

VIABILITY OF *IN VITRO* PRODUCED EMBRYOS OF GYR CATTLE (*Bos indicus*) AFTER CRYOPRESERVATION BY VITRIFICATION UNDER FIELD CONDITIONS¹

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ABSTRACT: The aim of this study was to evaluate the pregnancy rate of *in vitro* produced embryos of Gyr cattle (*Bos indicus*) after cryopreservation by the vitrification method under field conditions. Blastocysts in different developmental stages were transferred to recipient cows either fresh (n = 140) or warmed after vitrification (n = 138). The pregnancy rates obtained for fresh embryos were 46.15% (initial blastocyst), 46.93% (blastocyst) and 50.0% (expanded blastocyst) at 35 days post-fertilization, and 43.58% (initial blastocyst), 46.93% (blastocyst) and 50.0% (expanded blastocyst) at 60 days. The pregnancy rates after embryo vitrification were 35.0% (initial blastocyst), 42.30% (blastocyst) and 43.47% (expanded blastocyst) at 35 days post-fertilization, and 32.50% (initial blastocyst), 38.46% (blastocyst) and 43.47% (expanded blastocyst) at 60 days. Embryo vitrification or blastocyst developmental stage did not affect pregnancy rates or incidence of embryonic death. In conclusion, vitrification of Gyr (*Bos indicus*) embryos under field conditions is an efficient method that can be implemented to use surplus *in vitro* produced embryos without affecting pregnancy rates.

Keywords: *Bos indicus*, cryobiology, cryotolerance, Zebu.

VIABILIDADE DE EMBRIÕES GIR (*Bos indicus*) PRODUZIDOS *In vitro* APÓS CRIOPRESERVAÇÃO EM CONDIÇÕES DE CAMPO PELA VITRIFICAÇÃO

RESUMO: Objetivou-se com o trabalho avaliar a taxa de prenhez a partir de embriões de bovinos da raça Gir (*Bos indicus*) produzidos *in vitro* após criopreservação em condições de campo pelo método de vitrificação. Blastocistos em diferentes estádios de desenvolvimento foram transferidos a fresco (n = 140) ou aquecidos após a vitrificação (n = 138). As taxas de prenhez de embriões frescos foram 46,15% (blastocisto inicial), 46,93% (blastocisto) e 50,00% (blastocisto expandido) aos 35 dias e 43,58% (blastocisto inicial), 46,93% (blastocisto) e 50,00% (blastocisto expandido) aos 60 dias pós-fertilização, respectivamente. As taxas de prenhez após a vitrificação foram 35,00% (blastocisto inicial), 42,30% (blastocisto) e 43,47% (blastocisto expandido) aos 35 dias e 32,50% (blastocisto inicial), 38,46% (blastocisto) e 43,47% (blastocisto expandido) aos 60 dias pós-fertilização, respectivamente. A vitrificação do embrião ou estágio do desenvolvimento não afetaram as taxas de prenhez ou incidência de morte embrionária. Em conclusão, a vitrificação de embriões de bovinos da raça Gir (*Bos indicus*) sob condições de campo é um método eficiente que pode ser implementado para aproveitar os embriões produzidos *in vitro* excedentes sem afetar a taxa de prenhez.

Palavras-chave: *Bos indicus*, criobiologia, criotolerância, Zebu.

INTRODUCTION

In vitro production (IVP) of embryos permits to increase the number of descendants of high genetic merit females, prepubertal or old females, and females with acquired reproductive disorders. Despite the intensive application of IVP embryos under commercial conditions (THIBIER, 2006), these embryos display significant differences compared to *in vivo* produced embryos, such as metabolic alterations, aberrant gene expression, excessive lipid accumulation, an increased incidence of apoptosis, and reduced cryotolerance (GJORRET *et al.*, 2003).

The cryopreservation of embryos permits to conserve the physical and functional properties of cells for undefined periods of time (MASSIP, 2001). Cryopreservation can be used for preserving the germplasm of important animals or endangered species or for germplasm transportation within countries. Embryos can be cryopreserved using two main strategies: conventional freezing and vitrification (LEIBO and LOSKUTOFF, 1993; MARA *et al.*, 2013). Conventional freezing consists of the progressive reduction in freezing temperature and the use of cryoprotectants of low molecular weight and reduced toxicity (LEIBO and LOSKUTOFF, 1993). This method shows good efficiency with *in vivo* produced embryos. In contrast, vitrification is characterized by rapid cryopreservation using high concentrations of cryoprotectants and direct transfer of the embryos to liquid nitrogen (VAJTA *et al.*, 2000).

