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Original research article

Pregnancy and delivery rates after vitrification of *in vitro*-produced Nelore (*Bos indicus*) embryos under field conditions

Tasas de preñez y nacimientos después de la vitrificación de embriones Nelore (Bos indicus) producidos in vitro bajo condiciones de campo

Taxas de prenhez e nascimentos após vitrificação de embriões Nelore (Bos indicus) produzidos in vitro sob condições de campo

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Abstract

Background: Cryopreservation preserves cellular viability under low temperatures, resulting in diminished intracellular enzymatic activity and reduced cellular metabolism that ultimately allows preserving genetic material for indefinite periods of time. Embryos submitted to cryopreservation suffer from considerable morphological and functional damage, resulting in reduced survival and development rates.
Objective: To evaluate pregnancy and delivery rates of *in vitro*-produced (IVP) Nellore (*Bos indicus*) embryos after vitrification under field conditions.
Methods: The IVP embryos at blastocyst (Bl) and expanded blastocyst (Bx) were transferred fresh (n= 137) or after vitrification (n= 127).
Results: Pregnancy rates at 35 d for fresh embryos were lower in Bl (41.6) than in Bx (60.9) ($p<0.05$). After vitrification, pregnancy rates were similar at 35 d between Bl (38.0) and Bx (47.6) ($p>0.05$). Pregnancy loss at 60 d were similar ($p>0.05$) for both fresh (Bl: 3.1 and Bx: 4.8) and vitrified embryos (Bl: 1.9 and Bx: 4.7). Delivery rates were similar between groups ($p>0.05$). **Conclusion:** Both pregnancy and delivery rates of *Bos indicus* IVP embryos vitrified under field conditions are indistinguishable from fresh embryos.
Key words: *cattle reproduction, cryobiology, cryoinjury, cryotolerance, embryo surplus, embryo transfer.*

Resumen

Antecedentes: La criopreservación se caracteriza por el mantenimiento de la viabilidad celular a bajas temperaturas, resultando en reducido metabolismo y actividad enzimática intracelular, lo que permite la preservación del material genético por períodos de tiempo indefinidos. Los embriones sometidos a ésta técnica sufren daños morfológicos y funcionales considerables, dando como resultado una sobrevivencia y tasas de desarrollo reducidas.

Objetivo: Evaluar la tasa de preñez a partir de embriones Nelore (*Bos indicus*) producidos *in vitro* (IVP) después de la vitrificación bajo condiciones de campo.
Métodos: Embriones IVP en los estadios de blastocisto (Bl) y blastocisto expandido (Bx) se transfirieron en fresco (n= 137) o después de la vitrificación (n= 127).
Resultados: La tasa de preñez a los 35 d fue menor para los embriones transferidos en fresco en fase Bl (41,6) en relación con los Bx (60,9) ($p<0,05$). Después de la vitrificación, las tasas de preñez a los 35 d fueron similares entre Bl (38,0) y Bx (47,6) ($p>0,05$). Las pérdidas de preñez a los 60 d fueron similares ($p>0,05$) tanto para embriones en fresco en Bl (3,1) y Bx (4,8) como para los vitrificados (Bl: 1,9 y Bx: 4,7). Las tasas de nacimiento fueron similares entre los grupos ($p>0,05$).
Conclusión: Las tasas de preñez y nacimiento de embriones IVP vitrificados de Nelore (*Bos indicus*) bajo condiciones de campo son semejantes a las de embriones en fresco.
Palabras clave: *criobiología, crioinjuria, criotolerancia, embriones sobrantes, reproducción de ganado, transferencia de embriones.*

