate [Figure 4b; 27.9 (347/1254) and 29.3% (356/1213) for high and low O_2 group, respectively]. Nonetheless, when 477 embryos were individually transferred to recipient cows, pregnancy rate was significantly higher in low O_2 group [39.9% (111/278)] compared to high O_2 group [31.7% (63/199)] at the day 60 after E.T (Figure 4c).

Table 4 –Mean percentage and standard deviation of cleavage, blastocyst production and 60-day pregnancies derived from bovine embryos cultured in atmosphere with high (20%) or low (5%) oxygen tension.

	No.	**Nc.**	**Cleav rate (±SD)**	**Nb.**	**Blast rate (±SD)**	**N. ET**	**N.60d preg**	**60d preg rate (±SD)**
High O_2	1254	719	57.3 (17.3)	347	27.9% (15.5)	199	63	31.7% [a] (10.0)
Low O_2	1213	639	57.1% (13.5)	356	29.3% (17.0)	278	111	39.9% [b] (9.2)
Total	2467	1558	-	703	-	477	174	-

[a,b] Different superscripts within columns indicate difference ($P<0.05$). No= number of oocytes; Nc = number of cleaved embryos; Cleav rate = cleavage rate; Nb = number of blastocysts; Blast rate = blastocyst rate; N. ET = number of embryos transferred; N. 60d preg = number of pregnancies at day 60 after ET; 60d preg rate= pregnancy rate at day 60 after ET

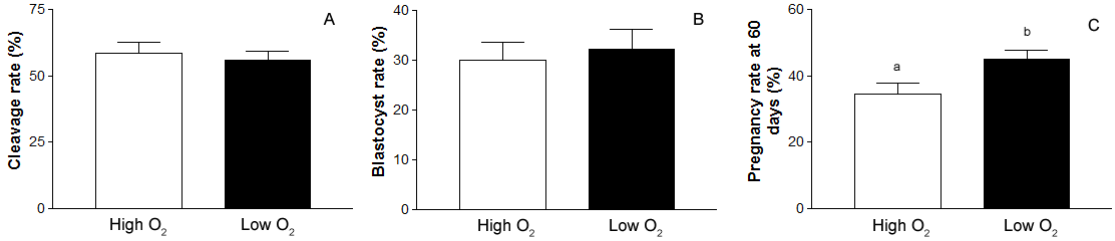

Figure 1 – Cleavage (A), blastocyst (B) and pregnancy (C) rates for bovine embryos cultured in high (20%) or low (5%) tension of oxygen. Different letters (a, b) above bars indicate statistical difference ($p<0.05$). Bars depict mean and whiskers depict SEM.

Discussion

In our professional experience with commercial bovine IVP, embryo *in vitro* development success may not be correlated with pregnancy outcome after ET Nonetheless, embryo transfer to recipient cows is usually restricted or strong limited due to its high cost and the need of infrastructure to manage and keep a herd.

The objective of this work was to improve the efficiency of our commercial bovine embryo IVP system based on literature information by using the strategy of carefully testing different in *vitro* culture conditions regarding the oxygen tension, the culture medium in the commercial routine. The experimental groups proposed have been previously proven not to have detrimental effects on the embryo development of oocytes derived from slaughterhouse ovaries (data not shown). This condition was important to assure that company results would not be compromised.

Results presented involve a high number of repetitions (10 to 45, depending on the experiment) and high number of oocytes (803 to 1254 in each group), as well as an expressive number of ET procedures (213 to 278). Therefore, not only embryo

preimplantation development from OPU oocytes is evaluated, but also embryo post-implantation viability.

We confirmed with robust data that embryo viability after ET is not fully mirrored by embryo developmental rates, and that since so many factors (oxygen tension, FCS supplementation, culture medium, cattle breed) may interfere in each specific laboratory results, this strategy is believed to bring consistent improvement in commercial efficiency without large investments on specialized and laborious assays.

The detrimental effects of atmospheric oxygen tension on cleavage and blastocyst development has been first observed in mouse embryos even after a short period exposure of the embryos (18, 19). These effects are generally believed to involve the decreased production of reactive oxygen species or ROS (20), but the redox state of the cells may be also significant in regulating no-toxic ROS concentrations, which are considered to be important to regulate various cell functions in preimplantation embryos (21). Most experiments on the bovine model confirmed that also for this species reduced oxygen tensions (5-7%) are most favorable for obtaining higher blastocyst development, as well as embryos with higher cell number (9, 22). Additionally, apoptotic cell proportion in bovine embryos produced at 5% oxygen tension was reduced compared to embryos produced at 20% oxygen tension (23). Nonetheless, the beneficial effects of low oxygen tension for embryo *in vitro* preimplantation development rate have not been confirmed by all works (24, 25) nor by our results from the experiment 1. We have only observed the beneficial effect of low oxygen tension on postimplantation embryo survival.

There are some factors which may be involved in the conflicting results of different oxygen tensions on embryo development. Peculiarities in bovine embryo IVP protocols occur among laboratories and may contribute to cause conflicting experimental results.

Regarding the experiments reported in this work, for the commercial bovine embryo oocyte source and quality are not the same as in most previously published works. Our results are based on OPU-derived oocytes and not on oocytes derived from slaughterhouse ovaries. When using oocytes obtained post mortem, normally a highly selected population of good quality oocytes is used for experiments. OPU-derived oocytes usually present less *cumulus* cells layers due to the increased manipulation compared to oocytes aspirated *post mortem*. Regarding oocyte morphological quality, only atretic oocytes are not submitted to IVF.

Another point to consider is that the SOF medium was supplemented with BSA (0.6%) as well as with FCS (2.5%) in both oxygen tensions tested. Also, even tough for low oxygen tension culture presumptive embryos should be stripped from granulosa cells, we normally observed few cells attached to the bottom of the droplet at day 7 of IVC, maybe cause the presence of coculture at the end of embry IVC at 5% oxygen tension. Embryo coculture with cells is usually performed at 20% oxygen tension (26). Nonetheless, the optimal culture conditions for embryo coculture with cells have not been defined (27).

