Research, Society and Development, v. 9, n. 6, e134963615, 2020
(CC BY 4.0) | ISSN 2525-3409 | DOI: http://dx.doi.org/10.33448/rsd-v9i6.3615

Estágio de desenvolvimento no envase afeta a viabilidade de embriões bovinos produzidos *in vitro*

Development stage at packaging affects viability of *in vitro* produced bovine embryos

Etapa de desarrollo en llenado afecta la viabilidad de los embriones bovinos producidos *in vitro*

Recebido: 08/04/2020 | Revisado: 18/04/2020 | Aceito: 19/04/2020 | Publicado: 21/04/2020

Mariana da Mata Silveira
ORCID: http://orcid.org/0000-0002-5990-6857
Instituto Federal de Educação Ciência e Tecnologia Goiano – Campus Rio Verde, Brasil
E-mail: damattamari@gmail.com
Thaisa Campos Marques
ORCID: http://orcid.org/0000-0003-1112-6699
Instituto Federal de Educação Ciência e Tecnologia Goiano – Campus Rio Verde, Brasil
E-mail: thaisacm@hotmail.com
Marco Antônio Pereira da Silva
ORCID: http://orcid.org/0000-0002-3875-1104
Instituto Federal de Educação Ciência e Tecnologia Goiano – Campus Rio Verde, Brasil
E-mail: marco.antonio@ifgoiano.edu.br
Karen Martins Leão
ORCID: http://orcid.org/0000-0001-5236-7558
Instituto Federal de Educação Ciência e Tecnologia Goiano – Campus Rio Verde, Brasil
E-mail: karen.leao@ifgoiano.edu.br

Resumo

O objetivo deste estudo foi avaliar a viabilidade de embriões produzidos *in vitro* (IVPE) bovinos em diferentes estágios de desenvolvimento, mantidos a 36ºC em diferentes tempos de transporte. No sétimo dia de cultura *in vitro*, os embriões grau I e II nos estágios iniciais do blastocisto, blastocisto e blastocisto expandido foram selecionados, envasados e distribuídos aleatoriamente por três tempos de transporte em uma incubadora portátil a 36°C: 6, 9 e 12 horas. Após o tempo de tratamento, os embriões foram re-cultivados em meio de cultivo por 72 horas e avaliados quanto à qualidade e desenvolvimento. Os dados de taxa de eclosão e degeneração foram analisados pelo teste do qui-quadrado. Os embriões envasados no estágio de blastocisto

expandido apresentaram maiores taxas de eclosão e menores taxas de degeneração. Os embriões envasados no estágio de blastocisto expandido mostraram maior viabilidade em comparação com os do blastocisto inicial, independentemente do tempo de transporte, e os blastocistos transportados por 6 e 9 horas.

Palavras-chave: Desenvolvimento embrionário; Transporte embrionário; Fertilização *in vitro*; OPU-FIV.

Abstract

The objective of this study was to evaluate the viability of *in vitro* produced embryos (IVPE) bovine at different developmental stages, maintained at 36ºC at different transport times. On the seventh day of *in vitro* culture, grade I and II embryos at early blastocyst, blastocyst, and expanded blastocyst stages were selected, packaged and randomly distributed to three transport times in a portable incubator at 36°C: 6, 9 and 12 hours. After the treatment time, embryos were re-cultured in culture medium for 72 hours and evaluated for quality and development. Hatching and degeneration rate data were analyzed by Chi-square test. Embryos packaged at the expanded blastocyst stage showed higher hatching rates and lower degeneration rates. Embryos packaged at the expanded blastocyst stage showed higher viability compared to those at early blastocyst, regardless of transport time, and blastocysts transported for 6 and 9 hours.

Keywords: Embryonic development; Embryonic transport; i*n vitro* fertilization; OPU-IVF.