Vitrification is becoming the main embryo cryopreservation technique due to its fast protocol while not requiring sophisticated equipment as does conventional freezing (ARAÚJO-LEMOS *et al.*, 2014, 2015). Moreover, several studies have shown increased survival of IVP embryos and higher viability measured at the molecular level (KUWAYAMA *et al.*, 2005; VAJTA *et al.*, 2006; STINSHOFF *et al.*, 2011; MARINHO *et al.*, 2015; LEME *et al.*, 2016). However, the efficiency of embryo vitrification remains variable and the influence of several important factors remains to be established. Bovine embryos produced *in vivo* from different cattle subspecies (*Bos taurus* and *Bos indicus*) differ significantly in their resistance to cryopreservation (VISINTIN *et al.*, 2002). However, the efficiency of vitrification of *Bos indicus* IVP embryos remains poorly described.

The aim of this study was to evaluate the viability of IVP blastocysts of Gyr cattle (*Bos indicus*) after cryopreservation by vitrification under field

conditions and the embryo transfer to synchronized recipient cows.

MATERIAL AND METHODS

The procedures performed throughout the experiment were formally approved by the Veterinary Medicine Ethics Committee of PIO DÉCIMO (Protocol 05/2011). The experiments were conducted at Nordeste In Vitro, Maceió, AL, Brazil.

Oocyte retrieval

Ovum pick-up was performed as described previously (ZHAO *et al.*, 2009). Ovarian follicles with a diameter > 2 mm were aspirated by ultrasound (DP 2200 VET, Mindray, Nanshan, Shenzhen, China) using a 7.5-MHz micro convex ultrasound transducer, a vacuum pump (WTA VET), and a hypodermic needle (20 G x 2", 0.9 x 50 mm) (Terumo Europe, Leuven, Belgium). This needle was connected to a 50-mL conical tube (Corning, Acton, MA, USA) through a silicon tube (0.8 m long, 2 mm internal diameter). The medium used for ovum pick-up was TCM-199 (Gibco Life Technologies, Grand Island, NY, USA) supplemented with 25 mM HEPES (Sigma-Aldrich, St. Louis, USA), 5% fetal bovine serum (FBS), 50 µL/mL gentamycin sulfate (Schering-Plough, São Paulo, SP, Brazil), and 10,000 IU/L sodium heparin (Sigma-H3149).

In vitro maturation (IVM)

Oocytes were selected based on their morphology, with least three compact cumulus cell layers and classified as grade 1 as described previously (MORATÓ *et al.*, 2008). The oocytes were washed three times in TCM-199/HEPES medium (Gibco Life Technologies) supplemented with 10% FBS, 0.20 mM sodium pyruvate, and 83.4 µg/mL amikacin. For IVM, TCM-199 supplemented with 10% FBS (Gibco Life Technologies), 1 µg/mL FSH (Folltropin, Bioniche Animal Health, Belleville, ON, Canada), 50 µg/mL hCG (Profasi, Serono, São Paulo, SP, Brazil), 1 µg/mL 17-β estradiol, 0.20 mM sodium pyruvate, and 83.4 µg/mL amikacin was used. Drops of embryo culture medium (100 µL) containing 25 to 30 oocytes were covered with mineral oil and incubated at 39°C in a humid atmosphere of 5% CO₂ for 22 to 26 hours.

In vitro fertilization (IVF)

The semen used was collected from bulls of proven *in vitro* fertility. Semen straws (2 x 10⁷/dose) were thawed for 30 seconds at 35°C. The semen

sample was washed twice by centrifugation at 200 g for 5 minutes in 2 mL TALP medium supplemented with 10 mM HEPES (Gibco, Life Technologies), 0.2 mM sodium pyruvate, and 83.4 g/mL amikacin. Spermatozoa were capacitated with 10 µg/mL heparin and motility was stimulated by the addition of 18 M penicillamine, 10 M hypotaurine, and 8 M epinephrine (PHE). After visual appraisal of motility and concentration adjustment to 25×10^6 viable spermatozoa/mL, semen was placed in medium containing 90 µL TALP-IVF supplemented with 10 µg/mL sodium heparin and PHE at 1×10^5 spermatozoa per drop (VIANA *et al.*, 2010).