Regarding the experiment 1, the effect of medium (SOF and G1/G2) were compared. Synthetic oviductal fluid (SOF), originally based on the biochemical analysis of ovine oviductal fluid and modified by the addition of amino acids, is a widely used medium for bovine IVC (15). Sequential media concept is based on the fact that embryo requirements vary according the embryo stage of development. Regarding energy metabolism, for example, significant changes occur from the zygote to the blastocyst stage. Bovine zygotes rely mainly on glycolisis to produce ATP, whereas in the blastocyst stage, ATP production increases by oxidative phosphorylation and therefore the glucose uptake increase is also observed (6, 8). Our results were produced under

high oxygen tension in SOF medium and low oxygen tension in G1/G2 medium, and show higher cleavage rates for embryos cultured in G1/G2 medium compared to SOF, but no difference has been observed on the blastocyst rates. On the pregnancy rate, the use of G1/G2 resulted in higher pregnancy rates on day 30 and 60 after ET

The culture medium in a general way, and the FCS in a specifically way exert significant impact on the gene expression pattern in the bovine embryo preimplantation stage (29). In fact, one of the most controversial issues regarding bovine embryo IVP strategies is the use of fetal calf serum (FCS) as culture media supplement. Even though FCS allows satisfactory rates of blastocyst development, it is an undefined culture medium supplement, which may present batch efficiency variation and sanitary concerns. It's known that FCS stimulates embryo glycolisis, alters mitochondrial metabolism, increases cytoplasmic lipid accumulation (30), alters gene expression patterns (31), and speeds blastocele formation (32). We believe that due to the low level of FCS added to SOF medium (2.5%), the negative effects of this supplement of embryo development have not occurred. In order identified if the high pregnant rates in G1/G2 medium come from the medium composition or from the low oxygen tension, we elaborated a second experiment using only SOF medium in a high and low oxygen tension. Our results show a positive effect in a pregnancy rates when the embryos are cultured in a low oxygen tension, regardless the medium culture used.

In conclusion, we show that positive effects of low oxygen tension culture on our *in vitro* embryo development process do not reflect on embryo development rates, but in pregnancy outcome. Also, the use of G1/G2 medium affects positively embryo cleavage. Nonetheless, positive effects of G1/G2 in embryo culture under low oxygen tension were seen in pregnancy rates only . These results emphasize the need of evaluating embryo viability by their transfer to recipient cows. The strategy of carefully

introducing IVC optimization procedures regarding the oxygen tension, culture medium and the influence of sex-sorted semen in the commercial routine allows fast and applicable improvement of commercial cattle embryo IVP efficiency.

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Conclusões

- A análise de um expressivo número de procedimentos de aspiração folicular e produção *in vitro* de embriões a partir de fêmeas Nelore mostrou: 1) uma média de oócitos recuperados superior aos valores da literatura para animais *Bos taurus*; 2) uma expressiva variação individual na produção de oócitos e subsequente produção embrionária.

- Para fêmeas Nelore, o método *in vitro* de produção de embriões permitiu maior número de prenhezes, em um dado intervalo de tempo, quando comparado ao método *in vivo.*

- Fêmeas Girolanda produziram mais oócitos do que doadoras Gir e Holandês. O uso de sêmen sexado apresentou resultados similares aos do laboratório para sêmen convencional. A estratégia de finalizar o desenvolvimento embrionário em estufas portáteis permitiu sucesso na utilização de receptoras distantes mais de 2.000 km do laboratório.

- Alterações nos meios de cultivo e na atmosfera gasosa apresentaram resultados similares de produção de embriões, mas houve variações nas taxas de prenhez.

Considerações finais

No primeiro artigo desta tese, mostrou-se por um número expressivo de dados, aspectos aplicados da raça Nelore na produção *in vitro* de embriões. Embora o artigo ainda esteja na fase de correções, as avaliações positivas dos revisores nos permitem acredita que será o primeiro artigo a trazer dados específicos do número de oócitos de *Bos indicus* em programas de larga escala. A importância desse artigo é a precisão dos dados, pois as citações anteriores, na sua maioria, referem-se às estimativas e comunicações pessoais. Embora os valores dessas estimativas fossem bem próximos dos valores aqui descritos, fazia-se necessário um artigo detalhado sobre o tema, considerando-se a importância desse aspecto para o cenário nacional e internacional do segmento comercial da produção *in vitro* de embriões. Também se demonstrou a importância da variação individual, retratando outro aspecto: a seleção, direta ou indireta, daquelas fêmeas com maior produção de oócitos para a aspiração folicular. Visando um rendimento maior nas etapas laboratoriais, tanto pecuaristas quanto veterinários naturalmente se inclinaram a buscar as doadoras com melhor potencial para a obtenção de oócitos. Embora a quantidade de oócitos da raça Nelore realmente tenha se mostrado alta, foi possível demonstrar uma média geral mais real, reduzindo-se números especulativos e, ao mesmo tempo, mostrando a viabilidade de se buscar fêmeas com melhor perfil para a produção *in vitro*.

No artigo de número dois, comparamos as técnicas de MOET e PIVE realizadas simultaneamente nas mesmas vacas Nelore. Ao demonstrarmos um maior número de prenhezes obtido pela técnica *in vitro*, ficou claro o motivo pelo qual o método *in vitro* tem ocupado um espaço substancial do segmento de produção de embriões no Brasil. Esses dados, já publicados, representam um cenário de mais de década da biotecnologia

da produção de embriões do Brasil, permitindo à comunidade internacional uma real compreensão de aspectos importantes: 1) a eficiência dos laboratórios nacionais nas etapas de produção embrionária e; 2) a particularidade fisiológica das fêmeas *indicus* quanto à produção de oócitos. Recordando o ceticismo da comunidade internacional quanto aos primeiros relatos da PIVE no Brasil, consideramos esse artigo bastante esclarecedor quanto aos dois itens citados. Destaca-se ainda o interesse acadêmico despertado quanto à foliculogênese em *Bos indicus*. Neste contexto científico, o maior desafio referente à PIVE no Brasil refere-se, possivelmente, à questão da grande produção de oócitos em zebuínos. Esta elevada quantidade é algo digno de nota, especialmente porque, considerando outros aspectos reprodutivos, as divergências entre *Bos taurus* e *Bos indicus* existem, mas não são tão discrepantes. A partir desses dados de campo, motivou-se a investigação científica para uma melhor compreensão dos fatores fisiológicos da população folicular ovariana em *Bos indicus*, conforme o artigo anexado como suplemento nesta tese, fruto do trabalho de uma mestranda da UEL.