Resumo
Antecedentes: A criopreservação é caracterizada pela manutenção da viabilidade celular em baixas temperaturas, resultando em atividade enzimática intracelular e metabolismo celular reduzido, que permite a preservação do material genético por períodos indefinidos de tempo. Embriões submetidos à criopreservação sofrem danos morfológicos e funcionais consideráveis, resultando em sobrevivência reduzida e menores taxas de desenvolvimento.
Objetivo: Avaliar a taxa de prenhez a partir de embriões Nelore (*Bos indicus*) produzidos *in vitro* (IVP) após a vitrificação sob condições de campo.
Métodos: Embriões IVP nos estádios de blastocisto (Bl) e blastocisto expandido (Bx) foram transferidos a fresco (n= 137) ou depois da vitrificação (n= 127).
Resultados: A taxa de prenhez aos 35 d foi menor para os embriões transferidos a fresco na fase de Bl (41,6), em relação aos Bx (60,9) ($p<0,05$). Apos a vitrificação, as taxas de prenhez foram semelhantes aos 35 d entre Bl (38,0) e Bx (47,6) ($p>0,05$). As perdas de prenhez aos 60 d foram semelhantes ($p>0,05$) tanto para embriões a fresco nos estádios de Bl (3,1) e Bx (4,8), e vitrificados em Bl (1,9) e Bx (4,7). As taxas de nascimentos foram semelhantes entre os grupos ($p>0,05$).
Conclusão: As taxas de prenhez e nascimentos dos embriões IVP vitrificados de Nelore (*Bos indicus*) sob condições de campo é semelhante àquela dos embriões a fresco.
Palavras-chave: c*riobiologia, crioinjúria, criotolerância, embriões excedentes, reprodução de gado, transferência de embriões.*

Introduction

Cryopreservation is a method that maintains cellular viability under low temperatures, resulting in diminished intracellular enzymatic activity and reduced cellular metabolism that ultimately allows preserving genetic material for indefinite periods of time (Seidel, 1996). This technique has applicability both on scientific and commercial settings (Dattena *et al*., 2004). Despite the differences in cryopreservation methodologies, all include temperature lowering, dehydration by cryoprotectants, freezing and thawing (Mara *et al*., 2013). Vitrification was developed as an alternative to increase cryotolerance for early cleavage embryos. This process consists of embryo dehydration by rapid exposure to a cryoprotectant solution of high molecular weight and direct immersion in liquid nitrogen (Vajta *et al*., 2000). Embryos submitted to cryopreservation suffer from considerable morphological and functional damage, resulting in reduced survival and developmental rates. Embryonic viability after cryopreservation depends upon embryo source (*in vivo* vs *in vitro*), quality, and developmental stage (Leibo *et al*., 1993, 1996). *In vitro* produced (IVP) embryos submitted to cryopreservation yield lower pregnancy rates than their *in vivo* counter-parts, possibly due to increased lipid content and smaller inner cell mass (Massip, 2001). Bovine embryo production system (*in vitro* vs *in vivo*), and breed (*Bos indicus* or *Bos taurus* breeds) are two major factors that affect embryo cryotolerance (Visitin *et al.*, 2002). Embryo vitrification has received great attention in the last decade as an attractive alternative to conventional freezing due to reduced costs, labour and overall simplicity (Kuwayama *et al.*, 2005; Vajta *et al.*, 2006; Sanches *et al.*, 2013; Kocyigit and Cevik, 2015). Moreover, it is considered an efficient technique by avoiding the formation of intra and extracellular ice crystals, diminishing damage to membranes and cellular organelles. However, despite significant progress in recent years, embryo cryopreservation in different domestic species does not hold standard practices and similar results (Prentice and Anzar, 2010), since IVP embryos remain less

tolerant to cryopreservation. Due to the widespread of IVP system worldwide, and increasing demand for vitrification of surplus embryos from cattle producers at increasing distances from IVP laboratories, the development of protocols under less rigorous conditions (i.e. field conditions) remains as a research topic worth exploring (Pereira *et al.*, 2016). Therefore, the objective of this study was to evaluate both pregnancy and delivery rates of *in vitro* produced (IVP) Nellore (*Bos indicus*) embryos after vitrification under field conditions.

Materials and Methods

Oocyte recovery

Ovum pick-up (OPU) was performed as described by Pereira *et al.* (2016). Ovarian follicles >2 mm diameter were aspirated by ultrasound (DP 2200 VET, Mindray, Nanshan, Shenzhen, China) using a 7.5 Mhz micro transducer convex, vacuum pump (WTA VET), and a 0.9 x 50 mm hypodermic needle 20G x 2" (Terumo Europe, Leuven, Belgium). The needle was connected to a 50 mL cone through a silicon tube (0.8 m long; 2 mm internal diameter). The medium used on OPU was TCM-199 (Gibco Life Technologies, Grand Island, NY, USA) supplemented with 25 mM HEPES (Sigma, St Louis, MO, USA), 5% fetal bovine serum (FBS), 50µg/mL gentamycin sulfate (Schering Plough, São Paulo, SP, Brazil), and 10 IU/mL sodium heparin (Sigma, St Louis, MO, USA).