Oocytes were washed three times after IVM in pre-IVF medium consisting of TCM-199 supplemented with 25 mM HEPES and 0.3% BSA (TCM-HEPES). 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°C under 5% CO₂ in saturated humidity for 18 to 20 hours.

***In vitro* embryo culture (IVC)**

The presumptive zygotes had their cumulus cells removed, were transferred to 100-µL drops of culture medium (SOFaa containing 0.5% BSA and 2.5% FBS), and incubated under mineral oil at 39 °C in a humid atmosphere of 5% CO₂, 5% O₂ and 90% N₂. Embryo development was assessed on days 3, 5 and 7 of IVC. On days 3 and 5, 50% of the culture medium was replaced with fresh medium (feeding). Fresh controls were randomly selected (n = 140) and evaluated on day 7.

Blastocyst vitrification

Blastocysts (n = 130) were submitted to vitrification by the open pulled straw (OPS) method as described previously (KUWAYAMA *et al.*, 2005). Grade I blastocysts were placed in 10% ethylene glycol (WakoPure Chemical Industries Co., Osaka, Japan) and 10% DMSO (WakoPure Chemical Industries Co.) in TCM-HEPES supplemented with 20% FBS for 1 minute at room temperature. Embryos were further transferred to a vitrification solution containing 20% ethylene glycol, 20% DMSO and 0.5 M sucrose, and incubated for 20 seconds at room temperature. During this incubation, three to five blastocysts were loaded into OPS straws (In Vitro Brasil, Mogi Mirim, Brazil) containing a minimum quantity of vitrification solution and stored in liquid nitrogen (-196°C).

Blastocyst warming

The blastocysts were removed from liquid

nitrogen, exposed for 4 seconds to air at room temperature, and warmed by immersing the OPS straws in warming medium (TCM-HEPES + sucrose) at 35°C for 1 minute. Blastocysts were then gradually incubated in warming medium containing 0.3 and 0.15 M sucrose for 5 minutes each at room temperature (MORATÓ *et al.*, 2008; VAJTA *et al.*, 1998). Embryos were loaded into 0.25-mL straws in HEPES-buffered SOF medium (*In Vitro* Brasil) and transferred to synchronized recipient cows after warming.

Embryo transfer

A total of 278 recipient cows were synchronized for fixed-time embryo transfer. 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 eCG in combination with 150 µg prostaglandin. The progesterone implant was removed on day 8 and a 1-mg estradiol benzoate shot was applied. Estrus detection was performed on days 9, 10 and 11 in the morning and afternoon by trained personnel. Before transfer, the ovaries were evaluated by transrectal ultrasound (DP 2200 VET, 5-MHz linear transducer, Mindray) for detection of the corpus luteum. Only recipients with a corpus luteum received an embryo on day 17. Pregnancy was diagnosed by transrectal ultrasound (DP 2200 VET, 5-MHz transducer linear, Mindray) on days 35 and 60 after IVF. Embryonic loss was calculated by subtracting the number of pregnancies on day 60 from the number of pregnancies on day 35.

Statistical analysis

Fresh embryos were used as the control group, while embryos submitted to vitrification comprised the experimental group. Embryos were the experimental units. Descriptive statistics reporting pregnancy rates as percentage was used. Inferential analysis of binomial data was performed using the chi-square test (samples of 6 or more records) or Fisher's exact test (five or fewer records), considering a level of significance of 5%.

RESULTS

Embryo viability after vitrification was evaluated by direct transfer to synchronized recipient cows (Table 1). Pregnancy rates on day 35 and day 60 after IVF were similar in the control and vitrification groups. The pregnancy rates

of the different blastocyst stages did not differ between groups (Table 1). The overall incidence of embryonic loss was low and losses were similar for blastocysts transferred fresh (1.49%) and after vitrification (5.35%). Moreover, the blastocyst stage did not affect the rate of pregnancy loss (Table 1). This low embryonic loss may be due to specific unidentified factors of *Bos indicus* that increase embryo viability after cryopreservation or improve intrauterine embryo synchronization, since embryo transfer was performed in a fixed-time manner.