O terceiro artigo enfeixa vários aspectos interessantes da técnica de PIVE. A comparação da produção de oócitos entre *taurus, indicus e taurus-indicus*, a utilização de sêmen sexado de Gir e Holandês e a logística de terminar o cultivo embrionário em estufas de transporte, com produção de milhares de prenhezes em menos de 12 meses. Um particular item desse artigo é a utilização direta da biotecnologia para a cadeia produtiva de um alimento nobre – o leite – em um país onde este aspecto se mostra tão necessário. A raça Girolanda representa a opção mais viável para a maior parte do território brasileiro, e gerar um montante expressivo de bezerras para serem futuras produtoras de leite, é certamente um dos grandes objetivos a tecnologia a serviço do homem. Apesar deste artigo ainda estar em correções, os revisores se mostraram extremamente inclinados à publicação, e vislumbra-se um impacto bastante favorável

desses dados no cenário internacional, tanto pelo interesse das técnicas descritas, quanto pelo interesse na aquisição da genética *indicus* brasileira. Cabe aqui, ainda, a constatação da urgente necessidade de padronização de protocolos sanitários para embriões produzidos *in vitro*, pois este representa o grande obstáculo para o trânsito internacional de embriões. Vislumbra-se, para um contexto imediato, a exportação de genética zebuína e seus cruzamentos, para vários países de clima tropical e subtropical, particularmente aqueles localizados nas Américas, África e Ásia.

No último artigo comparou-se várias condições de cultivo embrionário, com diferentes meios e concentrações de oxigênio. Um aspecto de destaque neste artigo é a continuidade dos experimentos até a fase gestacional, contexto bastante raro na maioria dos artigos atuais. Graças a tal particularidade, constatou-se que, apesar das taxas de desenvolvimento embrionário serem muito próximas, as condições de cultivo influenciaram diretamente as taxas de gestação. Esses resultados comprovam que, apesar da técnica de PIVE estar comercialmente estabelecida, os incrementos são possíveis e necessários.

Considerados de maneira conjunta, nossos resultados esclarecem o porquê da substituição da MOET pela PIVE na maior parte dos rebanhos de gado elite no Brasil. Demonstrou-se também que a PIVE pode ser uma ferramenta estratégica em projetos de multiplicação de larga escala de gado leiteiro, particularmente pelo uso bem-sucedido do sêmen sexado. Comprovou-se também que apesar da sua grande difusão e aplicação, a técnica de PIVE ainda pode ser melhorada. Essa melhoria se traduz em um melhor aproveitamento dos embriões transferidos, uma consequente diminuição dos custos, e uma possibilidade cada vez maior da utilização desta ferramenta nos processos de melhoramento genético de qualquer parte do mundo.

Uma lacuna quanto aos processos concernentes à PIVE refere-se à criopreservação dos embriões obtidos por esta técnica. Embora tenhamos resultados bastante interessantes de pesquisadores da área, protocolos mais eficientes e uma maior disseminação dos mesmos junto aos profissionais de campo se fazem necessários. Nosso grupo vem desenvolvendo pesquisas nesta área desde 2002 e recentemente obteve-se um sistema de cultivo embrionário alternativo, capaz de produzir embriões mais tolerantes ao processo de criopreservação. A tão reportada maior fragilidade de embriões zebuínos e as complicações decorrentes de todo o processo da PIVE não são mais barreiras intransponíveis. A viabilidade da técnica de congelamento, associada ao transporte de embriões frescos por longas distâncias, torna possível o atendimento de demandas nacionais e internacionais.

Nas décadas de 80 e 90, vimos a maioria dos países em desenvolvimento buscando genética bovina na América do Norte e Europa, apesar das óbvias restrições decorrentes das dificuldades de adaptação desses animais às zonas de clima quente. Hoje, estamos diante de uma grande possibilidade de ver o Brasil ser a fonte de biotecnologia e de genética pecuária para as futuras gerações. A magnitude desse cenário se mostra ainda maior, quando se considera a geração de alimentos nobres, justamente nos países onde são mais necessários.

Como consideração final, ressalta-se o caráter de pioneirismo de vários aspectos desse trabalho, culminando com a inserção do Brasil como um difusor de tecnologia na pecuária no cenário internacional. De igual importância é necessário ressaltar o idealismo de muitas pessoas, para transformar um trabalho de equipe em uma realidade de grandes proporções.

Anexo

"Estimate of the population of preantral follicles in the ovaries of *Bos taurus indicus* and *Bos taurus taurus* females"

O anexo a seguir, cujo texto está em fase final de elaboração, foi proposto a partir dos primeiros trabalhos desta tese. O artigo é parte do trabalho de mestrado da doutoranda Katia Cristina Fernandes da Silva. Está aqui incluído com o propósito de favorecer a discussão desta tese entre os membros da banca

Estimate of the population of preantral follicles in the ovaries of *Bos taurus indicus* and *Bos taurus taurus* females

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Abstract

Bos taurus indicus females provide a greater *in vivo* oocyte recovery (two to four times more) in comparison with *Bos taurus taurus.* This aspect has strongly contributed for the success of the embryo industry in Brazil. Little information is available to explain this difference. Our objective was to test the hypothesis that the difference in the oocyte yield is a result of higher numbers of preantral follicles in the ovaries of *B. indicus* females. Ovaries (n = 44) from Nelore fetuses (*n* = 10) and heifers (*n* = 12), and Aberdeen Angus fetuses (*n* = 10) and heifers (*n* = 12) were cut longitudinally into two halves, fixed in Bouin and processed for histological evaluation. The number of preantral follicles was estimated through counting of follicles in each histological section using the nucleus of oocyte as a marker and a correction factor. It was analyzed only one ovary per female. There was no difference ($P > 0.05$) between the average number of preantral follicles in the ovaries of *Bos indicus* and *Bos taurus* females. The average number of preantral follicles per female was 143,929 ± 253 (mean ± SD) and 285,155 ± 570, for *taurus* and *indicus* fetuses, and 76,851 ± 280 and 109,673 ± 293, for *taurus* and *indicus* heifers ($p > 0.05$). A large variation in numbers of preantral follicles was observed among individuals within the same category and between breeds. Our results suggest that there might be differences in mechanisms controlling follicle development after the preantral stage accounting for the greater oocyte yield from *Bos indicus* females.

Keywords: Preantral follicle, Fetus, Heifer, *Bos indicus, Bos taurus,* Cattle.

Introduction

In recent years, Brazil has become the leading country in the world for the number of embryos produced *in vitro* (Thibier, 2007) and this fact has been related with the number of follicles and oocytes from *Bos taurus indicus* females (Pontes et al., 2009). This aspect has strongly stimulated the emergence of researches to better understand differences between *indicus* and *taurus* females. It is accepted, for *indicus* females, a largest number of follicular waves (Figueiredo et al., 1997, Viana et al., 2000) and more follicles per wave than *Bos taurus taurus* females (Carvalho et al., 2008). Nelore (*Bos indicus*) cows have a higher population of antral follicles <5 mm (Segerson et al., 1984). Futhermore, dominant follicles and corpus luteum (CL) are smaller and estrus is shorter than for *taurus* females (Sartorelli et al., 2005; Rhodes et al., 1995).