Resumen

El objetivo de este estudio fue evaluar la viabilidad de embriones bovinos producidos *in vitro* (IVEP) en diferentes etapas de desarrollo, mantenidos a 36ºC en diferentes tiempos de transporte. En el séptimo día de cultivo *in vitro*, los embriones de grado I y II en las primeras etapas del blastocisto, blastocisto y blastocisto expandido fueron seleccionados, empaquetados y distribuidos aleatoriamente durante tres tiempos de transporte en una incubadora portátil a 36°C: 6, 9 y 12 horas. Después del tiempo de tratamiento, los embriones se volvieron a cultivar en medio de cultivo durante 72 horas y se evaluó su calidad y desarrollo. La tasa de eclosión y los datos de degeneración se analizaron mediante la prueba de chi-cuadrado. Los embriones empaquetados en la etapa de blastocisto expandido mostraron tasas de eclosión más altas y tasas de degeneración más bajas. Los embriones empaquetados en la etapa de blastocisto expandido mostraron una mayor viabilidad en comparación con los del blastocisto inicial, independientemente del tiempo de transporte, y los blastocistos transportados durante 6 y 9 horas.

Palabras clave: Desarrollo embrionario; Transporte embrionario; Fertilización *in vitro*; OPU-FIV.

1. Introduction

IVPE are more sensitive than those produced *in vivo* due to the production system with high oxygen concentrations and intracellular lipid accumulation, with consequent susceptibility to the action of reactive oxygen species (Agarwal et al. 2006). Such factors cause cell damage (Gupta et al. 2010) and alterations in the expression of genes for embryo quality and development (Stinshoff et al. 2011).

The maintenance medium is used to transport fresh embryo in the shortest possible time, allowing for continued development and preventing oxidative stress. However, embryonic viability, and consequently, pregnancy rate may be influenced by the time spent in transportation (Marinho et al. 2012).

Thus, the distance between laboratories and farms affects embryo quality when the embryo is transferred fresh (Teixeira 2013). An alternative used is the transport of embryos at the culture phase and at different stages, kept in a portable incubator with controlled atmosphere (Cavalieri et al. 2015).

Few studies investigated the effects of embryo development stage on embryo quality and development following transport. Thus, this study aimed to evaluate the viability of IVPE bovine, at different developmental stages, maintained at 36ºC during different transport times.

2. Materials and Methods

The present research is a laboratory research with a quantitative approach, as it used data collection, which were statistically analyzed to verify the relationship between the stage and embryonic viability (Pereira et al. 2018).

This study was approved by the Animal Ethics Committee, Rio Verde, Brazil.

All culture media used were purchased from Progest Biotechnology in Reproduction and Animal Health® (Botucatu, São Paulo, Brazil).

In vitro embryo production

Embryos were produced using cumulus-oocyte complexes (COC) harvested from local slaughterhouse derived ovaries of cows. Grade I and II COC (Stojkovic et al. 2001) were

cultured in drops (30-35 COCs/200 µL) of maturation media for 24 hours in an incubator at 38.5°C and 5% CO_2. Then, COCs were co-incubated with thawed motile sperm (1.0 x 10^6 cells/mL) in 200 µL fertilization medium (TALP-IVF supplemented with 6 mg/mL fatty acid-free BSA, 0.2 mM pyruvate, 30 µg/mL heparin, 20 µM penicillamine, 10 µM hypotaurine, 1 µM epinephrine and 75 µg/mL amikacin) at 38.5°C and 5% CO_2 for 24 hours. Possible zygotes were cultured in 200 µL culture medium (SOFaa, Holm et al. 1999) with 2.7 mM myo-inositol, 0.2 mM pyruvate, 2.5% FBS (v/v), 5 mg/mL fatty acid-free BSA and 75 µg/mL amikacin) for seven days (D7) at 38.5°C and 5% CO_2. On the third day (D3), half of the culture medium of each drop was replaced and the cleavage structures was evaluated.

