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FACULDADE DE VETERINÁRIA

PROGRAMA DE PÓS-GRADUAÇÃO EM CIÊNCIAS VETERINÁRIAS

CRIOPRESERVAÇÃO DE OVÓCITOS IMATUROS E EMBRIÕES BOVINOS PRODUZIDOS *IN VITRO*

ARNALDO DINIZ VIEIRA

Porto Alegre, 2006

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Autor: **ARNALDO DINIZ VIEIRA**[1]

Tese apresentada ao Curso de Doutorado do Programa de Pós-Graduação em Ciências Veterinárias da Universidade Federal do Rio Grande do Sul, como requisito parcial para obtenção do grau de Doutor em Medicina Veterinária na Área de Reprodução Animal.

Orientador: **Dr. José Luiz Rodrigues**

Porto Alegre, 2006

[1] Médico veterinário, Mestre em Fisiopatologia da Reprodução Animal.

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A comissão Examinadora, abaixo assinada,

Aprova a Tese de Doutorado

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Elaborada por

ARNALDO DINIZ VIEIRA

Como requisito parcial para obtenção do grau de

Doutor em Medicina Veterinária

COMISSÃO EXAMINADORA:

Dr. José Luiz Rodrigues
FAVET/UFRGS-RS
Orientador e Presidente da Comissão

Dra. Lígia Margareth Cantarelli Pegoraro
CPACT – Centro de Pesquisa Agropecuária de Clima Temperado
Membro da Comissão

Dr. Alceu Mezzalira
CAV/UDESC-SC
Membro da Comissão

Dr. Marcelo Bertolini
CAV/UDESC-SC
Membro da Comissão

Dr. Rui Fernando Félix Lopes
ICBS/UFRGS-RS
Membro da Comissão

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"Mais que proporcionar bons rebanhos, é importante um dia saciar a fome da humanidade." (Ulrich Lenk)

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Tese de Doutorado

Programa de Pós-Graduação em Ciências Veterinárias
Universidade Federal do Rio Grande do Sul

CRIOPRESERVAÇÃO DE OVÓCITOS IMATUROS E EMBRIÕES BOVINOS PRODUZIDOS *IN VITRO*

AUTOR: ARNALDO DINIZ VIEIRA
ORIENTADOR: Dr. JOSÉ LUIZ RODRIGUES
Porto Alegre, 01 de Dezembro de 2006.

A produção *in vitro* (PIV) de embriões bovinos é uma das biotécnicas de reprodução que mais evoluíram nas últimas duas décadas. No Brasil, a associação com a técnica de aspiração folicular guiada por ultra-som determinou uma significativa disseminação do uso comercial da PIV. A produção em larga escala de embriões PIV associada à dificuldade em criopreservá-los, determinou que um número significativo desses embriões passasse a ser descartado nas atividades de campo, gerando prejuízos aos produtores e técnicos envolvidos no processo. Os experimentos realizados no âmbito desta tese tiveram como objetivo identificar e propor soluções para um aproveitamento mais eficiente dos ovócitos e embriões PIV. Em virtude da qualidade dos embriões poder ser influenciada pelo sistema de produção *in vitro*, foi realizado um experimento (Capítulo 1) para determinar a capacidade dos embriões derivados de dois sistemas de PIV em resistir à vitrificação. Entretanto, nas condições testadas, não foram verificadas influências significativas do efeito do sistema de PIV. No segundo experimento (Capítulo 2), buscou-se identificar a influência da solução crioprotetora sobre a taxa de sobrevivência embrionária *in vitro* e *in vivo*. Os resultados obtidos revelaram um efeito significativo da solução de vitrificação sobre a sobrevivência *in vitro*, por outro lado as taxas de prenhez foram semelhantes. Estes resultados também comprovaram a viabilidade do método de transferência direta desenvolvido neste experimento. Finalmente, em um terceiro grupo de experimentos (Capítulo 3), foi determinada a viabilidade da vitrificação de ovócitos imaturos usando duas estratégias de resfriamento. Não foram observadas diferenças entre os tratamentos. Entretanto, a obtenção de produtos nascidos de embriões vitrificados, derivados de ovócitos imaturos vitrificados, confirma a viabilidade de criopreservação desses ovócitos como alternativa para programação das atividades de PIV.

Palavras chave: bovino, ovócito, embrião, *in vitro*, vitrificação, transferência direta.

ABSTRACT

Doctoral Thesis in Veterinary Medicine

Programa de pós-graduação em Ciências Veterinárias
Universidade Federal do Rio Grande do Sul

CRYOPRESERVATION OF IMMATURE OOCYTES AND IN VITRO PRODUCED BOVINE EMBRYOS

AUTHOR: ARNALDO DINIZ VIEIRA
ADVISER: Dr. JOSÉ LUIZ RODRIGUES
Porto Alegre - Dezember 01, 2006.

The *in vitro* production (IVP) of bovine embryos has achieved an important development during the last two decades. In Brazil the large scale use of the follicular aspiration guided by ultrasonography has determined a significant improvement in the *in vitro* embryo production as a commercial tool. The difficulties to cryopreserve bovine oocytes and IVP embryos are at the moment the greatest barrier impairing the efficient application of this reproductive biotechnology which results in the disposal of large numbers of bovine IVP embryos. Considering these factors, this Thesis aimed to highlight new approaches to reduce embryo disposal and improve the pregnancies rates after the cryopreservation of bovine immature oocytes and IVP blastocysts. The first experiment (Chapter 1) was designed to observe the cryotolorance of embryos produced by two different IVP systems. The tested *in-vitro* systems did not show differences in the cryotolerance by *in vitro* production system. A second experiment (Chapter 2), was carried out to determine *in vitro* and *in vivo* embryo survival rates of IVP blastocysts cryopreserved using different vitrifications solutions. The results showed a significant vitrification solution effect on *in vitro* survival, however, when *in vivo*, the pregnancy rates were not different. This experiment also verified the feasibility of the direct transfer method for vitrified embryos. Finally, in a third experiment (Chapter 3) the viability of the vitrification of immature oocytes was determined using two cooling strategies. Differences among the treatments were not observed. However, calves born from the transfer of vitrified embryos, derived from vitrified immature oocytes, highlighted that the increase in cryopreservation efficiency of immature oocytes may be an important alternative to improve the IVP commercial application.

Key Words: bovine, oocyte, embryo, *in vitro*, vitrification, direct transfer.

1- INTRODUÇÃO

Apesar dos índices comercialmente satisfatórios de nascimento obtidos com a transferência a fresco de embriões bovinos produzidos *in vitro* (PIV), o total aproveitamento da potencialidade da técnica é dependente da possibilidade da criopreservação tanto de ovócitos quanto de embriões.

O armazenamento dos ovócitos garantiria a criação de bancos de material genético de fêmeas zootecnicamente superiores ou de raças ou linhagens ameaçadas de desaparecimento, bem como, permitiria sua utilização em tecnologias associadas como a transferência nuclear. Na PIV, a criopreservação facilitaria a programação no início das atividades, possibilitando ainda contornar problemas de transporte observados em programas de aspiração folicular guiada por ultrasom realizados em locais distantes dos laboratórios.

Todavia, apesar de possibilitar certa programação, o simples uso de ovócitos criopreservados não eliminaria os problemas de variabilidade nos índices de desenvolvimento embrionário, determinando a necessidade de armazenar também os embriões excedentes. Esta condição é indispensável para o favorecimento da comercialização, da redução nos custos de sincronização e manutenção de receptoras, evitando especialmente as perdas de embriões observadas atualmente em função da reduzida taxa de sobrevivência embrionária obtida com métodos convencionais de congelamento.

A baixa viabilidade de ovócitos e embriões PIV submetidos à criopreservação se deve principalmente a sensibilidade que essas estruturas apresentam ao resfriamento. Quando expostos as temperaturas correspondentes à fase termotrópica de transição dos lipídios (ARAV *et al*., 1996), estas estruturas sofrem diferentes graus de alterações, de acordo com o tempo de exposição, característica que torna a vitrificação a técnica de criopreservação mais promissora, por possibilitar a rápida passagem através dessa faixa crítica de temperatura.

Apesar do incremento nos índices de desenvolvimento de blastocistos obtidos a partir de ovócitos imaturos vitrificados (YAVIN & ARAV, 2001; VIEIRA *et al*., 2002; ABE *et al*., 2005; SANTOS *et al*., 2006) e de elevação nas taxas de eclosão *in vitro*,

obtidas a partir de embriões PIV vitrificados (VAJTA *et al*., 1999), ainda se observa uma baixa correlação entre estes resultados e as taxas de prenhez observadas *in vivo*. Isto pode ser o reflexo não apenas do efeito de falhas metodológicas determinantes da exposição às temperaturas críticas, choque osmótico, efeitos tóxicos ou fraturas estruturais, mas também de deficiências no sistema de produção, antes ou depois das estruturas serem submetidas ao processo de vitrificação (VAJTA *et al*., 1999).

Como as intervenções na origem e qualidade intrínseca dos ovócitos resultam em pequenos incrementos de viabilidade, deve-se considerar o fato de que os maiores ajustes devem ser realizados no período pós-fecundação por ser a fase de cultivo mais crítica na determinação da qualidade dos embriões (LONERGAN et al., 2003).

Estes fatores determinam que além da necessidade em adequar os protocolos de vitrificação, sejam empregados sistemas de cultivo *in vitro* que possibilitem que as estruturas criopreservadas possam contornar possíveis danos, e expressar sua capacidade de regeneração e desenvolvimento *in vitro* e *in vivo*.

2- REVISÃO BIBLIOGRÁFICA

A partir do surgimento das chamadas tecnologias abertas de vitrificação (STEPONKUS et al., 1990; LANDA & TEPLA, 1990), a capacidade de obtenção de altas velocidades de resfriamento foi bastante incrementada, favorecendo a obtenção do estado vítreo.

Entretanto, o efeito do isolamento térmico criado pela liberação de vapor resultante da transferência de calor da amostra para o nitrogênio líquido tem afetado a homogeneidade do resfriamento. Esta limitação possibilita a ocorrência de danos durante a passagem através da zona de temperatura crítica e favorece a ocorrência de nucleação de gelo (desvitrificação).

O uso de nitrogênio super-resfriado pela ação do vácuo permite contornar estes problemas por proporcionar um marcante aumento na velocidade de resfriamento (HAN et al., 1995), especialmente na faixa entre +20 e –10^{o}C (ARAV *et al*., 2002).

A utilização de suportes de vitrificação com maior capacidade de condução térmica, como é o caso dos capilares de vidro usados em substituição às tradicionais palhetas de plástico (CHO *et al*., 2002; VIEIRA et al., 2006), pode atuar sinergicamente aumentando a velocidade de resfriamento. O emprego desses capilares também possibilita o aumento na velocidade de aquecimento, condição necessária para impedir a ocorrência de fraturas estruturais provocadas por desvitrificação durante esta fase do processo.