However, none of these comparisons is as relevant as the production of oocytes that can be obtained from *Bos indicus* (Zebu) females. Intriguingly, hundreds of oocytes can be obtained from *Bos indicus* females using only one ovum pick up (OPU). Our team harvested 251 oocytes from a Nelore cow using only one OPU session (Seneda et al., unpublished data) and higher means of up to 564 oocytes were reported from females of same breed (Santos et al., 2005). The average oocyte harvested from Nelore cows per OPU session, which ranges from 18 to 25 recovered oocytes, can be three to four times higher than the average described for *taurus* females (Machado et al., 2003; Rubin et al., 2005; Martins Jr et al., 2007). In a recent work, we demonstrated repeated harvests of 60 oocytes from Zebu donors using subsequent OPU sessions (Pontes et al., 2009), without any hormone stimulation or synchronization of follicular growth.

Despite this intriguingly high number of oocytes from Nelore females, there is no explanation for this difference among *Bos indicus* and *Bos taurus* females regarding reproductive physiology, which certainly motivates studies to better understand folliculogenesis in Zebu females. Among the probable hypothesis, possibly the first to merit investigation is the population of preantral follicles as they constitute the nonreplenishable pool of healthy follicles (ovarian reserve) that will be used throughout their reproductive life.

Around 2 million stem cells can be found in the ovaries of bovine fetuses at the end of the first trimester of pregnancy, but this number is drastic diminished at the last period of fetal stage to birth (Tanaka et al., 2001), when starts follicular activation and development (Nogueira et al., 2005). Effectively recruited follicles during the reproductive lifespan are those present at birth (Soto-Suazo & Zorn, 2005; van den Hurk & Zhao, 2005) and this ovarian reserve is highly variable at birth (Erickson, 1966ab; Ireland et al., 2009).

Since sexual maturity, ovarian follicles can follow two paths: ovulation or atresia. Repetition of these two processes during postnatal life leads to progressive reduction in the number of oocytes, as the reserve of preantral follicles is gradually being consumed (Erickson, 1966ab; Eggan et al., 2006; Ireland et al., 2009). Thus, the population of preantral follicles represents the ovarian reserve because they constitute more than 90% of all ovarian follicular population (Saumande, 1991; Figueiredo et al., 2007).

Little information is available about the population of preantral follicles in bovine females. A classic work quoted the number of 200,000 primordial follicles per ovary (Erickson, 1966). Taking into consideration Zebu females, studies on the total number of follicles with ovaries from *Bos indicus* females are even more rare (Lucci et

al., 2002), which difficult the analyze of the number of preantral follicles as a possible explanation for the distinguished oocyte production obtained *in vivo* from *Bos indicus* females.

The aim of the present work was to compare the population of preantral follicles from *Bos taurus indicus* and *Bos taurus taurus* females by assessing the number of preantral follicles in ovaries of fetuses and heifers.

Materials and Methods

Ovaries Collection

Ovaries (n = 44) from *Bos taurus indicus* (Nelore, n = 10) and *Bos taurus taurus* (Aberdeen Angus, n = 10) fetuses aging from 180 to 240 days, and *Bos taurus indicus* (Nelore, n = 12) and *Bos taurus taurus* (Aberdeen Angus, n = 12) heifers aging from 20 and 24 months were collected at slaughterhouse. Fetal age was estimated from the crown–rump length (Evans and Sack, 1973) and heifers' age according Faísca et al. (2002). There were analyzed only ovaries without the presence of corpus luteum and only one ovary per female.

Histological evaluation and follicle classification

Immediately before collection, the ovaries were washed in 0.9% saline solution, cut longitudinally into two halves and fixed in Bouin's solution for 24 hours. Then ovaries were washed in tap water, weighted with an analytical balance (Bel®, Monza-Italy) and placed in 70% alcohol. The ovarian halves were dehydrated in alcohol, cleared with xylene, embbebed in paraffin and serially sectioned at 7 μm with a rotating microtome (Leica®, Wetzlar- Germany). Every 120° histological seccion (Cahill et al., 1979) was mounted and stained with periodic acid Schiff (PAS) and haematoxylin to evaluate the number of health follicles. Preantral follicles were classified according to the developmental stage as primordial (one layer of flattened granulosa cells surrounding the oocyte), primary (one layer of cuboidal granulosa cells) and secondary (two or more layers of cuboidal granulosa cells; Hulshof et al., 1994), and as normal or degenerated according to their morphological appearance. Follicular morphology was analyzed according the integrity of the basement membrane, cellular density, presence or absence of pycnotic bodies in the granulose cells and integrity of the oocyte. Based on this parameters, there were evaluated only morphologically health follicles (Carambula et al., 1999). Sections were examined and photographed using a light microscope (Nikon®, Tokyo- Japan). Using an ocular micrometer, the average diameters of the oocytes were determined by measure of two follicles of each category (primordial, primary and secondary) per section where the nucleolus of the oocyte was observed (equatorial section). Each follicle and its associated oocyte were measured in two dimensions and it was performed the arithmetic mean of two measures. All procedures were performed by the same operator.

Quantification of follicles

The number of preantral follicles was estimated by counting of follicles in each histological section and it was done by only one operator. The nucleus of the oocyte was used as a marker, according the correction factor described by Gougeon e Chainy (1987) and the following formula:

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

*Nt = Estimated total number of follicle of each category; No = Number of follicles observed in the ovary; St = Total number of cuts done in the ovary; ts = Cutting thickness; So = Total number of sections observed; do = Mean diameter of the follicle nucleus of each category.

Statistical analysis

The number of preantral follicles of each category and the average number of follicles from each female category was not normally distributed and was compared using the Mann-Whitney test ($p < 0.05$). Correlation between ovarian weight and number of preantral follicles was assessed using Simple Linear Regression.

Results

Of the 44 ovaries examined, it was analyzed 411 histological sections, on average 1,498 follicles per section. The average number of preantral follicles per ovary for *Bos indicus* and *Bos taurus* females was similar ($p > 0.05$). There were on average 143,929 ± 253 preantral follicles in the ovaries from *indicus* fetuses, 285,155 ± 570 in *taurus* fetuses; 76,851 ± 280 in *indicus* heifers, and 109,673 ± 293 in *taurus* heifers (Table 1). Similarly, there was no difference between the average number of primordial, primary and secondary follicles from *Bos indicus* and *Bos taurus* females for both age assessed ($p > 0.05$; Table 1).

Table 1. Average number of preantral follicles per ovary from *Bos indicus* (Nelore) and *Bos taurus* (Aberdeen Angus) fetuses aging from 180 to 240 days and heifers from 20 to 24 months.

