STUDY OF TRANSPORT SYSTEMS FOR IN VITRO PRODUCED BOVINE EMBRYOS OVER LONG DISTANCES

Isabele Picada Emanuelli
Full professorCesumar University -UNICESUMAR, Brazil
Researcher at the Cesumar Institute of Science and Technology – ICETI, Brazil
isabele.emanuelli@unicesumar.edu.brORCID: https://orcid.org/0000-0002-1423-2748
55(44)999264438
*Corresponding author

Antônio Guilherme Roncada Pupulim
Professor Centro Universitário Integrado, Brazil
antonio.pupulim@grupointegrado.com.br
ORCID: https://orcid.org/0000-0002-7059-9456

Iamara Carvalho Sabatino Bento
Master in Clean Technologies, UNICESUMAR, Brazil.
iamarasabatino@outlook.comORCID: https://orcid.org/0009-0001-3317-3675

Raquel Zaneti Puelker
Progest Biotecnologia Pesquisa e Desenvolvimento
rapuelker@hotmail.com
ORCID: https://orcid.org/0000-0003-4753-9671

Josmar Mazucheli
Full professor
Department of Statistics, Universidade Estadual de Maringá, Brazil.
jmazucheli@gmail.com
ORCID: https://orcid.org/0000-0001-6740-0445

ABSTRACT
This study compared the effectiveness of transport medium and temperature on embryo transport. Embryos were produced in vitro until day 7 and transported for 12 hours. In the medium test, they were divided into two groups: GMan, maintained in embryo maintenance medium; and GHSOF, maintained in growth medium with FBS and amino acids, both at 36°C. To test the temperature, blastocysts were transported in HSOF medium, divided into G36 (36°C) and G38 (38°C). After transport, the blastocysts were evaluated and re-cultured, with hatching rates analyzed on day 10. The post-transport viable blastocyst rate was higher in the growth medium (GHSOF: 91.43% vs. GMan: 75.65%). There was no significant difference between the evaluated temperatures. It was concluded that the growth medium reduces embryo degeneration, increasing viability and hatching, while elevated temperatures during transport impair blastocyst hatching.

KEYWORDS: cattle; reproductive biotechnology; in vitro embryo production; transport.

1. INTRODUCTION

Livestock plays a crucial role in Brazil's economy, not only meeting the population's food demands but also generating employment and revenue for the country. According to the Brazilian Institute of Geography and Statistics (IBGE, 2022), Brazil is a global leader in cattle production, boasting the world's largest commercial herd with 224.6 million animals. This privileged position highlights the strategic importance of the Brazilian livestock sector in the global market.

Livestock production is essential in meeting the growing global demand for animal protein. Reproductive efficiency is key to enhancing productivity, yet reproductive inefficiency remains a significant challenge worldwide, leading to substantial economic losses. To address future needs and ensure sustainable agricultural production, agricultural research must harness emerging technologies, including modern reproductive biotechnology (VERMA et al., 2021). Reproductive biotechnologies are promising tools for overcoming productivity challenges in livestock systems (HADGU; FESSEHA, 2020). Among these, in vitro embryo production (IVP) stands out as one of the most effective methods for accelerating the production of genetically superior animals (VERMA et al., 2021; ARENDONK; BIJMA, 2003).

Research aimed at improving IVP outcomes includes studies on in vitro maturation, fertilization, embryo culture, and the metabolism of gametes and embryos, as well as the formulation of suitable culture media (VARAGO et al., 2008). However, the transport systems for in vitro-produced embryos from the lab to recipient cow centers deserve more attention, as there are few scientific studies published on this topic (CAVALIERI et al., 2015; LOIOLA et al., 2014). Beyond the stages of IVP, the transport of embryos to the transfer site is vulnerable to thermal stress, which can affect embryo quality (MIRANDA et al., 2007). Proper maintenance of the embryonic stage depends not only

on temperature but also on the transport medium. Growth media containing fetal bovine serum (FBS) and amino acids support embryo development, unlike maintenance media, which lack components that promote growth (CAVALIERI et al., 2015; LOIOLA et al., 2014).