A alta concentração de crioprotetores presentes na solução de vitrificação atua reduzindo a possibilidade de desvitrificação. Entretanto, a capacidade de indução e manutenção do estado vítreo difere entre os crioprotetores, em função de suas características químicas. Assim, soluções compostas por crioprotetores considerados como sendo fracos indutores da vitrificação, como o etileno glicol (EG) (BAUDOT *et al*., 2000), necessitam ser utilizados em concentrações elevadas, aumentando o risco de toxidade. Este problema pode ser minimizado pela associação com crioprotetores classificados como fortes indutores do estado vítreo, como o dimetil sulfóxido (DMSO) (BAUDOT *et al*., 2000). A associação dos crioprotetores permite a obtenção de uma solução com boa capacidade de vitrificação, aliada à redução na toxidade da solução. A

capacidade de vitrificação também pode ser influenciada pela adição de açúcares ou macromoléculas, que atuam aumentando a molaridade da solução, favorecendo a obtenção do estado vítreo de forma semelhante ao aumento da pressão hidrostática (RALL, 1987). Outra alternativa para obtenção do estado vítreo é o uso de aditivos que atuam como bloqueadores da nucleação de gelo, como é o caso das "proteínas anticongelantes" (ARAV et al., 1994) e dos polímeros sintéticos como o álcool polivinílico (WOWK *et al*., 2000).

Os efeitos osmóticos determinados pela alta concentração da solução de vitrificação podem ser minimizados mediante à exposição dos ovócitos e embriões a soluções menos concentradas, reduzindo as lesões estruturais causadas por choque osmótico (ABE *et al*., 2005). O uso de um período longo de exposição a uma solução de 2 a 4% de EG previamente a exposição às soluções mais concentradas aumenta a viabilidade das estruturas vitrificadas (PAPIS *et al*., 2000), provavelmente por permitir uma adaptação osmótica e saturação mais homogênea do crioprotetor dentro das células.

Apesar de terem sido testados uma série de ajustes, que potencialmente reduzem danos determinados pelas metodologias de vitrificação, a manifestação de seus benefícios é dependente de uma associação com possíveis fatores de incremento na capacidade de suporte dos meios de cultivo.

O desenvolvimento dos meios utilizados na PIV está baseado em duas correntes: a) a que busca a determinação de um meio definido criado com base nas necessidades dos embriões, e b) a que busca simular as condições fisiológicas de desenvolvimento embrionário. A partir desta última, foi formulado o fluido sintético de oviduto (SOF) (TERVIT *et al*., 1972), um dos meios de uso mais difundido na PIV. Com objetivo de incrementar as taxas de desenvolvimento embrionário, o SOF foi a base para diversos ajustes, dentre os quais, a adição de aminoácidos (TAKAHASHI & FIRST, 1992) e fatores embriotróficos como o citrato e inositol (HOLM *et al.*,1999). Entretanto, apesar de terem sido realizados ajustes em sua formulação, muitos compostos ainda estão presentes em concentrações muito inferiores às observadas *in vivo*. Quando algumas destas distorções são corrigidas, pode-se observar um incremento na taxa de desenvolvimento embrionário. Isto pode ser confirmado pelo aumento no número de embriões resultante da adição de glicina ao meio de cultivo (MOORE & BONDIOLI, 1993), resultado que pode ser justificado pelo fato de que, de um total de 24 tipos de aminoácidos pesquisados nos fluidos do oviduto e útero, a concentração de glicina corresponde a 44% e 27%, respectivamente (ELHASSAN *et al*., 2001).

Em virtude da característica de acelerar a velocidade de desenvolvimento embrionário (KHURANA & NIEMANN, 2000; RIZOS *et al*., 2003), e permitir um aumento na taxa de eclosão *in vitro* de embriões vitrificados (VAJTA *et al*., 1999), o soro sanguíneo ainda é largamente utilizado no cultivo *in vitro*. Porém, Rizos *et al*. (2003) relataram uma redução na transcrição de alguns genes e na criotolerância de embriões produzidos na presença de soro. Esses efeitos podem ser em parte revertidos mediante o condicionamento do meio de cultivo com células da granulosa, em função de sua capacidade de aumentar a criotolerância e viabilidade *in vivo* dos embriões (RIZOS *et al*., 2001).

Todos estes resultados potencialmente eficientes em aumentar a viabilidade embrionária são oriundos de trabalhos realizados por diferentes grupos, não havendo trabalhos avaliando as respostas determinadas por possíveis efeitos sinérgicos entre modificações nos procedimentos de cultivo *in vitro* e procedimentos de vitrificação. Desta forma, faz-se necessária a determinação da viabilidade de uso das seguintes associações:

- Utilização de micropipetas de vidro como suportes de vitrificação em substituição às palhetas plásticas estiradas, em função da característica física de maior condutibilidade térmica do vidro.
- Realização de adaptação osmótica com baixa concentração de crioprotetores como forma de reduzir o choque osmótico gerado pela exposição às soluções de vitrificação.
- Adição de álcool polivinílico como estabilizador do estado vítreo nas soluções de vitrificação.
- Utilização de nitrogênio líquido super-resfriado pela ação do vácuo, como estratégia de acelerar a velocidade de resfriamento e reduzir a sensibilização ao resfriamento.
- Utilização do aminoácido glicina e do co-cultivo com células da granulosa como formas de ajuste do meio de cultivo as necessidades dos embriões.

3- CAPÍTULO 1

Bovine *in vitro* embryo production protocol: does it really influence embryo cryotolerance?

(Protocolo de produção *in vitro* de embriões bovinos:
este realmente influencia a criotolerância dos embriões?)

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Bovine *in vitro* embryo production protocol: does it really influence embryo cryotolerance?*

Protocolo de produção *in vitro* de embriões bovinos:
este realmente influencia a criotolerância dos embriões?

Arnaldo Diniz Vieira, Fabiana Forell, Cristiano Feltrin,
Lucila Carboneiro dos Santos & José Luiz Rodrigues

ABSTRACT

The protocol of *in vitro* production (IVP) of bovine embryos is one of the critical factors determining embryo viability after cryopreservation. In this study were used two differents protocols to produce IVP bovine embryos, with variations in protein source, oocyte/zygote density per media volume, with the aim to determine the *in vitro* and *in vivo* embryo survival after vitrification using hand-pulled glass micropipettes. Expanded blastocysts (D7) were morphologically selected by size (≥180 μm) and osmotic behavior before they were randomly allocated to sub-groups by protocol: non-vitrified embryos (control; C) and vitrified embryos (V). For the evaluation of the *in vitro* survival, control embryos and a group of warmed vitrified embryos were *in vitro*-cultured (IVC) for 72 h. Re-expansion rates of warmed embryos at 24 h of IVC were 94.8% and 93.2% for Protocols 1 and 2, respectively. Hatching rates at 72 h of IVC of embryos from Protocol 1 (C=80% and V=75.8%) tended to be higher (P=0.0561, Chi2 test) than those from Protocol 2 (C=67.2% and V=59.3%). For the evaluation of *in vivo* survival, 21 vitrified embryos per protocol were singly non-surgically transferred to synchronized recipients (n=42) after the in-straw cryoprotectant dilution, resulting in 4 (19%) pregnancies per group on Day 60 of gestation. In conclusion, despite a lower variation on *in vitro* embryo development between both IVP protocols, the use of different protocols under the same laboratory conditions did not affect the *in vitro* and *in vivo* embryo viability after vitrification into hand-pulled glass micropipettes.

Key words: vitrification, embryo survival, direct transfer.

RESUMO

O protocolo de produção *in vitro* (PIV) de embriões bovinos é um dos fatores críticos na determinação da viabilidade dos embriões após a criopreservação. Neste estudo foram utilizados dois protocolos de PIV com variações na fonte protéica, relação oócito/zigoto por volume de meio e condições de cultivo objetivando determinar a sobrevivência *in vitro* e *in vivo* dos embriões após vitrificação dentro de micropipetas de vidro estiradas à mão. Blastocistos expandidos (D7) oriundos dos dois protocolos foram selecionados morfologicamente pelo tamanho (≥180 μm) e comportamento osmótico antes de serem aleatoriamente distribuídos em dois sub-grupos: embriões não vitrificados (controle; C) e embriões vitrificados (V). Para a avaliação da sobrevivência *in vitro*, os embriões controle e grupos de embriões reaquecidos foram cultivados *in vitro* (CIV) por 72 h. As taxas de re-expansão após 24 h de CIV dos embriões re-aquecidos foram 94,8% e 93,2% para os Protocolos 1 e 2, respectivamente. As taxas de eclosão (72 h) dos embriões do Protocolo 1 (C = 80% e V = 75,8%) tenderam a ser maiores do que as do Protocolo 2 (C = 67,2% e V = 59,3%). Para a avaliação da sobrevivência *in vivo*, após a remoção dos crioprotetores dentro da palheta, 21 embriões vitrificados de cada protocolo foram transferidos por via transcervical para receptoras síncronas (n=42). Aos 60 dias de gestação, foram diagnosticadas por palpação retal 4 (19%) prenhezes em cada grupo. Em conclusão, apesar de uma pequena variação na taxa de desenvolvimento *in vitro* entre os diferentes protocolos, os resultados de sobrevivência *in vitro* e *in vivo* demonstraram que, sob condições laboratoriais similares, os diferentes protocolos não afetaram a viabilidade dos embriões após a vitrificação em micropipetas de vidro estiradas à mão.

Descritores: vitrificação, sobrevivência embrionária, transferência direta.

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CORRESPONDENCE:
A.D. Vieira [vieira_ad@yahoo.com.br ; Fax: + 55 51 3316 7305].

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INTRODUCTION

Bovine embryos generated by distinct *in vitro* production (IVP) systems can respond differently to cryopreservation approaches [1,5,36]. Consequently, it is difficult to compare data from different laboratories due to the diverse experimental and enviromental conditions. This variability may be determined by a multitude of factors such as water quality [21,22], type of oil overlay [36], oocyte quality [15,28], and culture systems [18,19,27], among others. The embryo cryotolerance largely depends on the IVP conditions, which is a determinant of embryo quality, but cryosurvival can also be improved by adjusting or improving the cryopreservation procedures. The OPS technology has been proven successful not only for the vitrification of bovine immature oocytes and IVP embryos [37], but also in combination with the in-straw cryoprotectant dilution method [16,34]. In addition, the rapid cooling and warming rates in the OPS system may be accompanied by variations in the vitrification container [3,20]. In this view, the use of glass micropipettes may offer physical advantages over plastic straws. However, methodologies that allow the direct transfer of the embryos after the in-straw cryoprotectant dilution [16,34] have not been tested with glass micropipettes.

The aim of this study was to determine the influence of two IVP protocols on the *in vitro* development and *in vitro* and *in vivo* survival of bovine expanded blastocysts after vitrification into hand-pulled glass micropipettes.

MATERIALS AND METHODS

Source of cumulus-oocyte complexes

Bovine ovaries were transported to the laboratory in PBS at 24-32ºC upon slaughter. *Cumulus oocyte complexes* (COCs) isolated by ovary slicing were evaluated under a stereomicroscope and only COCs with evenly granulated cytoplasm and surrounded by a compact and dense cumulus cell layer were used in two distinct IVP protocols.

In vitro Embryo Production Protocols

The IVP systems used in this study were based on established procedures by Rodrigues *et al*. [29] and Vieira *et al.*, [37], for Protocols 1 and 2, respectively, with minor modifications. Distinctions between IVP protocols during the various embryo production steps are depicted in Table 1.

• ***In vitro* maturation (IVM).** COCs were *in vitro* matured in TCM-199 supplemented with 25.0 mM NaHCO3, 0.2 mM pyruvic acid, 50 µg/mL gentamicin, 0.5 µg/mL FSH, 0.03 IU/mL

hCG and heat inactivated estrous serum (Table 1) for 24 h at 39ºC and 5% CO_2 in humidified air.

• ***In vitro*** **fertilization (IVF).** Frozen-thawed spermatozoa were segregated by swim-up in Sperm-TALP [25]. Capacitated sperm cells were added to Fert-TALP medium [25] at 10^6 cells/mL and co-cultured with COCs for 20 h.