	Average number of preantral follicles per ovary			
	Primordial	**Primary**	**Secundary**	**Total**
indicus fetuses	89,051	53,454	1,423	143,929
(*n* = 10)	± 200[a]	± 190[a]	± 41[a]	± 253[a]
taurus fetuses	234,570	46,414	4,172	285,155
(*n* = 10)	± 557[a]	± 133[a]	± 59[a]	± 570[a]
indicus heifers	47,436	25,351	4,063	76,851
(*n* = 12)	± 249 [b]	± 141[b]	± 54[b]	± 280[b]
taurus heifers	83,726	21,010	4,937	109,673
(*n* = 12)	± 292[b]	± 82[b]	± 86[b]	± 293[b]

* Averages with different superscripts differ at 5% level in the same column. Means followed by standard error.

It was observed a large variation in the number of preantral follicles among individuals within the same category and between breeds. The variation within Nelore fetuses ranged from 41,957 and 248,865 preantral follicles (Figure 1) and from 50,326 and 1,090,140 in Angus fetuses (Figure 2). For Nelore heifers, the variation ranged from 9,623 to 260,371 preantral follicles per ovary (Figure 3) and, for Angus heifers, from 33,798 to 320,729 follicles (Figure 4).

There was no correlation between the ovarian weight and the number of preantral follicles per ovary for both categories, except for Angus fetuses. Three Nelore fetuses with higher population of follicles (179,993; 220,602 and 248,865 follicles) presented lower ovarian weight (0.36, 0.19 and 0.19 g) than the average category weight (Figure 1).

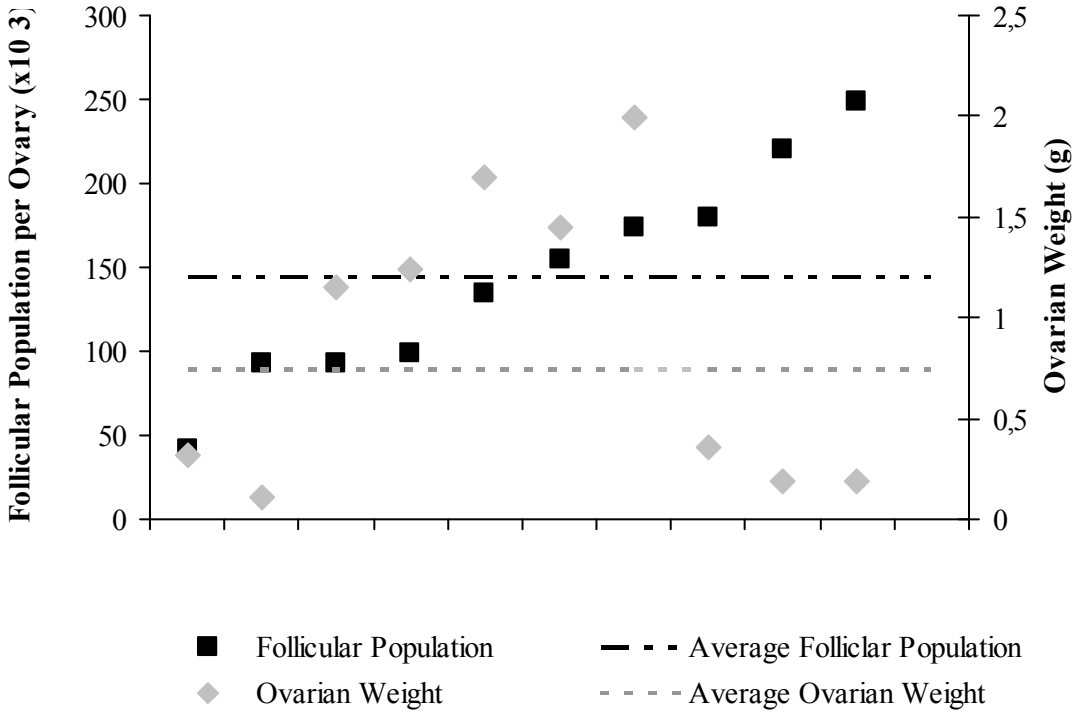

Figure 1. Average number of preantral follicles per ovary and ovarian weight of Nelore fetuses (r=0.15; p>0.01). Numbers 1 to 10 are ovaries.

Regarding Angus fetuses, there was correlation between the ovarian weight and the number of ovarian preantral follicles. Seven ovaries with lower ovarian weight than the average category weight also presented lower population of follicles than the average follicular population. Similarly, three animals that presented higher ovarian weight compared to the average also presented higher population of follicles than the average follicular population (Figure 2).

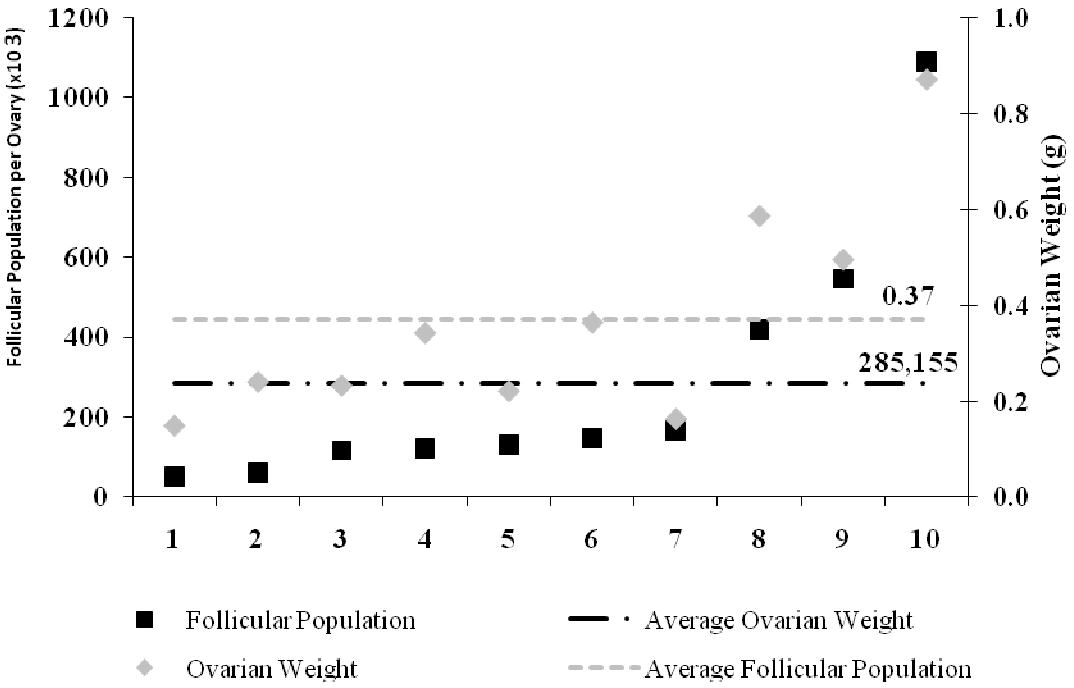

Figure 2. Average number of preantral follicles per ovary and ovarian weight of Angus fetuses ($r=0.94$; $p<0.01$). Numbers 1 to 10 are ovaries.