DISCUSSION

Modern cattle breeds are phylogenetically classified into two subspecies, namely *Bos taurus* and *Bos indicus*. More importantly, cattle reproductive physiology such as follicular dynamics and *in vivo* and *in vitro* embryo production efficiency differs between subspecies (VIANA *et al.*, 2010). Gene expression analysis has shown that these differences are more intense in embryos produced *in vivo* and that this variation is diminished during *in vitro* embryo culture by uncharacterized factor(s) (WOHLRES-VIANA *et al.*, 2011). The understanding of mechanisms governing subspecies differences in their reproductive physiology would have important scientific and commercial applications.

Vitrification is a practical cryopreservation

technique for somatic cells, gametes and preimplantation embryos and has important advantages over conventional freezing, such as its straightforward protocol and competitive costs while not requiring expensive equipment (VAJTA *et al.*, 1996; CHIAN *et al.*, 2004; MARINHO *et al.*, 2015; LEME *et al.*, 2016). Furthermore, vitrification has been extensively studied in an attempt to efficiently cryopreserve IVP embryos (ARAÚJO-LEMOS *et al.*, 2014, 2015) since these embryos have shown reduced cryotolerance compared to their *in vivo* counterparts (TOMINAGA, 2004; MUCCI *et al.*, 2006; LEME *et al.*, 2016). This fact is due, at least in part, to excessive lipid content under *in vitro* culture conditions (SUDANO *et al.*, 2011).

The overall outcome of vitrification depends on several factors such as freezing rate, viscosity, composition of the vitrification medium, and embryo-to-media ratio (YAVIN and ARAV *et al.*, 2007, 2009; HASLER, 2010; ARAV *et al.*, 2014). In addition to intrinsic technical variability, different biological factors can affect the efficiency of vitrification. The present study investigated the effect of embryo kinetics (blastocyst development stages on day 7) and source (e.g., *Bos indicus*) on pregnancy rates after embryo cryopreservation under field conditions. The pregnancy rates of vitrified embryos were similar to those of fresh non-cryopreserved controls, irrespective of blastocyst stage. This finding rules out any detrimental blastocyst stage-specific effect

Table 1. Effect of vitrification under field conditions on the pregnancy rate of *in vitro* produced embryos of Gyr (*Bos indicus*) cattle

Developmental stage	Pregnancy (35days) ¹	Pregnancy (60days) ²	Embryonic loss ³
Fresh (control)	N(%)	N(%)	N(%)
Initial blastocyst	18/39(46.15)	17/39(43.58)	1/18(5.55)
Blastocyst	23/49(46.93)	23/49(46.93)	0/23(0)
Expanded blastocyst	26/52(50.0)	26/52(50.0)	0/26(0)
Total	67/140(47.85)	66/140(47.14)	1/67(1.49)
Vitrification	N(%)	N(%)	N(%)
Initial blastocyst	14/40(35.0)	13/40(32.5)	1/14(7.14)
Blastocyst	22/52(42.3)	20/52(38.46)	2/22(9.09)
Expanded blastocyst	20/46(43.47)	20/46(43.47)	0/20(0)
Total	56/138(40.57)	53/138(38.4)	3/56(5.35)

¹35 days: 35 days after *in vitro* production of embryos. ²60 days: 60 days after *in vitro* production of embryos. ³Embryonic loss was calculated by subtracting the number of pregnancies on day 60 from the number of pregnancies on day 35.

Level of significance of 5%.

on cryotolerance. Pregnancy rates have been described with similar efficiencies for fresh IVP embryos, irrespectively of embryo developmental stage (SCANAVEZ *et al.*, 2013). Based upon these evidences, vitrification under field conditions is an attractive approach for cryopreservation of Gyr (*Bos indicus*) IVP embryos.

CONCLUSION

The pregnancy rate after fixed-time transfer of Gyr IVP embryos cryopreserved by vitrification under field conditions is similar to that of fresh embryos (non-cryopreserved controls), irrespective of blastocyst stage.

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