• ***In vitro*** **culture (IVC).** For Protocol 1, presumptive zygotes were cultured in modified SOF medium [6] at 39ºC for 168 h, under the conditions described in Table 1. Cleavage rates were recorded on Day 2 of development (IVF = Day 0). For IVC in Protocol 2, presumptive zygotes were incubated in SOFaaci medium [11] at 39ºC in 5% CO_2 in humidified air. Cleavage rates were recorded at 24 h of IVC, and at this time, uncleaved structures were removed from the wells and embryos were further cultured, under controlled atmosphere (Table 1) for additional 144h at 39ºC.

Table 1. Major distinctions between protocols (Prot.) for bovine embryo production

IVP Step	Prot.	E_2	Protein source	Structures	Media volume	Media	Oil overlay	O_2 tension
IVM	1	Yes	10% ECS	15 COC	100 µL	TCM 199	Yes	20%
	2	No	10% EMS	30-40 COC	400 µL		No	20%
IVF	1	-	BSA	15 COC	100 µL	Fert-TALP	Yes	20%
	2	-	BSA	30-40 COC	400 µL		No	20%
IVC	1	-	10% ECS	20 Emb.	80 µL	SOF	Yes	5%/168h
	2	-	5% EMS	30-40 Emb.	400 µL	SOFaaci	Yes	20%/24h 5%/144h

Embryo selection

At the end of the culture period (168 h = Day 7), blastocyst rates were recorded and only embryos reaching the expanded blastocyst stage (≥180 µm diameter) and classified morphologically as grade 1-2 [10] were selected for the experiments. The basic medium (BM) for embryo maintenance, vitrification and warming was PBS supplemented with 10% fetal calf serum (FCS). Embryos from both protocols that did not shrink after exposure to BM for 5 min were randomly allocated either to the non-vitrified control sub-group or to the vitrified sub-group. Embryos identified as controls were maintained in BM on a warm stage at 39ºC for approximately 60 min, time required for the vitrification and warming of both vitrification sub-groups. Twelve replicates were carried out to standardize procedures and minimize experimental variations.

Vitrification procedure

Embryos were exposed for 5 to 10 min to BM + 3% ethylene glycol (EG) for the evaluation of their osmotic behavior [13] and to promote an additional cryoprotectant cell influx to attain cellular saturation prior to vitrification [24]. Embryos that did not fully reexpand after shrinkage were discarded from the study. The vitrification procedure was based on methods originally described by Vajta *et al*. [35] using hand-pulled glass micropipettes (GMP), with outer diameters of approximately 0.6 mm, instead of plastic straws. GMPs containing vitrified embryos to be warmed for the *in vitro* development experiment were held vertically in a small plastic tube partially immersed in LN2, whereas GMPs containing vitrified embryos to be stored for their subsequent transfer to recipients (*in vivo* development experiment) were placed into pre-cooled and labeled 0.5 mL plastic straws [33], and stored in a LN2 tank.

Warming and in straw cryoprotectant dilution procedures

The warming procedure was based on the methods originally described by Isachenko *et al*. [12], using a warming solution (WS) composed of BM + 0.26 M sucrose. Following warming and pre-dilution [12], the contents of the GMPs were transferred into a plastic straw loaded with WS. For the *in vitro* development experiment, each straw loaded with a single embryo was held horizontally for 10 min at RT; then, embryos were expelled into a dish containing BM. Subsequently, vitrified and the non-vitrified control embryos were transferred to 100 mL drops of SOFaaci supplemented with 5% (v/v) of EMS, under oil, for additional 72 h of IVC for the determination of re-expansion and hatching rates. For the *in vivo* development experiment, each straw loaded with a single embryo was held horizontally for 5 min, at RT, prior to the direct non-surgical single embryo transfer (ET) to synchronous (± 24 h) recipient females. Pregnancy diagnosis was performed on Day 60 of gestation (53 days after ET) by rectal palpation.

Statistical analysis

Re-expansion, hatching and pregnancies rates were analyzed by the Chi-square test. Probability of $P<0.05$ was considered statistically significant.

RESULTS

After twelve replications, cleavage rates were different ($P=0.0002$) between protocols. However, the number of expanded blastocysts on D7 was similar between groups (Table 2). Re-expansion rates of vitrified embryos did not differ

(P>0.05) between protocols, but hatching rates of both vitrified and nonvitrified control embryos from Protocol 1 had a trend (P=0.0561) to be higher than embryos from Protocol 2 (Table 3). Embryo transfers after in-straw cryoprotectant dilution resulted in 4 (19%) pregnancies per experimental group at 60 Days of gestation.

Table 2. Comparative embryo yield efficiency between two IVP protocols (12 replications).

Protocol	IVC n	Cleavage n (%)	Embryos D7 n (%)	Expanded blastocysts n (%)
1	2155	1491[a] (69.2)	523[a] (24.7)	253[a] (11.7)
2	2605	1931[b] (74.1)	583[a] (22.4)	279[a] (10.7)

[ab] Columns without common superscripts differ (P<0.05)

Table 3. *In vitro* developmental rates of vitrified and non-vitrified (control) embryos produced by two different IVP protocols.

Protocol	Re-expansion at 24h		Hatching at 72h	
	vitrified	control	vitrified	control
1	55/58[A] (94.8%)	-	44/58[A] (75.8%)	48/60[A] (80.0%)
2	55/59[A] (93.2%)	-	35/59[B] (59.3%)	43/64[B] (67.2%)

[A,B] Columns without common superscripts differ (P<0.1)

DISCUSSION

The worldwide increase in the number of research groups adopting IVP technologies, along with the rise in its commercial applications, have tremendously diversified IVP procedures among different laboratories. Consequently, embryo production efficiencies between IVP protocols, or even laboratories, are very difficult to reconcile. In this study, embryo developmental rates were relatively similar between IVP protocols, despite differences regarding presence or absence of oil underlay and/or steroid hormone during IVM, or protein source, oocyte/embryo density, and media volume during the IVP steps. The medium droplet method used in Protocol 1 was first introduced by Gwatkin [9] for mammalian embryo culture and still remains very popular for the IVP of mammalian embryos. Nevertheless, it is known that the use of oil overlay determines a high contacting surface/volume ratio with the culture medium, resulting in an interaction, and potential exchange, of lipophilic substances (e.g., estradiol, progesterone) between both fluids, which may cause changes in medium composition [36], affecting embryo development. In this view, our results corroborated with Xu *et al*. [38], in which the absence of oil overlay during bovine IVM showed better cleavage rates than the maturation in medium under oil (Table 2). Such differences could well be attributed to better IVM conditions under Protocol 2 (no oil overlay). In addition, the use of larger numbers of oocytes [15] or medium volume during IVM may also be beneficial to maturation. However, despite the higher cleavage rate observed in Protocol 2, blastocyst rates were not different between IVP protocols. This effect could be associated with the embryo

incubation in 5% CO_2 in air (20% O_2) during the first 24 h of IVC, which is in accordance with results by others [40]. This biphasic gas atmosphere procedure was used in Protocol 2 to avoid the excessive manipulation of the dish prior to cleavage rate evaluation and removal of uncleaved structures from the wells [30]. Yuan *et al.* [40] demonstrated that the IVC at 20% O_2 for 72 h compromised the subsequent zygote development, even if the O_2 tension were lowered to 5% afterwards. In Protocol 1, the entire IVC period occurred under a low O_2 tension (5%), and in a higher embryo density [26] and serum concentration (10% vs. 5%) than Protocol 2. More serum in the medium provides more glucose levels and stimulates blastocysts development by accelerating the process of blastulation [17, 31, 39]. Despite these potential embryotrophic effects in Protocol 1, the use of EMS in Protocol 2 could also confer similar blastocyst yields, probably induced by embryotrophic factors (e.g., polyamine mitogenic factor) present in the equine serum [7]. The similar blastocyst rates observed between both IVP protocols showed compensatory differences during the distinct stages of the IVP process.

These data are in accordance with the observation that embryo production is also dependent on the origin and quality of the oocytes [15, 28], in parallel to the IVP protocol. During embryo selection by morphology and diameter, some embryos shrank upon exposure to PBS + FCS, despite medium isosmolarity. This osmotic behavior is thought to be an adjustment of the embryonic metabolic pathways to the abrupt change in substrate composition [8]. In this study, embryos that shrank in PBS or in a 3% EG solution were discarded; only embryos capable of activating osmotic control mechanisms necessary to tolerate the cryopreservation process [13] were selected. Interestingly, in some replications of this study, part of the embryos in the control group did not hatch after IVC, despite the selection of early blastulation and higher diameter criteria to improve embryo selection [23]. The lower hatching rate in non-vitrified embryos appeared to characterize metabolic alterations not identified or detectable during the selection process. In the vitrified groups, embryos from Protocol 1 had a trend for a better hatching rate ($P=0.0561$, Chi2 test). However, this trend observed in the *in vitro* survival study was not confirmed in the *in vivo* studies after the transfer of embryos to synchronous recipients ($P>0.05$). These results agree with Donnay *et al*. [4], i.e., that no correlation exists between hatching and pregnancy rates. The lack of correlation between *in vitro* and *in vivo* development could also be attributed to

the influence of the IVC conditions in the hatching rates post-warming [27]. If *in vitro*-produced bovine embryos are to be fully utilized in practice, the warming procedure must be simple and efficient enough to allow embryos to be handled and transferred in a fashion as simple as artificial insemination with thawed semen. In this study, embryos were successfully vitrified employing handpulled glass micropipettes (GMP) instead of hand-pulled plastic straws [35]. The use of a tube with a larger opening for the warming of vitrified samples [12] enhances the warming rate and facilitates the process when compared with the use of 0.25 mL straws [16,34]. In addition, the dilution of the cryoprotectant agents within plastic straws allowed embryos to be prepared, effectively and promptly, for direct transfer without the need of a stereomicroscope, which represents a considerable improvement and simplification for the direct transfer of bovine embryos vitrified into GMP or OPS.

The vitrification procedure usually results in cellular and subcellular damage, most of which are gradually restored over a 24 h period [32]. A reduction in the inner cell mass and trophectodermal cell number in vitrified embryos [14] might be one of the possible causes of placentary alterations, which may be associated with lower gestation rates after *in vitro* embryo manipulations [2]. In this study, pregnancy rates on Day 60 for both protocols were lower than those reported by Lewis *et al*. [16]. However, those authors transferred two embryos per recipient, and performed the pregnancy diagnoses 40 days after transfer, which should confer higher pregnancy rates than the data reported in this study. The transfer of a single embryo per recipient and the pregnancy diagnosis on Day 60 were chosen for practical and economical reasons, since the highest rates of embryonic and fetal losses in IVP embryos are known to occur between Days 30 and 44 of pregnancy [2]. After this period, pregnancy rates will reflect more closely the effectiveness of each IVP protocol.

This fact highlights the importance of a late gestation diagnosis, which is of economical importance to production *per se*, since losses after Day 60 of pregnancy do not appear to be higher in IVP pregnancies than controls [2].

CONCLUSIONS

Major variations in IVP protocols in this study included oil overlay and/or estradiol supplementation, protein source, oocyte/zygote density, medium volume, and culture atmosphere. Such differences in IVP protocols, under the same laboratorial conditions, did not affect

embryo development to the blastocyst stage, the cryotolerance of expanded blastocysts, and pregnancy rates of vitrified embryos following transfer to synchronous recipients. Further work is required to determine the effect of additional steps in different protocols on embryo yield, quality and cryotolerance. However, judging from these *in vitro* and *in vivo* experiments, the vitrification of embryos in hand-pulled glass micropipettes, combined with alternative warming and in-straw cryoprotectant dilution procedures, was proven effective for the direct transfer of IVP bovine blastocysts under field conditions, which has an important role in the practical implications for IVP research and its commercial application.