Packaging and simulated transport

On the D7, embryos were evaluated morphologically for developmental stage and embryonic quality. Grade I and II embryos at early blastocyst (Bi), blastocyst (Bl), and expanded blastocyst (Bx) stages were randomly selected and distributed to three transport times: 6, 9, and 12 hours. Embryos were packaged in 0.25 mL straws with maintenance medium (SOFaa, Holm et al. 1999) buffered with Hepes (HSOFaa) with 2.7 mM myo-inositol, 0.2 mM pyruvate, 2.5% FBS (v/v), 5 mg/mL fatty acid-free BSA and 75 µg/mL amikacin) and kept at 36°C in an portable incubator according to the time of treatment.

Unpackaging, re-culture and evaluations

After the time of the treatments, straws were removed from the portable incubator, unpackaging and re-culture of embryos in drops (25 µL) culture medium at 38.5°C and 5% CO_2 for 72 hours.

Embryos were evaluated for development and morphological quality upon unpackaging and at 24, 48 and 72 hours after packaging in re-culture.

Statistical analysis

Nine treatments were considered for the isolated evaluation, performing a chi-square test using the R Core 3.5.0 software (R Core Team 2018).

3. Results

Table 1 shows the percentage of hatched and degenerated embryos according to developmental stage at the time of packaging and transport time, evaluated at unpackaging and 24, 48 and 72 hours after packaging.

Table 1. Percentage of hatched (HAT-%) and degenerated (DEG-%) embryos according to developmental stage at the time of packaging (early blastocyst-Bi, blastocyst-Bl, expanded blastocyst-Bx) and transport time (6, 9 and 12 hours), evaluated at unpackaging and 24, 48 and 72 hours after packaging.

Stage	Time	Packaging	Unpackaging		24 horas		48 horas		72 horas	
		(N)	HAT	DEG	HAT	DEG	HAT	DEG	HAT	DEG
Bi	6h	77	0,0% b	0,0 % a	2,6% c	18,2% a	26,0% c	33,8% a	41,6% d	41,6% a
	9h	68	0,0% b	1,5% a	1,5% c	14,7% a	19,1% c	32,4% a	25,0% d	52,9% a
	12h	66	0,0% b	0,0 % a	1,5% c	9,1% a	27,3% c	21,2% a	45,5% d	34,8% a
Bl	6h	72	0,0% b	0,0 % a	29,2% b	6,9% a	61,1% b	12,5% b	69,4% c	20,8% b
	9h	90	0,0% b	1,1% a	16,7% b	2,2% b	66,7% b	12,2% b	67,8% c	23,3% b
	12h	80	1,3% b	0,0 % a	18,8% b	2,5% b	62,5% b	8,8% b	72,5% bc	16,3% bc
Bx	6h	94	1,1% b	1,1% a	69,1% a	2,1% b	86,2% a	6,4% b	89,4% a	8,5% c
	9h	90	6,7% ab	1,1% a	55,6% a	2,2% b	83,3% a	5,6% b	90,0% a	6,7% c
	12h	108	13,9% a	0,9% a	51,9% a	1,9% b	76,9% a	6,5% b	81,5% ab	9,3% c

Different letters indicate significant differences within a column ($P < 0.05$).

The different transport times had no influence at each developmental stage at the time of packaging ($P > 0.05$) in the evaluations of unpackaging, 24, 48 and 72 hours (Table 1).

In the unpackaging assessment, hatching rate of Bx stage embryos transported for 12h was higher than the other stages, regardless of transport time ($P < 0.05$). However, degeneration rate of Bx stage embryos was similar to Bi and Bl stages, regardless of transport time ($P > 0.05$).

Evaluations at 24 and 48 hours, regardless of transport time, Bx stage embryos showed higher hatching rates compared to the other stages at the time of packaging ($P < 0.05$).

In the 72-hour evaluation, Bx stage embryos had higher hatching rates and lower degeneration rates compared to those at the Bi, regardless of transport time, and at the Bl transported for 6 and 9 hours ($P < 0.05$).

4. Discussion

In the present study, embryos at a more advanced stage of development presented higher hatching rates and lower degeneration rates than embryos packaged at early stages.

Preimplantation embryonic development is a dynamic process that involves cell proliferation, differentiation, and death (Paula-Lopes & Hansen 2002). This process is regulated by signaling between the embryo and the maternal environment. An embryo´s ability to respond to changes in its environment is limited during the first cleavage divisions, when most of the embryonic genome is still inactive (Memili & First 2000).