Despite the strategic location of IVP labs, transport conditions often fail to support optimal embryo development, particularly over long distances like those in Brazil. During IVP and transport, maintaining the correct temperature is critical for embryo survival. Elevated temperatures can cause thermal stress, leading to the formation of free radicals and reducing the concentration of glutathione, a potent antioxidant (ANTUNES NETO et al., 2008).

Thermal stress is a major factor negatively impacting reproductive rates, typically occurring when the rectal temperature of breeding cows exceeds 41ºC. This can lead to decreased pregnancy rates, increased intervals between estrous cycles, anestrus, and poor embryonic and fetal development (TORRES-JUNIOR et al., 2008; HANSEN et al., 1999).

Heat stress can cause metabolic disturbances in oocytes and embryos, fundamentally affecting their developmental competence (MIĘTKIEWSKA et al., 2022). Exposure to high temperatures reduces embryonic development and may result in embryonic death during early divisions or within the first month of gestation (MIĘTKIEWSKA et al., 2022; AYALON et al., 1973).

During hot months, an early-stage embryo in the reproductive tract of a cow with elevated body temperature will have compromised physiological processes, reducing the viable blastocyst rate. Similarly, in vitro culture (IVC) under high temperatures decreases blastocyst rates due to thermal stress (RIVERA et al., 2001). Given that in vitro-produced embryos have lower quality, are susceptible to thermal and oxidative stress, and often endure long transport distances, this study aimed to investigate the effects of temperature and transport medium on bovine embryos produced in vitro during transportation.

2. MATERIALS AND METHODS

The experiments were conducted at the Reproductive Biotechnology Center (BIOTEC), at the Experimental Farm of the Centro Universitário de Maringá/CESUMAR, Maringá, Paraná. Ovaries were obtained from mixed-breed cows sourced from slaughterhouses in the Maringá/PR region and transported in 0.9% NaCl physiological solution at 36°C to BIOTEC.

In the laboratory, the ovaries were washed in 70% alcohol and physiological solution and maintained in a water bath at 36°C, with immediate follicular aspiration performed. Follicles between 2 and 8 mm in diameter were aspirated using an 18-gauge needle attached to a 10 mL syringe. The time from slaughter to the start of aspiration did not exceed 2 hours. All aspirated material was transferred to 50 mL Falcon tubes. The

sediment was transferred to 60 mm diameter polystyrene Petri dishes and assessed under a stereomicroscope at 50x magnification for oocyte selection and classification.

Oocytes were classified based on morphology (number of layers and degree of cumulus cell expansion, and the appearance of the cytoplasm in terms of color, homogeneity, and integrity) into grades I, II, III, and IV according to SENEDA et al. (2002). For this experiment, 823 COCs of the best quality, i.e., Grade I, were selected. These are characterized by a compact cumulus with more than three layers of cells, cytoplasm with fine and homogeneous granulations filling the zona pellucida, and a brown color. Grade II COCs have a compact cumulus partially surrounding the oocyte or completely around it, with fewer than three cell layers.

Selected COCs were matured in vitro in plates containing TCM 199 with Earles salts (Gibco®), glutamine (Sigma® cod: G8540), and NaHCO3 (Mallinckrodt®), supplemented with 10% FBS (Cultilab®), 22 µg/mL pyruvate (Biochemical cod: 44094), 50 µg/mL gentamicin (Sigma cod: G1272), 50 µg/mL LH (Lutropin - Bioniche®), 0.5 µg/mL FSH (Folltropin - Bioniche®), and 1 µg/mL estradiol, maintained at 38.5°C, 5% CO2, in air, with maximum humidity, for 22 to 24 hours.