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4- CAPÍTULO 2

In-straw cryoprotectant dilution of IVP bovine blastocysts vitrified in hand-pulled glass micropipettes

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In-straw cryoprotectant dilution of IVP bovine blastocysts vitrified in hand-pulled glass micropipettes

A.D. Vieira*, F. Forell, C. Feltrin, J.L. Rodrigues

Embryology and Biotechnology of Reproduction Laboratory, Faculty of Veterinary Medicine, UFRGS,Cx Postal 15004, 91501-970 Porto Alegre, RS, Brazil

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Abstract

The aim of this study was to determine the influence of two ethylene glycol-based vitrification solutions on *in vitro* and *in vivo* survival after in-straw cryoprotectant dilution of vitrified *in vitro*-produced bovine embryos. Day-7 expanded blastocysts were selected according to diameter (≥180 μm) and osmotic characteristics and randomly assigned to one of three groups (i) VSa: vitrification in 40% EG + 17.1% SUC + 0.1% PVA; (ii) VSb: vitrification in 20% EG + 20% DMSO; (iii) control: non-vitrified embryos. Vitrification was performed in hand-pulled glass micropipettes (GMP) and cryoprotectant dilution in 0.25 ml straws after warming in a plastic tube. Embryo viability was assessed by re-expansion and hatching rates after 72 h of IVC and by pregnancy rates after direct transfer of vitrified embryos. No differences in re-expansion rates were observed between vitrified groups after 24 h in culture (VSa = 84.5%; VSb = 94.8%). However, fewer VSa embryos (55.2%, $P < 0.05$) hatched after 72 h than the VSb (75.8%) and control embryos (80.0%). To evaluate *in vivo* viability, vitrified embryos (VSa = 20; VSb = 21) were warmed under field conditions and individually transferred to synchronous recipients. Pregnancy rates (day 60) were similar between groups (VSa = 20%; VSb = 19%). Greater hatching rates occurred after 72 h of IVC for EG +DMSO than EG + SUC + PVA vitrification solutions. However, using a GMP vitrification container and in-tube warming, both solutions provided similar pregnancy rates after the in-straw cryoprotectant dilution and direct embryo transfer.

Keywords: Cattle; Ice-blocking; Glass micropipettes; Direct embryo transfer

* Corresponding author. Tel.: +55 51 3316 6126; fax: +55 51 3316 7305.
E-mail address: vieira_ad@yahoo.com.br (A.D. Vieira).
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1. Introduction

In vitro-produced (IVP) bovine embryos are more sensitive to cryopreservation than their *in vivo*-derived counterparts (Enright et al., 2000). Efforts have focused on the adjustment of cryopreservation methods to special requeriments of IVP embryos.Vitrification procedures appear to be more efficient for the cryopreservation of IVP bovine embryos than other freezing methods (Agca et al., 1998; Kaidi et al., 2001). However, for the achievement of a glass-like solidification (vitrification), greater concentrations of cryoprotectants and greater cooling rates are required. The vitrification solutions (VS) composed of ethylene glycol (EG) + ficoll + sucrose (SUC) or trealose have been relatively effective for the cryopreservation of IVP bovine embryos (Tachikawa et al., 1993; Vajta et al., 1999a). However, because the use of ficoll had little or no effect on the glass transition and melting points of EG-based solutions (Shaw et al., 1997), the use of alternative additives to replace ficoll may improve embryo survival following vitrification. Polyvinyl alcohol (PVA) has been suggested as a potential additive to minimize the devitrification problem in EGbased solutions (Wowk et al., 2000).

The open-pulled straw (OPS) technology has been proven successful not only for the vitrification of bovine oocytes and IVP embryos (Vajta et al., 1998; Vieira et al., 2002) but also to be combined with the in-straw warming and cryoprotectant dilution method (Lewis et al., 1999; Vajta et al., 1999b; Tecirlioglu et al., 2003). However, the rapid cooling and warming rates in the OPS system may be altered by variations in the embryo vitrification container and warming method. For instance, glass micropipettes may offer physical advantages over plastic straws (Cho et al., 2002). In addition, a faster exposure of vitrified embryos to warming medium into a large tube (Isachenko et al., 2003) prior to the in-straw cryoprotectant dilution could facilitate procedures, preventing devitrification and improving embryo survival. The aim of the present study was to determine the influence of two VS composed of EG + SUC + PVA or EG +DMSO on the vitrification of bovine IVP expanded blastocysts stage embryos selected

by diameter and osmotic behavior. Results were assessed in terms of re-expansion, hatching and pregnancy rates following vitrification in hand-pulled glass micropipettes using an alternative warming and in-straw cryoprotectant dilution procedure for direct embryo transfer (ET).

2. Materials and methods

2.1. Bovine in vitro embryo production

Embryos were produced based on our previously established procedures (Oliveira et al., 2006), with minor modifications. Briefly, bovine ovaries were transported from a local abattoir to the laboratory at 23–32 ºC in Dulbecco's phosphate buffered saline (PBS). Cumulus-oocyte complexes (COCs) were recovered by ovary slicing 3–5 h after slaughter. A total of 2730 COCs were *in vitro*-matured for 24 h in bicarbonate-buffered TCM-199 + 10% heat inactivated estrous cow serum (ECS), 17β-estradiol and gonadotropins. Fertilization (day 0) was performed by frozen-thawed, swim-up-segregated spermatozoa (Coscioni et al., 2001). After 20 h, denuded presumptive zygotes were cultured in groups of 20 in 80µl droplets of synthetic oviduct fluid (SOF) + 10% ECS, under oil, at 39 ºC, 5% CO_2, 5% O_2 and 90% N_2 and maximum humidity. Cleavage rates were recorded on day 2 and blastocyst rates on day 7 of development. From 2155 presumptive zygotes placed in *in vitro* culture (IVC), 1491 cleaved (69.2%) and 523 (24.7%) developed to the blastocyst stage.

2.2. Embryo selection procedure

Twelve replicates were conducted including both vitrification groups and the control (nonvitrified) group to standardize procedures and minimize experimental error. Only embryos reaching the expanded blastocyst stage and ≥180µm diameter on day 7, and classified morphologically as grade I–II (Hasler et al., 1995), were selected (n = 253; 11.7%), pooled and randomly distributed among experimental groups. Basic medium (BM) used for embryo holding, vitrification and warming solution preparation was PBS + 10% fetal calf serum (Nutricell, Campinas, SP, Brazil). All equilibrations were performed on a 39 ºC heated stage in a warm room (25–27 ºC); under these conditions, temperature of the solutions was approximately 35 ºC. Throughout the experiment, collapsed and non-fully re-expanded embryos of all groups were discarded and only embryos with a greater survival probability were selected for continued experimental

purposes (Kaidi et al., 2000). Embryos in both vitrification groups were first subjected to pre-equilibration in diluted (3%) ethylene glycol (EG) solution for 5 min for promoting an initial cryoprotectant cell influx to facilitate cell saturation (Papis et al., 1999) prior to vitrification. Embryos allocated to the control group were maintained in BM during the period required for the vitrification and warming of embryos in both vitrification groups.

2.3. Vitrification procedure

The vitrification procedure was based on the OPS technology originally described by Vajta et al. (1999a), with modifications. OPS plastic straws were replaced by glass micropipettes (GMP) made from capillary tubes (1.5mm o.d.) softened by heat, and pulled manually until the outer diameter reached approximately 0.6 mm. The hand-pulled GMPs were broken at the narrowest point, after being scribed with a diamond tip, and were sterilized by heat. For the VSa vitrification group (test solution), the equilibration and vitrification solutions were composed of 20% EG, and 40% EG + 17.1% SUC + 0.1% PVA, respectively, whereas for the VSb vitrification group (control solution), those solutions were composed of 10% EG + 10% dimethyl sulphoxide (DMSO), and 20% EG + 20% DMSO, respectively. In both vitrification groups, each embryo was treated with one of the equilibration solutions for 60 s before being transferred to VSa or VSb solutions. Embryo loading was performed by capillarity by placing the narrowest end of GMP into 1–2 µl VS droplets. Embryos were exposed for 25 s to the VS, from first contact to plunging into liquid nitrogen (LN_2). GMPs with vitrified embryos that were immediately warmed after the end of vitrification procedures (*in vitro* survival study) were held vertically in a small plastic tube partially immersed in LN_2; GMPs to be stored for the further ET to recipients (*in vivo* survival study) were placed into pre-cooled and labeled 0.5 ml plastic straws, and stored in a LN_2 tank.

2.4. Warming and in-straw cryoprotectant dilution

For the in-straw cryoprotectant dilution, a 0.25 ml French straw connected to a 1-ml syringe was pre-loaded, as follows: 4 cm column with ~100µl BM, 1 cm column with air, 3 cm column with ~70µl warming solution (WS; BM+ 0.26M SUC), and 0.5 cm air column at the open end. For warming, the GMP was placed directly into the WS, after 3 s in air. In this process, once inserted into a tube filled with 1.2 ml of WS at 39 ºC, the

vitrified medium liquefies within 1–2 s, to be immediately diluted by the WS that enters the GMP by capillarity. Next, the GMP was introduced through its narrowest end into the open end of a plastic straw previously loaded, as described above. Then, the medium inside the GMP was aspirated into the plastic straw with a 1ml syringe (Fig. 1). For the *in vitro* survival study, each straw loaded with a single embryo was held horizontally for 10 min at room temperature (RT); then, embryos were expelled into BM and transferred to drops of SOF medium for additional 72 h of IVC. For the *in vivo* survival study, each straw loaded with a single embryo was held horizontally for 5 min at RT prior to the direct ET to synchronous recipient females.

2.5. Statistical analysis

Re-expansion, hatching and pregnancy rates were analyzed using the χ^2-test, for a level of significance of $P < 0.05$.