In vitro maturation (IVM)

Only oocytes with normal morphology, with at least 3 compact layers of cumulus cells and homogenous cytoplasm, were used (Pereira *et al.*, 2016). Immediately after, the oocytes were washed 3 times in HEPES-containing TCM-199 medium (Gibco Life Technologies, Grand Island, NY, USA) supplemented with 10% FBS, 0.20 mM sodium pyruvate and 83.4 µg/mL amycacyn (Sigma, St Louis, MO, USA). For IVM, TCM-199 (Sigma, St Louis, MO, USA) supplemented with 10% FBS, 1µg/mL FSH (Sigma, St Louis, MO, USA), 50 µg/mL hCG, 1 µg/mL 17-β estradiol (Sigma, St Louis, MO, USA), 0.20 mM sodium pyruvate (Sigma, St Louis, MO, USA) and 83.4 µg/mL amycacyn (Sigma, St Louis, MO, USA) were used. Drops (100 µL) of embryo culture medium containing 25 to 30 oocytes were placed under mineral oil at 39 ºC in a humid atmosphere of 5% CO_2 during 22 to 26 hours.

In vitro fertilization (IVF)

The semen used was collected from bulls of proven *in vitro* fertility. Semen straws (25 x 10^6 spermatozoa per straw) were thawed for 30 seconds at 35 ºC. Semen samples were washed twice by centrifugation at 200 *g* for 5 minutes in 2 mL TALP medium supplemented with 10 mM HEPES (Sigma, St Louis, MO, USA), 0.2 mM sodium pyruvate (Sigma, St Louis, MO, USA) and 83.4 g/mL amycacyn (Sigma, St Louis, MO, USA). Spermatozoa were capacitated with 10µg/mL heparin and motility was stimulated with addition of 18 M penicillamine (Sigma, St Louis, MO, USA), 10 M hypotaurine (Sigma, St Louis, MO, USA), and 8 M epinephrine (Sigma, St Louis, MO, USA) (PHE). After visual appraisal of sperm motility, spermatic concentration was adjusted to 1 x 10^6 viable spermatozoa/mL, and placed in medium containing 90µL TALP-IVF supplemented with 10 µg/mL sodium heparin (Sigma, St Louis, MO, USA) and PHE with 1x10^5 spermatozoa per drop (Viana *et al.*, 2010).

Oocytes were washed 3 times after IVM in pre- IVF medium composed of TCM-199 (Gibco Life Technologies, Grand Island, NY, USA) supplemented with 25 mM HEPES (Sigma, St Louis, MO, USA), and 0.3% BSA (Sigma, St Louis, MO, USA). Additionally, oocytes were co-incubated with spermatozoa in FERT- TALP medium supplemented with 10 µg/mL sodium heparin and PHE under mineral oil at 39 ºC under saturated humidity and 5% CO_2 during 18 to 20 hours.

In vitro embryo culture (IVC) and blastocyst vitrification

The presumptive zygotes had their cumulus cells removed and were transferred to 100 µL drops of culture medium (SOFaa containing 0.5% BSA and 2.5% FBS) under mineral oil at 39 ºC in a humid atmosphere of 5% CO_2, 5% O_2 and 90% N_2. Embryo development was assessed on day 3 (D3), day 5 (D5), and day 7 (D7) of IVC. At D3 and D5, 50% of culture medium was replaced with fresh medium (feeding). Fresh controls were randomly selected (n = 140) at day 7 (D7) and transferred to synchronized recipients, as described below.