Subsequently, oocytes were placed in a plate with 35 drops of 75 µL of maturation medium with 10 oocytes per drop, covered with mineral oil. Fertilization was carried out in 75 µL drops of Talp-FIV medium (Parrish et al., 1986) with bovine serum albumin (BSA; Sigma® cod: A3311), 50 µg/mL gentamicin (Sigma® cod: G1272), PHE solution (2 µM penicillin (Sigma® cod: P4875), 1 µM hypotaurine (Sigma® cod: H1384), and 0.25 µM epinephrine (Sigma® cod: E4250), 10 µg heparin (Sigma® cod: H3149), and 22 µg/mL pyruvate (Biochemical® cod: 44094). Semen from a Nelore bull was thawed in a water bath at 35°C for 30 seconds. To select motile sperm and remove diluents, a single centrifugation in Percoll gradients of 45% and 90% was performed for 9 minutes. The sperm concentration used was $1x10^6$ sperm/mL.

After 24 hours of fertilization, the possible zygotes were cultured in SOF (Synthetic Oviduct Fluid; TAKAHASHI & FIRST, 1992), supplemented with 5% FBS and 0.5% BSA. The culture was maintained for 24 hours after insemination in an incubator with a controlled gas atmosphere containing 5% CO^2, 5% O^2 balanced with 90% N^2. On day 7, embryos in culture were analyzed for quality as 1, 2, 3, or 4, corresponding to good, fair, poor, and degenerate, respectively, according to the International Embryo Transfer Society (IETS, 1999) manual, and for developmental stage. Only embryos of quality 1 and 2 in the early blastocyst and blastocyst stages were selected.

Embryos were then packaged and placed in transport conditions using the WTA® transporter (Watanabe Tecnologia Aplicada Ltda, Cravinhos, SP, Brazil) for 12 hours in the laboratory with different transport media and temperatures, as described in Experiments 1 and 2 below. In Experiment 1, embryos were divided into two groups: GMan - embryos maintained in a maintenance medium (VIGRO Holding Plus – Bioneche®), without FBS and amino acids (n=115 blastocysts) and GHSOF - embryos maintained in a growth medium "Synthetic Oviduct Fluid" (TAKAHASHI et al., 1992;

HSOF) supplemented with TCM 199 – Hepes buffer, containing FBS and amino acids (n=105 blastocysts). Both groups were kept at 36°C for 12 hours, with 36°C chosen as the standard transport temperature.

In Experiment 2, embryos were also divided into two groups: G38 - embryos transported at 38°C (n=81); and G36 - embryos transported at 36°C (n=65). Both groups were maintained in growth medium (HSOF). The temperature of 38°C was selected as it is used in in vitro culture, favoring development.

After 12 hours of transport in both experiments, embryos were retrieved from the straws, analyzed, and classified as viable blastocysts, degenerated, and hatched, then re-cultured in IVC medium until day 10 for final assessment, hatching rates, and degeneration.

Embryo viability and blastocyst hatching rates were analyzed using logistic regression methodology. The logistic model was used to estimate regression coefficients and the probability of regressor occurrence through maximum likelihood methodology. The software used was SAS, version 9.1. The independent variables tested in Experiment 1 were maintenance medium vs. growth medium; and in Experiment 2 were 36°C vs. 38°C transport temperatures. The dependent variables tested in both experiments were post-transport embryo viability at 12 hours, post-transport blastocyst hatching rates at 12 hours, and blastocyst hatching rates after 48 hours of in vitro culture.

3. RESULTS & DISCUSSION

The results from Experiment 1, which evaluated the viability rates of blastocysts after 12 hours of transport (Table 1) and the final hatching rates on day 10 (Table 2), for those maintained in maintenance medium (GMan) and growth medium (GHSOF), are presented below.

Table 1. Distribution of non-viable and viable blastocysts after 12 hours of transport in the groups maintained in maintenance medium (GMan) and growth medium (GHSOF) (n and %).

Groups	Non-Viable[1]	Viable[2]	Total
GMan	28 (24.35%)[a]	87 (75.65%)[a]	115
GHSOF	09 (07.57%)[b]	96 (91.43%)[b]	105

[1] Non-viable blastocysts and [2] Viable blastocysts after 12h of transport. Different superscripts in the same column differ significantly ($p<0.001$).

Table 2. Distribution of non-hatched and hatched blastocysts after re-culture on day 10, for groups maintained in maintenance medium (GMan) and growth medium (GHSOF) (n and %).