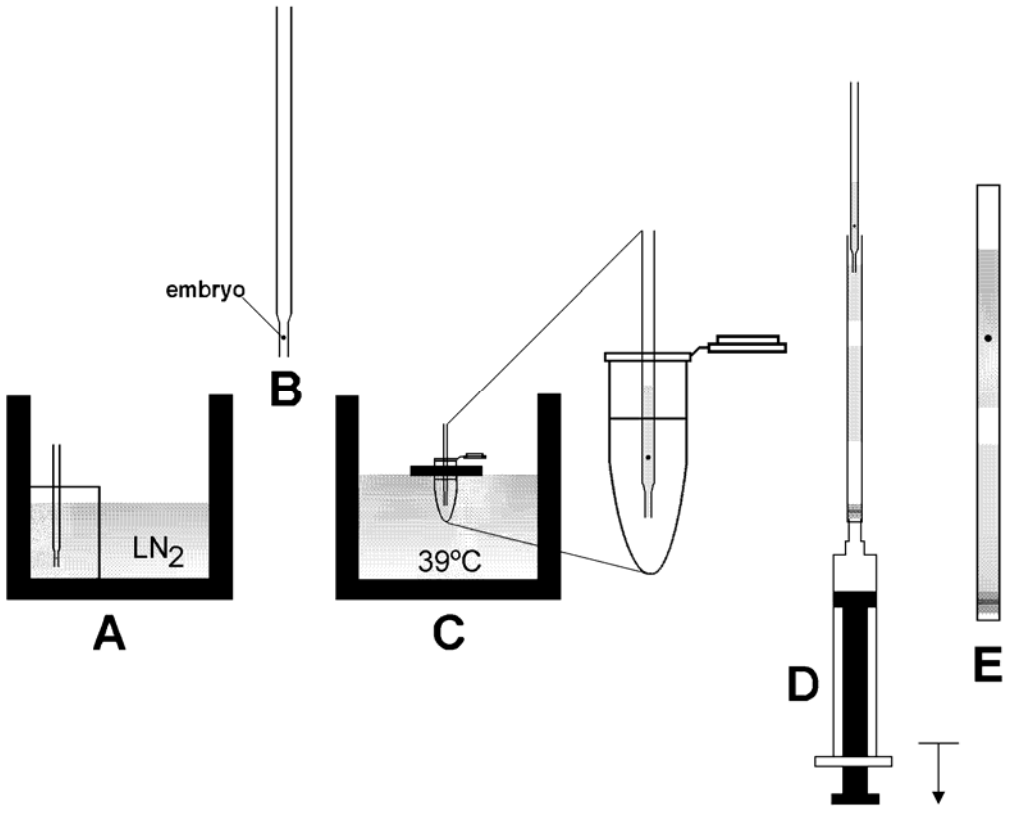

Fig. 1. Steps involving warming and in-straw dilution of GMP-vitrified embryos. (A) GMP into LN_2; (B) GMP exposure to air (3 s); (C) GMP plunged into tube with warming medium; (D) GMP contents transferred to 0.25 ml Straw; (E) 0.25 ml straw containing embryo for direct transfer.

3. Results

From the selected expanded blastocysts, 25 embryos collapsed and were discarded. In the first set of experiments (assessment of *in vitro* survival), a total of 176 expanded blastocysts were vitrified (VSa and VSb groups) or cultured as fresh (non-vitrified control group), as depicted in Table 1. No differences were observed between re-expansion rates of embryos vitrified in VSa and VSb groups at 24 h of IVC after warming (Table 1). However, at 72 h of IVC hatching rates Table 1. of VSa group was less ($P < 0.05$) than in VSb and control groups (Table 1). In the second set of experiments (assessment of *in vivo* survival), a total of 41 embryos were warmed and transferred individually to suitable synchronous recipient females. No differences in pregnancy rates were observed on day 60 of gestation between the VSa and VSb groups (Table 1).

Table 1

Developmental rates of selected embryos from vitrified and non-vitrified experimental groups

Experimental	*In vitro* evaluation			*In vivo* evaluation	
group	Embryos	Re-expanding (24h)	Hatching (72h)	Transferred embryos	Pregnancy (day 60)
VSa	58	49 (84.5%)	32 (55.2%)a	20	4 (20%)
VSb	58	55 (94.8%)	44 (75.9%)b	21	4 (19%)
Control	60	-	48 (80.0%)b	nt	-

VSa: vitrification solution, 40% EG + 17.1% SUC + 0.1% PVA; VSb: 20% EG + 20% DMSO; Control: non-vitrified embryos; nt: not transferred. Numbers in the same column with different letters differ significantly P<0.05

4. Discussion

In the present study, bovine IVP expanded blastocysts were successfully vitrified in GMP using a VS containing EG as a single permeable cryoprotectant (VSa) solution. In addition, a practical warming method for the in-straw cryoprotectant dilution following vitrification was also used. This method may further encourage the widespread use of the direct ET of IVP bovine embryos vitrified with OPS technology using glass or plastic embryo containers. The lack of differences in hatching rates between the non-vitrified and VSb-vitrified embryos (Table 1) supports the effectiveness of the non-invasive embryo quality evaluation systems used in the present study. However, despite the absence of differences in embryo re-expansion rates at 24 h post-warming between vitrification groups, the lesser hatching rate in the VSa-vitrified embryos could be attributed to the toxicity of the cryoprotectant additives. In the VSa solution, EG toxicity may have been increased by a prolonged treatment with warmer temperatures (~35 ºC) during the whole vitrification procedure. Such toxicity was minimized in the

VSb solution due to the use of two permeable cryoprotectants (EG + DMSO), at lesser individual concentrations (20%). The use of both cryoprotectants tends to minimize their individual toxicities, even at warmer temperatures, while providing the cryoprotective effect during cooling/warming. The incorporation of 0.1% PVA to the VSa solution to minimize the devitrification problem (Wowk et al., 2000) did not improve *in vitro* embryo survival in the present study; however, the similar pregnancy rates from both vitrified groups (Table 1) corroborates with the observation that VS comprising 40% EG + 17.1% SUC do not depend on ficoll to achieve a vitreous state (Shaw et al., 1997).

Current techniques for the direct transfer of vitrified embryos by the OPS method (Lewis et al., 1999; Vajta et al., 1999b; Tecirlioglu et al., 2003) require trained operators to handle properly embryo-container during direct in-straw warming. This procedure requires the introduction of the OPS-container directly into the warming straw after exposure to air for 3–4 s. If the operator fails to insert the OPS into the straw, the warming rate will be reduced, which may cause embryo damage by devitrification and/or VS toxicity. The procedure described by Isachenko et al. (2003), and used in the present study, circumvents this problem, facilitating and expediting the warming process when compared with the use of the 0.25 ml straw. The amount of medium (1.2 ml) contained in the tube yields a fast warming rate and efficient dilution of the VS. After the column of the vitrified solution melts and the warming medium fills the GMP, the operator has sufficient time to transfer the GMP's contents into a 0.25 ml straw for the completion of the cryoprotectant dilution. In the present study, no embryos remained trapped inside the GMP after warming (*in vitro* survival study). Furthermore, the dilution of the cryoprotectant agents within plastic straws allowed embryos to be promptly prepared for direct ET without the need of laboratory equipment. The use of GMP associated with the warming in a plastic tube and subsequent in-straw cryoprotectant dilution was demonstrated in the present study to be a valuable alternative to facilitate the application of the direct ET of vitrified bovine IVP embryos under field conditions.

In summary, VS composed of EG +DMSO enabled more bovine IVP expanded blastocysts to hatch after warming and IVC for 72 h than embryos vitrified in EG + SUC + PVA. However, with using a GMP container and tube warming, both VS provided similar pregnancy rates after the in-straw cryoprotectant dilution and direct ET.

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5- CAPÍTULO 3

Calves Born After Direct Transfer of Vitrified Bovine IVP Blastocysts Derived From Vitrified Immature Oocytes

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Calves Born After Direct Transfer of Vitrified Bovine IVP Blastocysts Derived From Vitrified Immature Oocytes

AD Vieira, F Forell, C Feltrin and JL Rodrigues

Laboratório de Embriologia e Biotécnicas de Reprodução Animal, FAVET-UFRGS, Porto Alegre, RS, Brazil.

Contents

Vitrification has been proposed as the method of choice for the cryopreservation of bovine oocytes, as rapid cooling decreases chilling sensitivity. The aim of this study was to determine the in vitro and in vivo survival of immature oocytes vitrified using super-cooled liquid nitrogen (at the "boiling off" state, i.e., at ≤-200ºC). Immature oocytes were randomly allocated to three groups: (1) non-vitrified control group, (2) vitrification in liquid nitrogen at -196ºC, and (3) vitrification in super-cooled liquid nitrogen (≤-200ºC). Open-pulled glass micropipettes were used as vitrification containers. Immature oocytes were in vitro-matured (IVM), fertilized (IVF) and cultured (IVC) to the blastocyst stage. In vitro viability was assessed by cleavage and blastocyst rates on Days 2 and 7 of IVC, respectively. Vitrified blastocysts derived from immature vitrified oocytes were transferred using a modified direct embryo transfer approach to synchronous recipients. The in vitro embryo development of vitrified immature oocytes was not influenced by the liquid nitrogen state. After direct transfer (one embryo per recipient) of vitrified embryos (n=16) derived from immature vitrified oocytes, two healthy calves were obtained per group. These results indicated that vitrification of immature bovine oocytes using glass micropipettes under normal or super-cooled liquid nitrogen, resulted in viable blastocysts and live calves following in vitro embryo production.

Introduction

One of the greatest challenges to reproductive cryobiologists today is to develop an adequate cryopreservation method for immature bovine oocytes. The cryostorage may help researchers to overcome obstacles they face through fluctuations in oocyte availability, seasonal variations in quality, or stringent sanitary constraints. Furthermore, it may overcome logistical problems associated with oocyte aspiration by

transvaginal ultrasonography and may facilitate a better matching of recipient availability in commercial in vitro embryo production programs.

The oocyte cryopreservation feasibility has been confirmed by the production of healthy calves born from frozen (Otoi et al. 1995, Suzuki et al. 1996, Kubota et al. 1998, Yang et al. 1998) or vitrified (Vieira et al. 2002, Abe et al. 2005) immature bovine oocytes. This feasibility was further demonstrated by the birth of calves from cryopreserved embryos, derived from both frozen matured oocytes (Otoi et al. 1992) and from vitrified immature oocytes (Vieira et al. 2002). However, despite such encouraging results, the cryopreservation of oocytes and IVP embryos is not yet an established efficient technology. The high lipid contents of these structures may account for the apparent increased chilling sensitivity (Zeron et al. 1999).

The high cooling speed obtained by vitrification has been regarded as a possible way to minimize the problems with cryopreservation. However, until recently, the highest cooling rate of common vitrification procedures was limited to that obtained by plunging samples into liquid nitrogen (LN_2) at normal saturated boiling point (-196ºC). When a warm object is plunged into LN_2 at normal boiling state, the heat conductance briefly leads to the evaporation of the nitrogen around the sample, resulting in an insulated layer. This thermal insulation reduces heat transfer, which hinders a uniform and rapid cooling of the sample. One way to boost the cooling speed is to avoid the formation of the vapor layer around the sample by using super-cooled LN_2. A very fast cooling rate was obtained when the temperature of LN_2 was decreased below -196ºC by applying negative pressure (Han et al., 1995). Under this condition, the forced convection produces an unsaturated boiling state as LN_2 is super-cooled (≤-200ºC = "boiling off"), allowing a high heat conductance to increase dramatically the cooling rate. The super-rapid cooling into LN_2 at the "boiling off" demonstrates the effectiveness of such approach for the vitrification of mature (Martino et al. 1996, Arav et al. 1997, 2000) and immature (Yavin and Arav 2001, Sanjay et al. 2002, Santos et al. 2006) oocytes. However, the limited available data supporting this notion still show low embryo yield after vitrification. The aims of this study were to determine the in vitro and in vivo survival and viability of immature oocytes vitrified in open-pulled glass micropipettes using super-cooled LN_2.

Materials and methods

Media and reagents

All chemicals were from Sigma Chemical Co. (St. Louis, MO, USA), unless stated otherwise. All solutions were prepared using water from a Milli-Q Synthesis System (Millipore, Bedford, MA, USA).

Source of oocytes

Bovine ovaries obtained from a local abattoir were transported to the laboratory within 5-6 h after slaughter, at 24-32ºC, in a thermos flask containing Dulbecco's phosphate buffer saline supplemented with penicillin and streptomycin. After ovary slicing, cumulus-oocyte complexes (COCs) were collected and washed in holding medium (HM; medium 199, M-2520) added with 2.14 mM $NaHCO_3$ (S-5761), 2.0 mM pyruvic acid (P-2256), 50 mg/mL gentamycin (G-1264), and 10% (v/v) FCS (Nutricell®, Campinas, SP, Brazil). Selected COCs were classified according to morphological characteristics and only those with evenly granulated cytoplasm and surrounded by a compact and dense cumulus cell layer were used.