Two Nelore heifers that had greater ovarian weight (6.47 and 6.78 g) presented lower population of follicles (36,789.24 and 44,433.27 follicles) compared to the average. Among Nelore heifers, the female with greater population of follicles (260,371.06 follicles) presented lower ovarian weight (2.23 g) than the average, as shown in Figure 3.

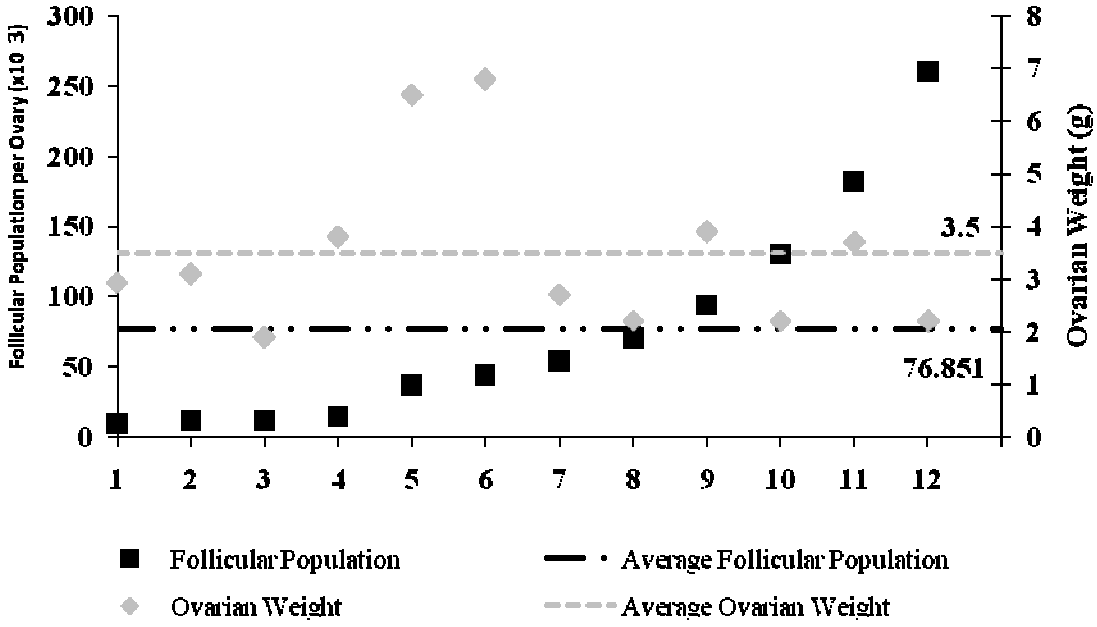

Figure 3. Average number of preantral follicles per ovary and ovarian weight of Nelore heifers (r=0.24; p>0.01). Numbers 1 to 12 are ovaries.

Among Angus heifers, the female that presented greater population of follicles (320,728.06 follicles) had lower ovarian weight (4.84 g) compared to the average. The Angus heifer with greater ovarian weight (9.30 g) had the lowest ovarian population (33,797.87; Figure 4).

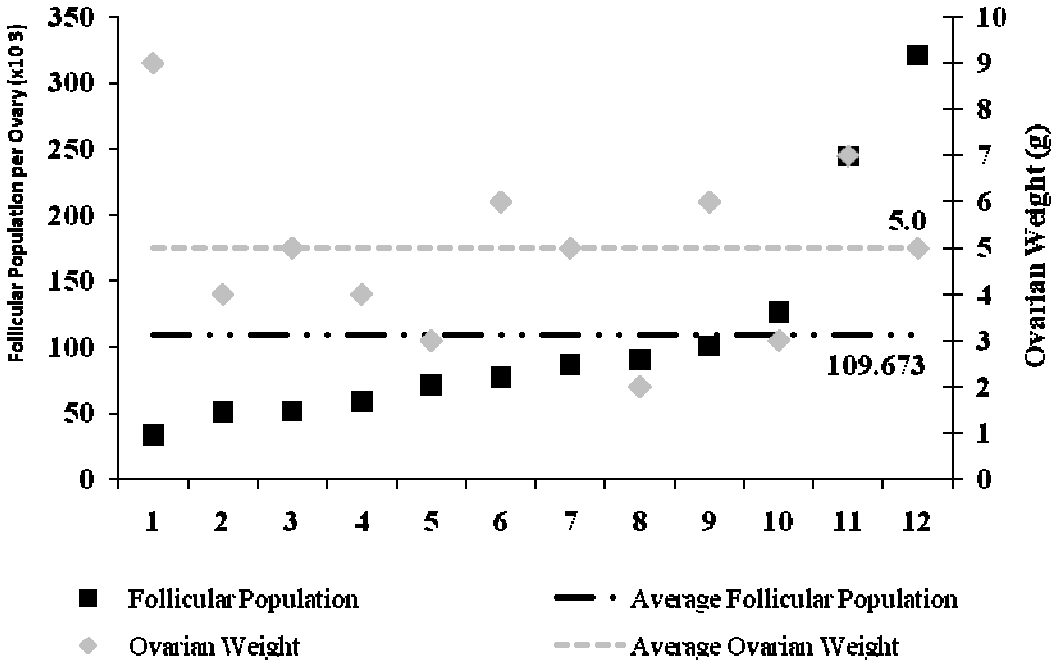

Figure 4. Average number of preantral follicles per ovary and ovarian weight of Aberdeen Angus heifers (r=0.14; p>0.01). Numbers 1 to 12 are ovaries.