Groups	Non-Hatched[1]	Hatched[2]	Total
GMan	75 (65.22%)[a]	40 (34.78%)[a]	115
GHSOF	29 (27.62%)[b]	76 (72.38%)[b]	105

[1] Non-hatched blastocysts and [2] Hatched blastocysts after re-culture on day 10. Different superscripts in the same column differ significantly ($p<0.001$).

Logistic regression modeling considering the medium as an independent variable revealed a significant difference in the proportion of viable embryos (Table 1) ($p<0.001$). The chance of viable embryos after 12 hours of transport in the GHSOF group (91.43%) was 4 times higher than in the GMan group (75.65%). Literature reports (TAKAHASHI et al., 1996) confirm these findings, showing that embryos transported for 18.3 hours in a maintenance medium without any antioxidants had 30% of embryos degenerated, with only 50% of the remaining embryos considered viable blastocysts (quality 1 and 2, according to IETS, 1999).

In contrast, Ramos et al. (2006) found that the transport of fresh embryos for 6 or 12 hours in maintenance medium with temperatures between 33 to 35°C did not affect the hatching and degeneration rates when compared with fresh IVF embryos not subjected to transport. Likewise, Loiola et al. (2014) observed that in a bovine IVF program, where embryonic culture occurred partially during an average transport time of 18 to 24 hours in a portable incubator at 38°C after day 6 of IVC, the blastocyst and hatched blastocyst rates (33%) were similar to the national average (35%; BRUM et al., 2002). Furthermore, Cavalieri et al. (2015) subjected embryos to 24 and 48 hours of transport in an incubator with a gas mixture containing 5% O_2, 5% CO_2, and 90% N_2 at 38.4°C from day 5 and 6 of culture and found no difference in blastocyst rates between the evaluated periods.

Regarding the hatching rate after re-culture (Table 2) on day 10, logistic regression modeling revealed a statistical difference ($p<0.001$) between the groups, with the hatching rate in the GHSOF group (72.38%) being 5 times higher than in the GMan group (34.78%). However, Zanenga et al. (2008), evaluating pregnancy rates between Syngro® medium, similar to the GMan group in this experiment, which can be used for transport for approximately 9 hours at temperatures up to 34°C, and H-SOF, similar to the GHSOF group, obtained different results, with a pregnancy rate of 45% in Syngro® medium and 42% in H-SOF medium.

The results from Experiment 1 demonstrated better outcomes for the GHSOF medium, likely because this medium acts as a protective agent against free radicals, in addition to providing embryotrophic substances such as albumin, fatty acids, growth factors, amino acids, vitamins, insulin, and IGF-1, which are essential for blastocoel

formation and favor development up to the hatched blastocyst stage, thus increasing subsequent viability, as noted by Bavister et al. (1995) and Lim et al. (1999).

Data on viable blastocyst rates after 12 hours of transport (Table 3) and final hatching rates on day 10 (Table 4), maintained in growth medium at 36°C (G36) and 38°C (G38), are shown in Table 3.

Table 3: Distribution of non-viable and viable blastocysts after 12 hours of transport in groups maintained in growth medium at 36°C (G36) and 38°C (G38) (n and %).

Groups	Non-Viable[1]	Viable[2]	Total
G36	05 (07.69%)[a]	60 (92.31%)[a]	65
G38	14 (17.28%)[a]	67 (82.72%)[a]	81

[1] Non-viable blastocysts and [2] Viable blastocysts after 12h of transport. Different superscripts in the same column differ significantly ($p<0.001$).

Logistic regression modeling considering temperature as an independent variable revealed that the proportion of viable blastocysts (Table 3) was similar at both temperatures ($p>0.09$), indicating no significant difference.

Table 4: Distribution of non-hatched and hatched blastocysts after re-culture on day 10, in groups maintained in growth medium at 36°C (G36) and 38°C (G38) (n and %).