Experimental groups

To determine the effect of the super-cooled LN_2 ($CoolLN_2$) on cleavage and blastocyst rates obtained after immature oocyte vitrification, selected COCs were randomly allocated to three groups: 1) non-vitrified control group, 2) vitrification in LN_2 at -196^{o}C (boiling point), and 3) vitrification into $CoolLN_2$ at ≤-200ºC. Open-pulled glass micropipettes (GMP; Vieira et al. 2006a) were used as vitrification containers for immature oocytes and IVP blastocysts.

Procedures and animal use were approved by the Animal Ethics Committee of the Federal University of Rio Grande do Sul.

Quenching into "boiling off" LN_2

A homemade vacuum pump device was used for $CoolLN_2$ production. The pump was connected through two-way silicon hoses: one way connected to an outside valve and the other connected to an acrylic recipient with an open/closed hermetic cover containing a styrofoam cup filled with ~250 mL LN_2. The vacuum pump was started at a time so that formation of unsaturated boiling LN_2 at ≤-200ºC coincided with the end of

vitrification solution exposure time, allowing at least 40 sec for CoolLN_2 to form. At this time, the pump was turned off, and the vacuum was eliminated by opening the outside valve, when the recipient was opened and the sample quenched into super-cooled LN_2. Subsequently, the vitrified sample was transferred from CoolLN_2 to normal LN_2 before the preparation of the next sample.

Immature oocyte vitrification and warming

The vitrification protocol was adapted from Vieira et al. (2002), with minor modifications. Briefly, COCs were successively suspended in HM, containing 3% ethylene glycol (EG, E-9129) for 5-10 min for initial oocyte cryoprotectant saturation (Papis et al. 2000). Then, groups of 5-6 COCs were placed in HM + 10% EG + 10% dimethyl sulphoxide (DMSO: D-2650) for 30 sec. Subsequently, COCs were suspended in vitrification solution (VS) composed of HM + 20% EG + 20% DMSO + 17.1% sucrose (SUC, S-1888), supplemented with 0.1% polyvinyl alcohol (PVA, P-8136) as an ice blocking nucleation agent (Wowk et al. 2000). COCs were sequentially transferred from 100 μL to 2 μL VS drops. Loading was performed by placing the narrow end of the GMP in the 2 μL droplet and aspirating COCs in a 1-2 mm long column by capillarity. Total exposure time to the VS, from COCs loading into GMPs until plunging and swirling into LN_2 or CoolLN_2, was 25 sec. The GMP containing vitrified oocytes were held vertically in a small plastic tube. Ten minutes after the end of vitrification procedures, warming was started with a different group in each replicate. For warming, GMPs with the COCs were exposed to air for 3 sec, and then the narrow end was plunged into HM containing sucrose, where COCs were expelled. The cryoprotectants were diluted by two steps of decreasing gradients of sucrose in HM (0.26 M and 0.16 M) for 5 min each, and subsequently transferred to HM. Non-vitrified control COCs were maintained in HM during the vitrification/warming procedures. Sixty minutes after vitrification started, COCs from all groups were held in HM, and then subjected to in vitro maturation (IVM).

In vitro embryo production (IVP)

Embryos were produced in 11 replicates based on our established procedures (Vieira et al. 2006b), with minor modifications. Briefly, vitrified and fresh COCs (n=960) were subjected to IVM for 24 h in bicarbonate-buffered TCM-199 + 10% heat inactivated estrous mare serum (EMS), and gonadotropins. In vitro fertilization (IVF = Day 0) was

performed using frozen-thawed, swim up-segregated spermatozoa. Twenty four hours after IVF, presumptive zygotes were stripped of cumulus cells by vortexing (1800 rpm for 70 sec) and co-cultured with cumulus cells, sequentially washed and centrifuged for three times, under paraffin oil (M-8410), in SOFaaci culture medium supplemented with 10 mM Glycine (G-6388), 1.5 mM D-Glucose (G-6152) and 5% (v/v) of EMS. Cleavage and blastocyst rates were recorded on D2 and D7 of in vitro culture (IVC), respectively.

Embryo vitrification and warming

Selected blastocysts derived from vitrified immature oocytes from Groups 1 and 2 were vitrified according to their respective group (LN_2 or $CoolLN_2$), following our established vitrification procedures (Vieira et al. 2006a). For long term storage, GMPs with vitrified blastocysts were placed into pre-cooled labeled 0.5 mL plastic straws sealed with a metal sphere on the plug's end. After GMP insertion, the open end of the straw was sealed with another metal sphere to be stored in a LN_2 tank.

Assessment of oocyte meiotic stage

After IVM, 234 COCs (79 fresh, 83 LN_2 and 72 $CollLN_2$) were stained to assess the meiotic stage, evaluating nuclear maturation by the presence of a metaphase II plate (MII) and the first polar body. Cumulus cells were stripped by pippeting in a 3% (w/v) sodium citrate (S-4641) solution. Subsequently, denuded oocytes were fixed in ethanol:acetic acid solution (3:1) for 24 h. After this period, groups of 5 denuded oocytes were placed in the center of a clean glass slide into a minimum amount of medium. For staining, 20 μL of 0.25% (w/v) lacmoid (L-7512) on 45% aqueous acetic acid solution were dropped on the oocytes. Cover slips were placed onto the glass slides with a small amount of modeling clay on each corner and pushed down until fluid contacted the cover slip. Oocytes were watched carefully under a stereomicroscope while being pressed to prevent the breaking of the zonae pellucidae. To assess the meiotic stages, slides were examined under phase contrast microscope at a 400 x magnification, 5 min after staining.

Embryo transfer

A total of 16 embryos obtained from immature vitrified oocytes (8 from each vitrified group) were warmed and directly transferred (Vieira et al. 2006a) to individual recipient

females (1 embryo/recipient). Pregnancy diagnosis was performed by ultrasonography on Day 30 of pregnancy, with reconfirmations carried out by rectal palpation on Day 90 of gestation.

Statistical analysis

Meiotic stage, cleavage and blastocyst rates, and pregnancy rates were analyzed by the Chi-square test, for a level of significance of 5%.

Results

A relative low meiotic maturation rate was observed in stained IVM oocyte in all groups. The non-vitrified control group and the vitrified groups (LN_2 or $CoolLN_2$) showed a similar pattern of development to the MII stage after staining (Table 1).

Table 1: Meiotic resumption to MII after IVM of control non-vitrified oocytes and oocytes vitrified into LN_2 or $CoolLN_2$.

Treatment	Stained oocytes n	MII n (%)
Control non-vitrified	79	50 (63.3)
Vitrified - LN_2	83	50 (60.2)
Vitrified - $CoolLN_2$	72	40 (55.5)

Immediate morphological appearance of vitrified COCs upon warming was only altered by plane fractures comprising the cumulus cells and/or the oocytes. The incidence of fractures was similar for COCs vitrified into LN_2 or in $CoolLN_2$ (Table 2).

The embryo yield was not affected by vitrification into LN_2 or in $CoolLN_2$. However, for both groups combined, the blastocyst rates of vitrified COCs were significantly lower ($P<0.05$) than that of non-vitrified controls (Table 2).

From 16 recipients receiving vitrified/warmed blastocysts derived from vitrified oocytes (8 per group), a total of 3 (38%) and 2 (25%) from Groups 1 (LN_2) and 2 ($CoolLN_2$ groups), respectively, were diagnosed as pregnant on Day 30 of gestation. On Day 90 of gestation, 2 pregnancies were reconfirmed in each group, resulting in 4 healthy calves born at term (calving rate of 25%).

Table 2: Embryo development after IVM, IVF and IVC of fresh or vitrified immature oocytes.

Treatment	COCs n	Fracture rate n (%)	IVC n	Cleavage rate (D2) n (%)*	Blastocyst rate (D7) n (%)*
Control non-vitrified	320	-	317	212[a] (66.8)	88[a] (27.7)
Vitrified - LN_2	320	13 (4.0)	290	138[b] (47.5)	17[b] (5.86)
Vitrified - CoolLN_2	320	19 (5.9)	275	123[b] (44.7)	12[b] (4.36)

[a,b]: Numbers in the same column without common superscripts differ ($P<0.05$).

*Based on the initial number of IVC presumptive zygotes within the same group.

Discussion

The emergence of new vitrification methods making use of increased cooling rate strategies has created a new wave of experimentation in cryobiology. However, only a few studies describe procedures to circumvent the thermal-insulation effect determined by the immersion of warm objects into LN_2. Dropping oocytes/embryo-containing vitrification solution on cold metal surfaces (Steponkus et al. 1990, Dinnyés et al. 2000) is one way to achieve an efficient heat exchange by eliminating the insulation effect, however the handling of the vitrified drops is not practical. An alternative way is the "boiling off" nitrogen slush (Mazur et al. 1992, Steponkus and Caldwell 1993, Martino et al. 1996, Arav and Zeron 1997, Arav et al. 2000, Sanjay et al. 2002). Still, despite the achievement of very high cooling rates, the use of a vacuum pump connected to a glass vacuum desiccator (Martino et al. 1996), or the use of the Vit-IMT device (Arav et al. 2000) were proven non-practical or cost-limiting. Our simplified device circumvents these problems and permits the achievement of improved heat exchange rates into super-cooled LN_2.

In this study, the in vitro embryo yield after oocyte vitrification was not influenced by the LN_2 boiling state (Table 2). Based on the limited cooling rate gain in relation to the characteristics of vitrification containers (Arav et al. 2000), we observed that increments on cooling rate obtained with super-cooled LN_2 were not significantly different than those with normal LN_2. Such results may be explained by the high thermal conductivity of glass as opposed to other containers, such as plastic straws.

Following warming, the immediate morphological aspect of vitrified oocytes was only altered by plane fractures. Relatively high rates of nuclear maturation were observed in both vitrified treatments, demonstrating that the vitrification procedures did not compromise the extrusion of the first polar body (Table 1). However, the assessment of nuclear maturation suggested vitrified/warmed oocytes to be more fragile, with a need for extra care for the removal of cumulus cells and during cover slip mounting.

After fertilization, presumptive zygotes derived from vitrified/warmed oocytes showed a higher rupture rate than fresh, non-vitrified oocytes, after cumulus cells removal by vortexing. This can be observed by the reduction of the total number of IVC presumptive zygotes following cumulus cells removal (Table 2).

Until recently, typical vitrification systems employed a very short equilibration period into intermediate solutions containing relatively higher cryoprotectant concentrations prior to oocyte suspension into VS. Such procedure provides a certain level of cell saturation and osmotic shock reduction prior to the vitrification procedure per se. However, for immature oocytes, the actin microfilament organization, distributed as a continuous layer under the plasma membrane, limits cryoprotectant permeability, compromising the initial cell saturation (Le Gal et al. 1994). Even though the permeability of oocytes is higher for DMSO than for EG (Agca et al. 1997), in our study, oocytes were pre-equilibrated in a diluted (3%) EG solution for a relatively large period for the achievement of an additional cell influx of this cryoprotectant (Papis et al. 2000). Blastocyst rates similar to the one presented in this study (4-5%) have been demonstrated by others (3-8%) after minor modifications of the OPS vitrification technology (Le Gal and Massip 1998, 1999, Vieira et al. 2002, Santos et al. 2006). Such findings suggest that the cryoprotectant associations and exposure times are still the main limiting factors determining lower cleavage and blastocyst rates after vitrification. The exact mechanism of the damage is unknown, but the disruption of gap junction channels (Fuku et al. 1995), combined with lipid and mitochondria alterations (Fuku et al. 1995, Abe et al. 2005) have been previously suggested by others.