Among the ovaries assessed, 38.6% (17/44) had multinucleate follicles (primordial and primary). Multinucleate follicles were observed in the ovaries from Nelore fetuses (*n* = 3) and heifers (*n* = 3) and Angus fetuses (*n* = 6) and heifers (*n* = 5). The number of oocytes within a follicle ranged between two to nine (Figure 5). Of the ovaries that presented multinucleate follicles, approximately half of them (8/17) had higher population of ovarian follicles than the average, between 33,798 (Nelore heifer) and 1,090,140 (Angus fetus) preantral follicles. It was also observed cell cords in two Nelore heifers and one Angus fetus and one Angus heifer. It was not observed relationship between the presence of cell cords and the number of follicles per ovary. The average number of preantral follicles in the ovaries that showed these structures was smaller than the average follicular population. There were on average 36,789 and 182,189 preantral follicles per ovary from Nelore heifer, 121,529 follicles from Angus fetus, and 91,673 from Angus heifer (Figure 5).

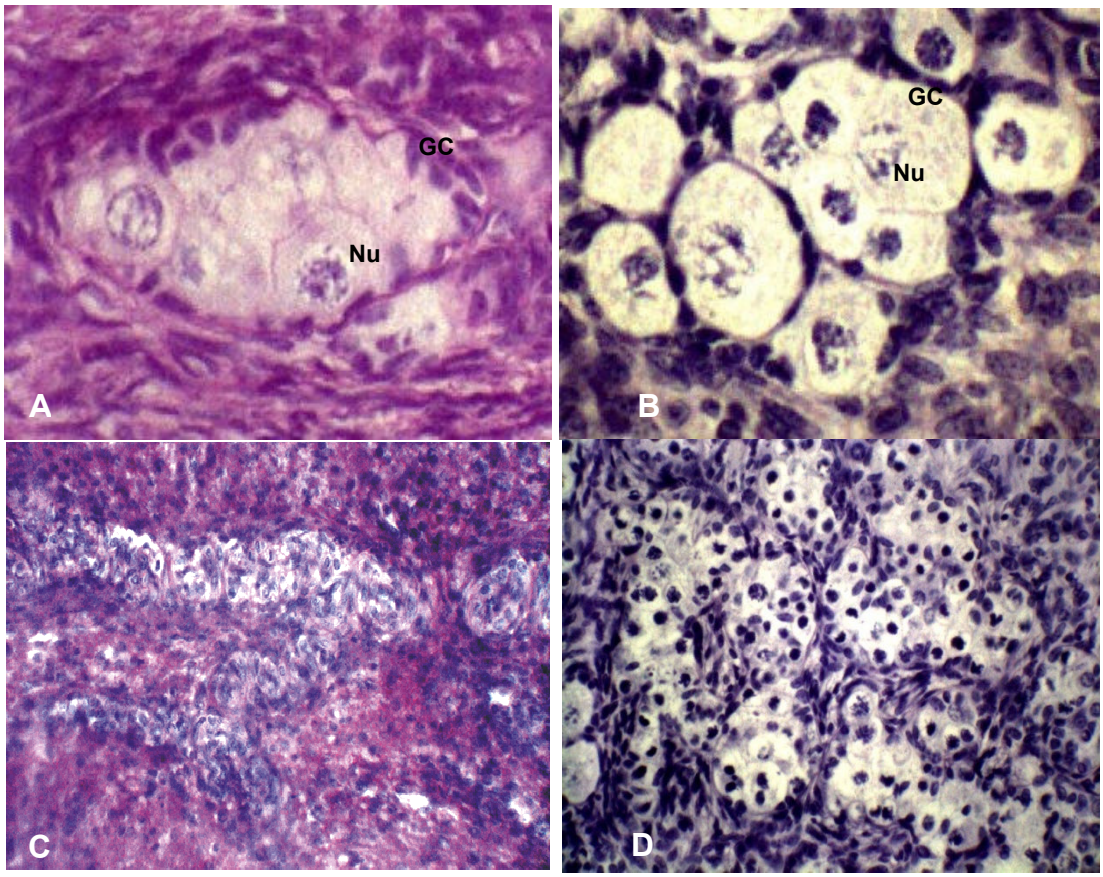

Figure 5. Histological classification of multinucleate follicles and cell cords. Multinucleate follicles in Nelore (A) and Aberdeen Angus (B) heifer. Cell cords in the ovary from Nelore heifer (C) and Aberdeen Angus fetus (D). Presumptive nucleus of oocytes (Nu) enclosed within a follicle-like cell with a single layer of granulosa cells (GC). Sections were stained with periodic acid Schiff (PAS) and haematoxylin. The scale bars represents X µm in A, B, C and D. Original magnifications X400.

Discussion

We first time presented a comparative study between the population of preantral follicles in ovaries from *Bos indicus* and *Bos taurus* fetuses and heifers. Besides contributing to a better understanding of number and morphology of preantral follicles, it was showed no difference between *indicus* and *taurus* females. Thus, it remains uncertain how it can be obtained vastly superior numbers of oocytes from *indicus* females using ovum pick up (OPU).

The follicular population in the fetal period in *Bos indicus* (143,929 follicles) presented in this work is closer to 163,216 follicles in Nelore fetuses aging 180 days (Diniz et al., 2005). For *taurus* females, our results (285,155 follicles) were higher than the approximately 102,000 follicles described by Tanaka et al. (2001) for Holstein fetuses at the end of pregnancy. However, the number of follicles in the fetal period is controversial, because while some authors suggest significant variation in the number of these structures in different gestational periods (Tanaka et al., 2001, Diniz et al., 2005), others indicate the opposite (Carambula et al., 1999), particularly after the fifth month of pregnancy. Despite this, we obtained fetal ovaries aged between six and eight months of gestation, period of shorter trend of variations in the population of preantral follicles (Muranishi et al., 2002).

Fetal ovarian weight is established after the sixth month of pregnancy, because growth of stroma, proliferation of oogonia and formation of blood vessels occur before this period (Muranishi et al., 2002). Regarding ovarian weight and number of preantral follicles, our results showed very low correlation for Nelore females and Angus heifers. Little information is available about ovarian weight and preantral follicles, but regarding antral follicles, ovaries from *indicus* females showed equally low correlation between ovarian weight and number of oocytes produced (Fernandes et al., 2001).

Despite this context for categories mentioned, Angus fetuses presented a high correlation ($r = 0.94$, $p = 0.0006$) between ovarian weight and number of preantral follicles (Figure 2). It is interest to notice that *taurus* fetuses also presented the highest number (1,090,140) of follicles from all categories mentioned. However, for Nelore fetuses it was observed a large range in values of ovarian weights (Figure 1), without any relationship with follicular population. Considering that similar reports are scarce in literature and the complexity of the actives mechanisms in fetal gonads during this period (Diniz et al., 2005), it is difficult to propose an explanation for this discrepancy observed between *taurus* and *indicus* fetuses and even between Angus fetuses and heifers.