Blastocyst vitrification

Blastocysts (n= 130) were submitted to vitrification by the "Cryotop" method described by Kuwayama *et al.* (2005). Grade I initial blastocysts, blastocysts, and expanded blastocysts were placed in 10% ethylene glycol (EG) (Sigma, St Louis, MO, USA) and 10% DMSO (Sigma, St Louis, MO, USA) in HEPES- containing TCM-199 ((Sigma, St Louis, MO, USA) supplemented with 20% FBS (HTCM-FBS) during 1 minute at room temperature (RT). Embryos were

transferred to a vitrification solution containing 20% EG, 20% DMSO and 0.5 M sucrose (Sigma, St Louis, MO, USA) for 20 seconds in RT. During this incubation, blastocysts were loaded on top of polypropylene Cryotop tips (3 to 5 embryos) with minimum quantity of vitrification solution and immersed in liquid nitrogen.

Blastocyst warming

Blastocysts were removed from liquid nitrogen, exposed for 4 seconds in RT and subsequently warmed by immersion of polypropylene Cryotop tip (In Vitro Brasil, Mogi Mirim, Brazil) in medium (TCM- HEPES + sucrose) at 35 °C for 1 minute. Blastocysts were gradually transferred to HTCM-FBS medium containing 0.3 and 0.15 M sucrose (Sigma, St Louis, MO, USA) during 5 minutes each in RT (Morató et al, 2008; Vajta et al, 2010; Pereira et al, 2016). Embryos were loaded in 0.25 mL straws with HTCM-FBS medium and transferred to recipients after warming.

Embryo transfer

Embryo transfer was performed in a fixed-time manner. Recipients received intravaginal progesterone implants (CIDR, Pfizer, Hamilton, New Zealand) and 2 mg estradiol benzoate (Estrogin, Farmavet, São Paulo, SP, Brazil) on day 0. On day 7, recipients received 400 IU equine chorionic gonadotropin (eCG; Sincro eCG®; Ourofino Agronegócio) in association with 150 µg synthetic prostaglandin (D-Cloprostenol; Ciosin®; MSD Saúde Animal). The progesterone implant was removed on day 8, associated with an application of 2 mg estradiol. Before transfer, ovaries were evaluated by transrectal ultrasonography for the detection of corpus luteum (CL). Only recipients with a CL received an embryo on day 17. Pregnancy diagnosis was performed by transrectal ultrasonography on days 35 and 60 after IVF. Furthermore, all pregnancies were monitored monthly until delivery.

Statistical analysis

Analysis was performed by Fisher's Exact test using Prophet 5.0 Software for Windows 95, considering 5% ($p<0.05$) significance.

Results

<u>In vitro</u> production of bovine blastocysts

A total of 750 oocytes were retrieved from 30 donor cows by OPU (mean of 25.0 oocytes/cow). After IVF, 638 presumptive zygotes were cultured (85.06%). The cleavage rate was 73.66% (470/638), and blastocyst development rate at day 7 was 41.37% (264/638).

Pregnancy and delivery rates after transfer of fresh or vitrified blastocysts

The overall pregnancy rates on days 35 and 60 were similar between embryos transferred either fresh or after vitrification ($p>0.05$; Table 1). Overall pregnancy loss at 60 d was similar between fresh (7.69%) and vitrified embryos (6.00%; Table 1).

Table 1. Pregnancy and delivery rates for Nellore (*Bos indicus*) *in vitro*-produced embryos transferred fresh or after vitrification.

Embryos		Pregnancy rate at day 35	Pregnancy loss at day 60	Delivery rate
Embryo type	Transferred (n)	n (%)	n (%)	n (%)
Fresh	137	65 (47.44)	5 (7.69)	60 (43.79)
Vitrified	126	50 (39.68)	3 (6.00)	47 (37.30)

No statistical difference was observed between pregnancy rates ($p>0.05$).

Regarding fresh embryos, the stage of embryonic development affected pregnancy rate at day 35 (Table 2). Blastocysts transferred fresh displayed lower pregnancy rate when compared to expanded blastocysts ($p<0.05$; Table 2). The pregnancy rate after transfer of vitrified embryos was not affected by the stage of blastocyst development on both time points of development (Table 2). No difference was observed on pregnancy loss between blastocyst and expanded blastocyst stages, irrespectively if transferred fresh or after vitrification (Table 2). Moreover, delivery rates were similar between fresh and vitrified embryos (Table 1).

Table 2. Effect of blastocyst developmental stage on pregnancy rate for Nellore (*Bos indicus*) *in vitro* produced embryos transferred fresh or after vitrification.