More advanced stage embryos after the first cell division have a greater development potential and likelihood of pregnancy than late cleaving embryos (Carrocera et al. 2016, Fenwick et al. 2002). According Sugimura et al. (2012), slowly cleaving embryos had a higher incidence of abnormal chromosomes and downregulation of interferon-tau (*IFNT)* expression. Abnormal chromosomal may play a role and likely reflects the quality of spermatozoa and oocytes (Magli et al. 2007) and apoptosis is a form of cell death that can function to eliminate cells damaged by environmental stress (Paula-Lopes & Hansen 2002). These facts explain the higher degeneration rate of Bi and Bl embryos presented in this study in relation to Bx stage.

Similar results to this study evaluating the pregnancy rate of beef and dairy cows as a function of the stage of *in vitro* embryo development at the time of transfer. In beef cows, the pregnancy rates of 51%, 42% and 39.5% for Bx, Bl and Bi, respectively. In dairy cows, the rates were 43% (Bx), 40% (Bl) and 23.5% (Bi) (Pacheco et al. 2018). This relationship was also reported by Silva (2010) with Bx (44%) and Bl (32.7%). However, there is an indication that hatched blastocyst or morula have a lower pregnancy rate than other developmental stages (Jainudeen et al. 2004).

The moment of formation of the blastocyst is a predictor of the quality of the embryo, since the embryos that form the blastocoel earlier are superior to the late ones in relation to the total number of cells, allocation of internal cell mass and trophectodermal and cryosurvival (Mahmoudzadeh et al. 1995; Van Soom et al. 1997). Thus, the developmental stage of IVPE on the seventh day indicates quality, since the more advanced stages presented higher pregnancy rate due to the higher activity and superior quality of the embryos (Farin et al. 2004, Sanches et al. 2013, Munoz et al. 2014). This is due to several factors, among which the expression of the IFNT gene, that is involved in the establishment of pregnancy and signaling of maternal recognition of pregnancy in ruminants (Barnwell et al. 2016).

Thus, in this study, we observed that embryos packaged at an expanded blastocyst stage have greater viability. This suggests that the greater speed of embryonic development after cleavage may have a greater potential to promote pregnancy.

5. Conclusion

In conclusion, embryos packaged at the expanded blastocyst stage showed higher viability than at the initial blastocyst, regardless of the transport time and blastocysts transported for 6 and 9 hours, indicating higher hatching rate and lower degeneration rate in re-culture.

Future research with gene expression, cryopreservation and embryo transfer are suggested for further clarification regarding the development of bovine embryos produced *in vitro*.

Funding

Special thanks to Instituto Federal Goiano – Campus Rio Verde, CNPQ and CAPES.

References

Agarwal, A., Said, T. M., Bedaiwy, M. A., Banerjee, J., & Alvarez, J. G. (2006). Oxidative stress in an assisted reproductive techniques setting. *Fertility and Sterility, 86* (3), 503-512. doi: 10.1016/j.fertnstert.2006.02.088.

Barnwell, C. V., Farin, P. W., Ashwell, C. M., Farmer, W. T., Galphin, S. P., Jr & Farin, C. E. (2016). Differences in mRNA populations of short and long bovine conceptuses on Day 15 of gestation. *Molecular, Reproduction and Development*, *83* (5), 424–441. doi:10.1002/MRD.22640.

Carrocera, S., Caamaño, J. N., Trigal, B., Martín, D., & Díez, C. (2016). Developmental kinetics of in vitro-produced bovine embryos: An aid for making decisions. *Theriogenology 85* (5), 822–827. doi:10.1016/j.theriogenology.2015.10.028.

Cavalieri, F. L. B., Andreazzi, M. A., Colombo, A. H. B., Emanuelli, I. P., Moreski, D. A. B., & Silva, W. M. (2015). Estudo sobre o cultivo in vitro de embriões bovinos durante o transporte. *Ars Veterinaria*, *31*(1), 7-11. doi: 10.15361/2175-0106.2015v31n1p07-11.