The average number of preantral follicles (109,673) in *Bos taurus taurus* heifers was slightly smaler than that described originally by Erickson (132,000), in 1966, for 14 *taurus* heifers (Hereford) aging on average between 19 and 24 months. The approximate difference of 20,000 follicles can be considered small, because it is an evaluation of hundreds of thousands of structures in the ovary, besides the difference between breeds. Considering the uniformity of number of animals and age group, we admit agreement between our results and those from Erickson (1966), with an estimate around 120,000 preantral follicles as being representative for *Bos taurus* heifers until 24 months old.

Regarding the population of preantral follicles in *Bos taurus indicus* cows, Lucci et al. (2002) reported an average of 70,576 follicles per ovary from Nelore cows, which is close to 76,851 follicles observed in our work for heifers. Comparison of our data with those from these authors suggests an average between 70,000 and 80,000 follicles for *indicus* females after 12 months old. It is worth highlight that works on this

approach are scarce in the literature, and that there is no report specifically with *indicus* heifers.

The population of follicles in the ovaries is remarkably variable at birth. Consequently, adults also have a highly variable ovarian reserve throughout their reproductive lifespan (Erickson, 1966ab). This high individual variation makes difficult the application of any statistical model. Reports of variation between animals regarding the number of follicles include extremes from zero to 700,000 (Erickson, 1966) and similar situations were observed by Tanaka et al. (2001). In our work, we noted exactly the same situation, because extremes for *Bos indicus* fetuses were 41,958 and 248,865 follicles and, for *Bos taurus* fetuses, 50,326 and 1,090,140 follicles. For heifers, we also identified large variation, with follicular population between 9,623 and 260,371 for *Bos indicus* and 33,798 and 320,729 follicles for *Bos taurus*.

This high individual variation in the number of ovarian follicles seems to be constant throughout the reproductive lifespan, because there is high repeatability in the number of follicles per wave in adulthood (Burns et al., 2005). The same observation was done to obtain oocytes *in vivo* using OPU (Pontes et al., 2009). These ranges in the number of preantral follicles facilitates the understanding why some animals can produce hundreds of oocytes in a single OPU procedure, such as 251 (Seneda et al., unpublished data) and 564 (Santos et al., 2005), although these high numbers of *in vivo* oocytes have only been reported for *indicus* females (Pontes et al., 2009). In zebu females, recent studies demonstrated that the individual variation in the numbers of oocytes seems to be related to genes GDF9, BMP15 and FGF8 (Biase et al., 2008). These authors reported an increase of 2.26 ± 1.08 oocytes considering only the effect of FGF8, as well as a possible increase of 7.36 ± 1.12 oocytes when all genes were considered together. Besides the promising aspects described by Biase et al. (2008), we acknowledge that other factors and/or genes may be involved, because the individual variations have numbers quite higher than those reported by these authors.

Considering only values, *Bos taurus* females are superior in number of preantral follicles. Probably, individual variation have masked this difference, which is quite interesting because opposite situation is observed on *in vivo* oocyte recovery, when *Bos indicus* females have unquestionable superiority on oocyte production, which tends to be four times higher than those from *Bos taurus* females (Thibier, 2004, Rubin et al., 2005, Pontes et al., 2009). According to our results, the number of follicles established in the fetal period does not explain *indicus* females superiority on *in vivo* oocyte production and probably there are another mechanisms involved.

Bos indicus females have more follicular waves (Viana et al., 2000) and more follicles per wave (Carvalho et al., 2008), which makes the matter more intriguingly because this would mean a greater number of preantral follicles since there is high repeatability in the number of follicles per wave (Burns et al., 2005). A possible explanation could be different rates of follicular atresia between *taurus* and *indicus* females. Diminishment on follicular reserve is associated with high rates of follicular atresia (Krysko et al., 2008). It is possible that *taurus* females present higher rates of follicular atresia, although this has not been extensively studied.

In this context, it seems appropriate to mention the hypothesis of neofolliculogenesis or postnatal follicular renewal presented recently by Johnson et al. (2004 and 2005). These authors suggested differentiation of bone marrow's stem cells into germline stem cells. Although the controversy over this subject (Eggan et al., 2006), the formation of viable oocytes from stem cells has been already shown (Dyce et al., 2006).

Among the ovaries from fetuses and heifers, *taurus* and *indicus*, evaluated, 38.6% (17/44) showed structures similar to multinucleate follicles (Figure 5). For Nelore females, such structures have already been described in cows as “polyovular” follicles, with a 83% (five/six) frequency, by Lucci et al. (2002), and in fetuses in the first trimester of pregnancy, during oogenesis, as “nests of oogonia” (Diniz et al., 2005). In the early fetal life, such structures have also been reported in humans (Gondos et al., 1971) and under name of "cysts" (Pepling et al., 2006). While the germ cells are dividing to form cysts, they also interact with epithelial pregranulosa cells, becoming organized into ovigerous or ovarian cords, which remain until primordial follicles begin to form (Odor & Blandau, 1969; Byskov, 1986; Hirshfield, 1991; Guigon & Magre, 2006). Thus, it is indeed intriguingly to observe such structures during the late fetal period and especially in heifers, as evidenced in this work for *taurus* and *indicus* females, and for *indicus* cows, as reported by Lucci et al. (2002).

Besides multinucleate follicles, we also observed cell cords in Nelore heifers and Angus heifers and fetuses (Figure 5), similar to follicular cords described to germ cells and pregranulosa cells organized into ovarian cords. This occurrence is reported during fetal life (Diniz et al., 2005, Yang & Fortune, 2008), and it is not known possible explanations for this finding. Germ cells clusters appear in greater quantity at the beginning of pregnancy and gradually disappear as more primordial follicles are formed. At the end of pregnancy, the ovarian cords are almost completely disappeared and secondary follicles are present (Yang & Fortune, 2008).

Both multinucleate follicles and cell cords reported in the present study could suggest some follicular renewal activity, since these structures are typically described in early folliculogenesis. But more accurate methods are needed for these greatest investigations. It is important to notice that there was no relationship among these structures and the number of ovarian preantral follicles. However, recent studies showed that the high variation in the number of antral follicles among *Bos taurus* heifers showed high correlation with the number of polyovular follicles (Ireland et al., 2008).

Our work contributed to a better understanding of number and morphology of preantral follicles. The population of preantral follicles from *Bos indicus* heifers and fetuses did not differ from *Bos taurus* females, however, we observed an expressive individual variation. Probably, there is another difference among *Bos indicus* and *Bos taurus* females than the population of preantral follicles to explain the difference regarding oocyte production between this breeds. Atypical follicles clusters organized into nests and cords, which are classically cited during early fetal stage, suggest that more studies on this subject are needed.

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