The IVC system can have a marked effect on both the yield and, more importantly, the quality of the embryos produced (Rizos et al. 2001). In turn, embryo quality certainly affects cryotolerance. In an attempt to improve in vitro embryo development and embryo quality and, consequently, cryotolerance, we supplemented the IVC medium with glycine. Aside from being predominant into the oviductal and uterine fluids (Elhassan et al. 2001), the use of this amino acid for IVC resulted in further

improvement in embryo development in a dose-response manner (Moore and Bondioli 1993). In addition, we used a co-culture system with a granulosa cell monolayer in an attempt to improve survival after cryopreservation, as also demonstrated by others (Rizos et al. 2001). Despite all IVC modifications, improvements in blastocyst yield from vitrified oocytes were not observed. Nonetheless, pregnancy rates observed on Day 30 and reconfirmed on Day 90 of gestation, and the birth of 4 healthy calves, confirmed the viability and cryotolerance of embryos produced in our IVP system. Regardless of the reduction in development to the blastocyst stage following the vitrification of immature oocytes, the attainment of pregnancies after direct transfer of single vitrified oocyte-blastocyst is encouraging for the development of further research in oocyte cryopreservation.

In conclusion, results from this study indicated that vitrification of immature bovine oocytes using glass micropipettes in boiling or super-cooled liquid nitrogen resulted in viable blastocysts that sustain another round of vitrification and the production of live offspring following transfer to female recipients.

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Author's address (for correspondence): Arnaldo Diniz Vieira, Faculdade de Veterinária, Laboratório de Embriologia e Biotécnicas de Reprodução Animal, Universidade Federal do Rio Grande do Sul, Av. Bento Gonçalves, 9090, CEP *91501-970*, *Cx. Postal 15004,* Porto Alegre, RS, Brasil. E-mail: vieira_ad@yahoo.com.br

6- DISCUSSÃO GERAL CONSIDERAÇÕES FINAIS

Neste trabalho foram desenvolvidos diferentes experimentos em busca da determinação de possíveis alternativas para uma eficiente criopreservação de ovócitos bovinos imaturos e embriões PIV.

No primeiro experimento, foram comparados dois sistemas de produção *in vitro* com o objetivo de determinar sua possível influência na viabilidade à crioconservação dos embriões. Foram observadas apenas pequenas variações entre as distintas etapas do processo, não havendo, no entanto, diferenças significativas nas taxas de desenvolvimento e na viabilidade embrionária *in vitro* e *in vivo*. Esses resultados podem ser justificados pela utilização de condições experimentais equivalentes, devendo-se considerar especialmente a qualidade da água usada na preparação dos meios e a equivalência da qualidade dos ovócitos utilizados, fatores que potencialmente afetam a taxa de desenvolvimento e viabilidade embrionária (NAGAO et al., 1995; LONERGAN et al., 2003).

No segundo experimento, foram comparadas duas soluções de vitrificação reconhecidas como eficientes para criopreservação de embriões PIV (TACHIKAWA et al., 1993; ISHIMORI et al., 1993). Na época em que foi utilizada, a solução composta por etileno glicol (EG) e sacarose (SAC) proporcionou taxas satisfatórias de sobrevivência embrionária (TACHIKAWA et al., 1993). Entretanto, o tipo de palheta utilizado pode ter limitado os resultados em função do comprometimento da velocidade de resfriamento, fato que justificou sua utilização, associada com uma metodologia que permite a obtenção de maiores velocidades de resfriamento, como à empregada no primeiro experimento deste trabalho. Adicionalmente, buscou-se aumentar a estabilidade da solução de vitrificação mediante a adição de álcool polivinílico (PVA), que em pequenas concentrações, atua bloqueando a nucleação de gelo em soluções compostas por EG (WOWK et al., 2000). Também se buscou a redução de um possível choque osmótico e a obtenção de uma saturação celular homogênea, mediante a exposição dos embriões a uma solução com 3% de EG.

Apesar da solução de vitrificação composta por EG e dimetil sulfóxido (DMSO) ter proporcionado uma maior de taxa de eclosão *in vitro*, as taxas de prenhez não

revelaram diferenças entre as duas soluções, resultado que pode ser justificado por alterações em diferentes pontos celulares de acordo com o tipo de solução crioprotetora. No caso dos embriões vitrificados com a solução EG+SAC+PVA, estas lesões provavelmente só foram regeneradas no ambiente uterino. Possibilidade que caracteriza a necessidade de adequação não só da metodologia de criopreservação, mas também do ambiente a que as estruturas, sensibilizadas pelo processo, vão estar expostas durante o período critico em que deve ocorrer a regeneração das lesões sofridas durante o resfriamento e/ou aquecimento.

No terceiro experimento, foram comparadas duas estratégias de resfriamento na vitrificação de ovócitos bovinos imaturos objetivando a determinação das taxas de desenvolvimento embrionário *in vitro* e *in vivo*. Para obtenção do resfriamento foi utilizado nitrogênio líquido em estado de ebulição (-196ºC) ou super-resfriado (≤-200ºC). O super-resfriamento obtido pelo efeito do vácuo sobre o nitrogênio líquido permite a eliminação do estado de ebulição facilitando a troca de calor entre o líquido e a amostra, aumentando a velocidade de resfriamento. Entretanto, neste experimento, não foi observada uma diferença significativa na taxa de desenvolvimento embrionário entre os dois grupos. Resultado que pode ser justificado pelo fato da capacidade de condução térmica da micropipeta de vidro ter suplantado o efeito do ganho de velocidade propiciado pelo super-resfriamento do nitrogênio líquido. Contudo, apesar de não ter sido possível a obtenção de um incremento nos índices de desenvolvimento embrionário, neste trabalho foi demonstrada a viabilidade de produção de embriões a partir de ovócitos imaturos vitrificados em micropipetas de vidro. Também foi demonstrado que os blastocistos derivados de ovócitos imaturos vitrificados possuem viabilidade suficiente para resistir a vitrificação e estabelecer prenhezes após transferência direta de um embrião por receptora. Estes resultados estimulam a realização de novos experimentos, direcionados para adequação da composição da solução de crioprotetores, do tempo de exposição e da velocidade de resfriamento, bem como, determinam a necessidade da adequação do ambiente de maturação, fecundação e cultivo *in vitro*. Neste aspecto, deve-se considerar a possibilidade de redução ou até eliminação do soro sanguíneo da composição dos meios e também a "estabilização da membrana" dos ovócitos e embriões, tornando-os mais resistentes a criopreservação (SEIDEL, 2006).

Apesar de não terem sido evidenciadas correlações significativas entre os resultados observados *in vitro* e *in vivo* nos três experimentos realizados, os dados

coletados permitem a realização de adequações que podem determinar o incremento na viabilidade embrionária. Esse incremento pode ser obtido mediante a associação entre as distintas etapas do processo de PIV, identificadas como mais eficientes na comparação entre os sistemas testados no primeiro experimento.

O emprego do procedimento de seleção de embriões baseado no comportamento osmótico, associado à seleção morfológica, mostrou-se um auxiliar na predição da capacidade de desenvolvimento *in vitro* dos embriões frescos e vitrificados. Os resultados observados nos experimentos sugerem que este procedimento pode ser incorporado a rotina de classificação dos embriões, tornando essa avaliação menos subjetiva.

A utilização do procedimento de vitrificação em micropipetas de vidro, armazenamento em palhetas de 0,5 mL seguido do emprego do protocolo de aquecimento descrito no segundo experimento também demonstrou grandes possibilidades de incorporação a rotina de transferência de embriões vitrificados em condições de campo. Este tipo de protocolo pode favorecer a implementação de trabalhos acadêmicos ou mesmo permitir a aplicação comercial da transferência direta de embriões PIV vitrificados.

A incorporação do co-cultivo celular associado à adição do aminoácido glicina ao meio de cultivo não proporcionou de aumento nas taxas de desenvolvimento *in vitro*. Entretanto, a obtenção do nascimento de quatro produtos derivados de ovócitos imaturos vitrificados sugere que o sistema de cultivo utilizado proporcionou um ambiente satisfatório para o desenvolvimento de embriões com qualidade suficiente para tolerar o processo de vitrificação e transferência direta.

O nascimento dos quatro produtos derivados da transferência de embriões vitrificados, produzidos a partir de ovócitos imaturos vitrificados demonstra que essa tecnologia é viável. Entretanto, os resultados obtidos no terceiro experimento sugerem que o protocolo de vitrificação necessita de adequação, sendo que, existem indicações de que a solução crioprotetora é um dos principais limitantes á sobrevivência de ovócitos imaturos vitrificados.

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8- ANEXO 1

CRIOPRESERVAÇÃO DE OÓCITOS BOVINOS

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CRIOPRESERVAÇÃO DE OÓCITOS BOVINOS

Vieira, A. D.[1]; Rodrigues, J. L.[2]
[1]MV. MSc. Aluno doutorado PPGCV/UFRGS
[2]Prof. Dr. Pesquisador CNPq
Laboratório de Embriologia e Biotécnicas da Reprodução UFRGS
Porto Alegre - RS. Caixa postal 15004.

O aumento na demanda de material genético para emprego nas diferentes biotécnicas de reprodução, gerou a necessidade da organização de bancos de germoplasma. Ao mesmo tempo, a crescente utilização comercial da produção *in vitro* de embriões bovinos (PIV) exige uma maior disponibilidade de oócitos. Uma das estratégias que vem sendo levada em consideração é o emprego de oócitos criopreservados em função da flexibilidade de aplicação e facilidade na programação das atividades. A maioria dos experimentos vem sendo realizada utilizando oócitos maturados (metáfase II), por proporcionarem maiores taxas de desenvolvimento em relação aos oócitos imaturos (vesícula germinativa). Entretanto, apesar da obtenção de taxas de 25% desenvolvimento embrionário a partir de oócitos maturados vitrificados (Vajta *et al*., Mol. Reprod. Devel., n.51, p.53-58, 1998) e de prenhez com o uso do mesmo tipo de material como citoplastos receptores na fusão nuclear (Booth *et al*., Theriogenology, n. 51, p. 999-1006. 1999), a ocorrência de alterações genéticas determinadas por falhas na reorganização dos fusos meióticos limitam a viabilidade da criopreservação destes oócitos. Por outro lado, do ponto de vista logístico os trabalhos com oócitos maturados dificultam o aproveitamento do potencial de animais criados em locais distantes de centros equipados para PIV, em função das limitações decorrentes da perda de viabilidade determinada pelo tempo de transporte e dependência do laboratório equipado para iniciar a maturação *in vitro*. Desta forma, a busca de soluções na criopreservação de oócitos imaturos justifica-se pelo fato de que neste estádio o sistema de fusos não está organizado e o material genético encontra-se descondensado e protegido dentro do envelope nuclear, permitindo contornar os problemas observados com oócitos maturados. Resultados preliminares, com taxas de 15% de desenvolvimento embrionário até o estádio de blastocisto (YAVIN & ARAV, Cryobiology. n. 43, p. 331. 2001) e nascimentos de produtos normais (VIEIRA *et al*., Cryobiology, n. 45, p. 91-94. 2002) a partir de oócitos imaturos vitrificados confirmam a potencialidade da técnica. O incremento nas taxas de sobrevivência pós criopreservação de oócitos imaturos em níveis que tornem os resultados comercialmente atrativos, vai depender da adoção de estratégias experimentais, que permitam modificações e ajustes dos protocolos empregados. O principal direcionamento é o da vitrificação com redução nos efeitos tóxicos e osmóticos das soluções crioprotetoras com o aumento na velocidade de resfriamento. A associação da criopreservação de oócitos imaturos acondicionados em palhetas, através da vitrificação realizada nas condições de propriedade rural, com a aspiração folicular guiada com o auxílio do ultrasom, permitirá um melhor aproveitamento do potencial reprodutivo de animais mantidos em locais distantes dos laboratórios equipados para PIV. O aumento na viabilidade pós-criopreservação de oócitos imaturos deverá também possibilitar a expansão da sua utilização em outras biotécnicas de reprodução.