Farin, C. E., Farin, P. W., & Piedrahita, J. A. (2004). Development of fetuses from *in vitro* -produced and cloned bovine embryos. *Journal of Animal Science, 82,* 53-62. doi: 10.2527/2004.8213_supplE53x.

Fenwick, J., Platteau, P., Murdoch, A. P. & Herbert, M. (2002). Time from insemination to first cleavage predicts developmental competence of human preimplantation embryos *in vitro*. *Human Reproduction, 17* (2), 407–412. doi: 10.1093/humrep/17.2.407.

Gupta, M. K., Uhm, S. J., & Lee, H. T. (2010). Effect of vitrification and beta-mercaptoethanol on reactive oxygen species activity and in vitro development of oocytes vitrified before or after in vitro fertilization. *Fertility and Sterility, 93* (8), 2602-2607. doi: 10.1016/j.fertnstert.2010.01.043.

Holm, P., Schimidt, M. H., Greve, T. & Callesen, H. (1999). High bovine blastocyst development in a static in vitro production system using SOFaa medium supplemented with sodium citrate and myo-inusitol with or without serum-proteins. *Theriogenology*, *52* (4), 683-700. doi: 10.1016/S0093-691X(99)00162-4.

Jainudeen, M. R., Wahid, H., & Hafez, E.S.E. (2004). Indução da ovulação, Produção e Transferência de Embriões. In HAFEZ, E.S.E & HAFEZ, B. (Ed.), *Reprodução Animal* (pp. 409-434). São Paulo, Brasil: Manole.

Magli, M. C., Gianaroli, L., Ferraretti, A. P., Lappi, M., Ruberti, A. & Farfalli, V. (2007). Embryo morphology and development are dependent on the chromosomal complement. *Fertility and Sterility, 87* (3), 534–541. doi: 10.1016/j.fertnstert.2006.07.1512.

Mahmoudzadeh, A. R., Van Soom, A., Bols, P., Ysebaert, M. T., & De Kruif, A. (1995). Optimization of a simple vitrification procedure for bovine embryos produced in vitro: effect of developmental stage, two-step addition of cryoprotectant and sucrose dilution on embryonic survival. *Reproduction, 103* (1), 33–39. doi:10.1530/jrf.0.1030033.

Marinho, L. S. R., Untura, R. M., Morotti, F., Moino, L. L., Rigo, A. G., Sanches, B.V., Pontes, J. H. F. & Seneda, M. M. (2012). Large-scale programs for recipients of in vitro-produced

embryos. *Animal Reproduction, 9* (3), 323-328. Retrieved February 13, 2020, from https://www.animal-reproduction.org/article/5b5a6059f7783717068b46ed.

Memili, E & First, N. L. (2000). Zygotic and embryonic gene expression in cow: a review of timing and mechanisms of early gene expression as compared with other species. *Zygote, 8* (1), 87-96. doi: 10.1017/s0967199400000861.

Munoz, M., Uyar, A., Correia, E., Diez, C., Fernandez-Gonzalez, A., Caamano, J. N., Martinez-Bello, D., Trigal, B., Humblot, P., Ponsart, C., Guyader-Joly, C., Carrocera, S., Martin, D., Marquant Le Guienne, B., Seli, E. & Gomez, E. (2014). Prediction of pregnancy viability in bovine in vitro-produced embryos and recipient plasma with Fourier transform infrared spectroscopy. *Journal of Dairy Science, 97* (9), 5497-5507. doi: 10.3168/jds.2014-8067.

Pacheco, S. L., Hoppen, A. R. & De Souza Rosa, F. (2018). Comparação da taxa de prenhez conforme o estágio de desenvolvimento de embriões produzidos in vitro e transferidos em bovinos de corte e leite. *Revista de Ciências Agroveterinárias e Alimentos*, 2. Retrieved March 16, 2020, from https://pdfs.semanticscholar.org/aae1/5af2be6b85e4f25c3b927310784c00558c86.pdf.