Developmental stage	Pregnancy rate at day 35				Pregnancy loss at day 60			
	Fresh embryos		Vitrified embryos		Fresh embryos		Vitrified embryos	
	n	%	n	%	n	%	n	%
Blastocyst	40/96	41.6[b]	40/105	38.0	3/96	3.1[a]	2/105	1.9[a]
Expanded blastocyst	25/41	60.9[a]	10/21	47.6[a]	2/41	4.8[a]	1/21	4.7[a]

Values with different superscript letters ([a, b]) within the same column indicate significant difference ($p<0.05$).

Discussion

Bovine breeds are grouped in two subspecies, namely *Bos taurus* and *Bos indicus*, which differ in various aspects of their reproductive physiology, including embryo production potential under *in vitro* and *in vivo* conditions (Viana *et al.*, 2010). Differences between these subspecies can also be observed at the gene expression level in preimplantation embryos. Strikingly, these differences are somewhat attenuated by *in vitro* culture, suggesting a capacity for adaptive plasticity during early development (Wohlres-Viana *et al.*, 2011). Moreover, embryo source (e.g. *in vitro* or *in vivo*) is a major factor to embryo cryotolerance (Mucci *et al.*, 2006). It has been shown that IVP embryos have increased lipid content compared to their *in vivo* counterparts, which is associated with reduced survival after cryopreservation (Sudano *et al.*, 2011).

Vitrification is being widely adopted as an alternative embryo cryopreservation method to conventional freezing, due to its simplicity, reduced costs, and less labor, while dispensing for sophisticated equipment (Vajta et al., 1996). Vitrification is characterized by high concentrations of cryoprotectants (ACP), resulting in high viscosity of the vitrification solution, which leads to physical properties similar to solid compounds, despite the absence of crystallization (Chian et al., 2004).

). The data described here demonstrated that vitrification was as efficient as fresh embryos to establish pregnancy in recipient cows. During vitrification, the freezing rate, viscosity and volume of vitrification solution where embryos are placed, are important factors to consider (Arav et al., 2002; Yavin and Arav, 2007). A small volume of embryos improves heat transfer, increasing cryopreservation rate (Yavin et al., 2009). The high concentration of cryoprotectants used during vitrification could cause toxicity; however, due to the small volume and short period of exposure to cryoprotectants in the present study, no effect was expected on pregnancy rate.

Our results show that embryos transferred fresh during blastocyst and expanded blastocyst stages contributed differently to the overall pregnancy rate at 35 d. However, this difference was not observed on day 60, where vitrification was as efficient as fresh embryos. This discrepancy is probably due to the smaller cell number of the blastocysts transferred. Alternatively, the increased time of exposure to supplemented embryo culture medium after warming may have offered increased energy support and higher cellular metabolism that ultimately led to improved embryo quality and higher survival *in vivo*.

Scanavez *et al.* (2013) did not observe any effect of developmental stage of IVP embryos (morulae and initial blastocyst) compared to embryos at later stages (blastocyst, expanded blastocyst and hatched blastocyst). In the present study, pairwise comparisons (vitrification and fresh controls) between developmental stages resulted in different pregnancy rates for blastocysts and expanded blastocysts. It is important to note that all blastocysts were transferred at day 7 of development, irrespectively of their developmental stage. Putative differences in cellular metabolism and proliferation between embryonic developmental stages may have resulted in such differences in pregnancy rates of fresh controls.

Pregnancy loss was similar after transfer of blastocysts and expanded blastocysts at 60 d, which disagrees with Andreoti et al. (2009), who reported that embryonic loss of d7 embryos at earlier stages was higher than more developed embryos. This result is probably due to the variable correlation between embryo development kinetics and developmental potential. Another factor that probably contributed to high pregnancy rates was the improved synchrony between uterine environment and embryonic stage due to embryo transfer in a fixed-time manner (Randi et al., 2015

In conclusion, both pregnancy and delivery rates of *Bos indicus* IVP embryos after vitrification under field conditions is

indistinguishable from fresh embryos.

Conflict of interest

The authors declare they have no conflicts of interest with regard to the work presented in this report.

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