Palavras chave: gameta feminino, vitrificação, armazenamento, bancos genéticos.

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CRYOPRESERVATION OF BOVINE OOCYTES

Vieira, A. D.; Rodrigues, J. L.

Biotechnology and Embryology Laboratory UFRGS Porto Alegre - RS. Mail Box 15004.
Author's electronic address: vieiraad@bol.com.br

The increasing demand of genetic material to be used in different biotechnologies created the necessity of germoplasm bancs. At the same time, the growing industry of *in vitro* produced (IVP) embryos demands a high disponibility of oocytes. One of the strategies that had been used is the cryopreservation of oocytes, because those oocytes are flexible to use and it is easy to program the activities. Most of the experiments used mature oocytes (metaphase II), since higher developmental rates were obtained comparing with the use of immature ones (germinal vesicle). However, although 25% developmental rate was obtained using mature vitrified oocytes (Vajta *et al.*, Mol. Reprod. Devel., n.51, p.53-58, 1998), and a pregnancy was achieved using the same oocytes as receptors for nuclear transfer (Booth *et al.*, Theriogenology, n. 51, p. 999-1006. 1999), genetic alteration due to misplacement of the meiotic fibers, impair the use those cryopreserved oocytes. By other hand, the utilization of mature oocytes impair the use of outstand genetic donors located in farms far way from the IVP centers, due to the limitation and loss of viability during transportation of the oocytes to the Laboratory. The search for solutions for cryopreserve immature oocytes are justified since, in this developmental stage, the meiotic fibers are not organized and the chromatin is still protected into the nuclear envelope, allowing to avoid the problems observed during cryopreservation of mature oocytes. Preliminary results with 15% development to blastocyst (YAVIN & ARAV, Cryobiology. n. 43, p. 331. 2001) and birth of normal calves (VIEIRA *et al.*, Cryobiology, n. 45, p. 91-94. 2002) were obtained using vitrified immature oocytes, confirming the applicability of this method. The improvement of survival rates after cryopreservation of immature oocytes to become commercially attractive depends on experimental strategies and adjustments on developed protocols. The main focus is to reduce the toxic and osmotic effects of cryoprotectors during vitrification increasing the cooling rate. The association of the vitrification of immature oocytes in straws and the ovum pick-up, performed in the farm, allowed a better use of genetically superior animals kept in distant locations. The increase in viability after cryopreservation of immature oocytes will, also, allowed the utilization of this material in other reproductive biotechnologies.

Key words: Female gamete, vitrification, storage, genetic bancs.

9- ANEXO 2

SOLUÇÕES PARA VITRIFICAÇÃO E AQUECIMENTO

Sol. de sacarose 1,0M

Produto	Quantidade
	5ml
Sacarose	1,712g
TCM Hepes / PBS	4,175ml (5x835µl)
Filtrar e fracionar = 1,0ml (armazenar no freezer)	

Sol. de sacarose 1,0M + qsp para 0,1% PVA na SV

Produto	Quantidade
	5ml
Sacarose	1,712g
Álcool polivinílico	0,011
TCM Hepes / PBS	4,175ml (5x835µl)
Filtrar e fracionar = 1,0ml (armazenar no freezer)	

10- ANEXO 3

CICLO DE VITRIFICAÇÃO

PREPARAÇÃO

- Preparar ponteiras para vitrificação
- Aquecer mesas térmicas
- Aquecer o meio de manipulação
- Descongelar uma alíquota de sol.sacarose e uma de soro

Meio de aquecimento

Produto	Quantidade
Sol. de sacarose	1,0ml
Soro de égua em estro (20%)	0,25ml

PLACA I (MANUTENÇÃO)

- Poço 1 = 400µl meio de manipulação (grupo 1)
- Poço 2 = 400µl meio de manipulação (grupo 2)
- Poço 3 = 400µl meio de manipulação (controle)
- Poço 4 = 400µl meio de manipulação (outro)
- **Obs**: No caso de embriões, dar um banho com meio de manipulação (TCM-hepes ou PBS) em uma placa de 11mm para evitar a presença de óleo no poço da placa de manutenção.

PLACA II (AQUECIMENTO)

- Poço 1 = 800µl meio de manipulação + 400µl meio de aquecimento
- Poço 2 = 400µl meio de manipulação + 200µl meio de aquecimento
- Poço 3 = 400µl meio de manipulação + 100µl meio de aquecimento
- Poço 4 = 400µl meio de manipulação

PLACA III (VITRIFICAÇÃO)

- Poço 1 = 400µl meio de manipulação
- Poço 2 = 485µl meio de manipulação
- Poço 3 (SV50) = 400µl meio de meio de manipulação
- Poço 4 (SV100) *

*Ovócitos = 300µl meio de aquecimento
*Embriões = 300µl meio de manipulação

VITRIFICAÇÃO DE BLASTOCISTOS

Passos:

- Preparar o nitrogênio e ponteiras para manipulação e vitrificação (numerar)
- Constituir os grupos em cada tratamento
- Transferir os embriões para placa de manutenção
- Retirar as amostras de crioprotetor do freezer para aquecimento em banho-maria
- Adicionar o Etileno glicol aos respectivos poços, homogeneizando a solução:
  Poço 2 = 15µl EG (colocar os embriões = 5min de exposição)
  Poço 3 = 50µl EG
  Poço 4 = 100µl EG
- No momento de início adicionar o DMSO aos respectivos poços, homogeneizando a solução (reação térmica ao contato com o EG = evitar precipitação do soro):
  Poço 3 = 50µl DMSO
  Poço 4 = 100µl DMSO

- Formar uma gota de 100µl de SV100 no meio da placa (ajustar lupa em menor aumento)
- Iniciar a vitrificação ao final de 5min de exposição à solução 3% EG
- Colocar 1 a 3 embriões na SV50. Manutenção por **60seg**
- Formar uma gota de 2-4µl de SV100 ao lado da gota central
- Transferir os embriões para gota central de SV100
- Transferir os embriões da gota central para gota pequena
- Tocar a gota com a ponteira aspirando os embriões
- Ao final de **25seg** mergulhar a ponteira no nitrogênio, mantendo uma posição inclinada e em sentido contrário a entrada da ponteira.
- Armazenar a ponteira em uma palheta de 0,5 previamente identificada.

VITRIFICAÇÃO DE OVÓCITOS

Passos:

- Após a seleção e constituição dos grupos de cada tratamento
- Preparar o nitrogênio e ponteiras para manipulação e vitrificação (numerar)
- Transferir os CCOs para placa de manutenção
- Retirar as amostras de crioprotetor do freezer para aquecimento em banho-maria
- Adicionar o Etileno glicol aos respectivos poços, homogeneizando a solução:
  Poço 2 = 15µl EG (colocar os CCOs = 5min de exposição)
  Poço 3 = 50µl EG
  Poço 4 = 100µl EG
- No momento de início adicionar o DMSO aos respectivos poços, homogeneizando a solução (reação térmica ao contato com o EG = evitar precipitação do soro):
  Poço 3 = 50µl DMSO
  Poço 4 = 100µl DMSO
- Formar uma gota de 100µl de SV100 no meio da placa (ajustar lupa em menor aumento)

- Iniciar a vitrificação ao final de 5min de exposição à solução 3% EG
- Colocar 5 CCOs na SV50. Manutenção por **30seg**
- Formar uma gota de 2-4µl de SV100 ao lado da gota central
- Transferir os CCOs para gota central de SV100
- Transferir os CCOs da gota central para gota pequena
- Tocar a gota com a ponteira aspirando os CCOs
- Ao final de **25seg** mergulhar a ponteira no nitrogênio, mantendo uma posição inclinada e em sentido contrário a entrada da ponteira.
- Armazenar a ponteira em uma palheta de 0,5 mL previamente identificada.

AQUECIMENTO EM PLACA

- Colocar as ponteiras em posição vertical dentro do nitrogênio
- Expor a ponteira ao ar por 4seg
- Mergulhar a ponteira no poço 1 da placa de aquecimento (iniciar cronometragem de 5min)
- Transferir as estruturas para o poço 2
- Ao final de 5min transferir as estruturas para o poço 3
- Ao final de 5min transferir as estruturas para o poço 4 e daí para placa de manutenção

AQUECIMENTO EM PALHETA

- Colocar 1,2ml de solução de aquecimento em um tubo de reação e aquecer em banho-maria ($40^{o}C$)
- Preparar uma palheta de 0,25ml com solução de aquecimento (manter conectado a seringa)
- Colocar as ponteiras em posição vertical dentro do nitrogênio
- Expor a ponteira ao ar por 4seg e mergulhar no tubo
- Inserir a extremidade da ponteira na boca da palheta e aspirar o conteúdo com o auxilio da seringa
- Completar a palheta e proceder a inovulação entre 5 e 10 minutos após o aquecimento.

11- ANEXO 4

Curriculun vitae

FORMAÇÃO ACADÊMICA/TITULAÇÃO

2003 Doutorado em Ciências Veterinárias.
Universidade Federal do Rio Grande do Sul, UFRGS, Rio Grande do Sul, Brasil.
Título: VITRIFICAÇÃO DE OVÓCITOS IMATUROS E EMBRIÕES BOVINOS PIV.
Orientador: JOSÉ LUIZ RIGO RODRIGUES.
Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPQ, Brasil.

2000 – 2002 Mestrado em Medicina Veterinária.
Universidade Federal de Santa Maria, UFSM, Rio Grande do Sul, Brasil.
Título: VITRIFICAÇÃO DE OÓCITOS BOVINOS IMATUROS.
Ano de obtenção: 2002.
Orientador: MARA IOLANDA BATISTELLA RUBIN.
Bolsista do(a): Conselho Nacional de Desenvolvimento Científico e Tecnológico, CNPQ, Brasil.

1991 - 1996 Graduação em Medicina Veterinária.
Universidade do Estado de Santa Catarina, UDESC, Santa Catarina, Brasil.
Título: Transferência de embriões em bovinos.
Orientador: ALCEU MEZZALIRA.
Bolsista do(a): Programa de Iniciação Científica - Universidade do Estado de Santa Catarina, PROBIC - UDESC, Brasil.

ÁREAS DE ATUAÇÃO

1 Reprodução Animal, Ginecologia e Andrologia Animal.
2 Reprodução Animal, Inseminação Artificial Animal.
3 Reprodução Animal, Fisiopatologia da Reprodução Animal.
4 Reprodução Animal, Reprodução assistida.

LINHA DE PESQUISA

1 Criopreservação de gametas e embriões bovinos PIV.

ATUAÇÃO PROFISSIONAL

Universidade do Estado de Santa Catarina - UDESC
Vínculo institucional
2002 - 2002 Vínculo: Professor colaborador
2005 - Atual Vínculo: Professor Colaborador
Disciplinas ministradas:
Fisiopatologia da Reprodução Animal I
Ginecologia e Obstetrícia