Paula-Lopes, F. F. & Hansen, P. J. (2002). Heat shock-induced apoptosis in preimplantation bovine embryos is a developmentally regulated phenomenon. *Biology of reproduction, 66* (4), 1169–1177. doi: 10.1093/biolreprod/66.4.1169.

Pereira, A. S., Shitsuka, D. M., Parreira, F. J. & Shitsuka, R. (2018). *Metodologia do trabalho científico* [e-book]. Santa Maria, RS: UFSM, NTE. Retrieved April 2, 2020, from https://repositorio.ufsm.br/bitstream/handle/1/15824/Lic_Computacao_Metodologia-Pesquisa-Cientifica.pdf?sequence=1.

R Core Team (2018). R: A Language and Environment for Statistical Computing. R Foundation for Statistical Computing, Viena, Áustria (2018). Retrieved July 3, 2019, from http://www.R-project.org.

Sanches, B. V., Marinho, L. S., Filho, B. D., Pontes, J. H., Basso, A. C., Meirinhos, M. L., Silva-Santos, K. C., Ferreira, C. R. & Seneda, M. M. (2013). Cryosurvival and pregnancy rates

after exposure of IVF-derived *Bos indicus* embryos to forskolin before vitrification. *Theriogenology*, *80* (4), 372-377. doi: 10.1016/j.theriogenology.2013.04.026.

Silva, L. A. (2010). Taxa de gestação e mortalidade embrionária em receptoras de embriões produzidos in vitro, após sincronização do estro com diferentes protocolos hormonais (dissertação de mestrado). Universidade Federal de Minas Gerais, Belo Horizonte, Brasil. Retrieved January 21, 2019, from https://www.vet.ufmg.br/DOWNLOAD.php?o=8&i=20140424144514&a=taxas_de_gestacao_e_mortalidade_embrionaria_em_receptoras_de_embriaes.

Stinshoff, H., Wilkening, S., Hanstedt, A., Brüning, K. & Wrenzycki, C. (2011). Cryopreservation affects the quality of in vitro produced bovine embryos at the molecular level. *Theriogenology, 76* (8), 1433-1441. doi: 10.1016/j.theriogenology.2011.06.013.

Stojkovic, M., Machado, S. A., Stojkovic, P., Zakhartchenko, V., Hutzler, P., Gonçalves, P, B. & Wolf, E. (2001). Mitochondrial distribution and adenosine triphosphate content of bovine oocytes before and after in vitro maturation: correlation with morphological criteria and developmental capacity after in vitro fertilization and culture. *Biology of Reproduction, 64* (3), 904-909. doi: 10.1095/biolreprod64.3.904.

Sugimura, S., Akai, T., Hashiyada, Y., Somfai, T., Inaba, Y., Hirayama, M., Yamanouchi, T., Matsuda, H., Kobayashi, S., Aikawa, Y., Ohtake, M., Kobayashi, E., Konishi, K. & Imai, K. (2012). Promising System for Selecting Healthy In Vitro–Fertilized Embryos in Cattle. *PlosOne, 7* (5), e36627. doi:10.1371/journal.pone.0036627.

Teixeira, J. R. (2013). Transporte e cultivo de embriões bovinos por 24, 48 e 72 horas antes da transferência (dissertação de mestrado). Universidade Federal de Uberlândia, Uberlândia, Brasil. Retrieved January 21, 2019, from https://repositorio.ufu.br/handle/123456789/13145.

Van Soom, A., Ysebaert, M. T., & de Kruif, A. (1997). Relationship between timing of development, morula morphology, and cell allocation to inner cell mass and trophectoderm in in vitro-produced bovine embryos. *Molecular Reproduction and Development, 47* (1), 47–56. doi:10.1002/(sici)1098-2795(199705)47:1<47::aid-mrd7>3.0.co;2-q.

Percentage of contribution of each author in the manuscript

Mariana da Mata Silveira – 50%

Thaisa Campos Marques – 20%

Marco Antônio Pereira da Silva – 5%

Karen Martins Leão – 25%