Groups	Non-Hatched[1]	Hatched[2]	Total
G36	16 (24.62%)[a]	49 (75.38%)[a]	65
G38	41 (51.00%)[b]	40 (49.00%)[b]	81

[1] Non-hatched blastocysts and [2] Hatched blastocysts after re-culture on day 10. Different superscripts in the same column differ significantly ($p<0.001$).

Regarding the hatching rate after re-culture on day 10 (Table 4), logistic regression modeling showed a statistical difference ($p<0.001$) between the groups, with the hatching rate in the G36 group (75.38%) being 3.5 times higher than in the G38 group (49.00%).

In Experiment 2, there was no significant difference in the rate of viable blastocysts on day 7, but a significant difference was observed in the rate of hatched blastocysts on day 10. This difference was likely a consequence of the 38°C temperature, as it increases metabolism, releasing toxic substances such as ammonia (GARDNER et al., 1994). Ammonia release is a result of amino acid presence in the GHSOF medium, particularly glutamine, which undergoes spontaneous breakdown at temperatures above 37°C, causing structural changes in blastomeres and significantly reducing their implantation capacity, leading to considerable fetal loss after pregnancy stabilization (LANE; GARDNER, 2007).

Additionally, under these conditions, free radicals are formed that damage cellular DNA, causing injury to embryonic cells (HALLIWELL et al., 1989). Thermal stress is associated with the production of ROS (reactive oxygen species), which causes alterations in embryos, decreasing their development to the blastocyst stage (SAKATANI et al., 2004) and potentially inducing cell death (LIN et al., 1991).

The rate of hatched blastocysts after 12 hours of transport in the GMan and GHSOF groups, as well as in the G36 and G38 groups, were also low. These results underscore the importance of considering environmental factors and transport conditions in the in vitro embryo production process to improve embryo viability and hatching capacity. They also highlight the ongoing need for research and development of new approaches to enhance the efficiency and quality of assisted reproduction.

Overall, this study contributes to the scientific understanding of embryo transport techniques and emphasizes the importance of applying good practices in embryo handling and transport. This information is valuable for professionals in the field and the livestock industry as a whole, providing insights for optimizing reproductive processes and contributing to the sustainable advancement of animal production.

4. CONCLUSIONS

Based on the conditions under which the experiment was conducted, the results demonstrated that the embryo culture medium containing FBS and amino acids reduced embryo degeneration post-transport, increasing viability and hatching compared to the embryo maintenance medium. It was observed that exposure to higher temperatures during transport did not impair the morphological quality of blastocysts on the seventh day, but it reduced their hatching ability.

In summary, this study contributes to the advancement of knowledge in the field of reproductive biotechnology, providing evidence that the proper selection of the embryo transport medium and temperature control are essential to preserve quality and enhance the hatching rate of embryos produced in vitro. These findings have practical implications for the animal reproduction industry and may contribute to the development of more effective and safer embryo transport strategies, resulting in improved success rates in assisted reproduction programs.

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REFERENCES

Antunes Neto, J. M. F., Silva, L. P., & Macedo, D. V. (2008). Proteínas de estresse “hsp70” atuam como marcadoras de estresse oxidativo em ratos “Wistar” submetidos a treinamento contínuo de corrida para indução de overreaching. Revista Brasileira de Educação Física, Esporte, Lazer e Dança, 3, 116-130.

Arendonk, J. A. M., & Bijma, P. (2003). Factors affecting commercial application of embryo technologies in dairy cattle in Europe—a modeling approach. Theriogenology, 59, 635-649.

Ayalon, N. (1978). A review of embryonic mortality in cattle. Journal of Reproduction and Fertility, 54, 483-493.

Bavister, B. D. (1995). Culture of preimplantation embryos: facts and artifacts. Human Reproduction Update, 1, 91-148.

Brum, D. S., Leivas, F. G., Bernardi, M. L., & et al. (2002). Cultivo individual de blastocistos bovinos produzidos in vitro. Brazilian Journal of Veterinary Research and Animal Science, 39(2), 87-92.

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.

Confederação de Agricultura e Pecuária do Brasil. (2017). CNA: Comissão Nacional Pecuária de Corte. http://www.cnabrasil.org.br/central-comunicacao/comissoes-nacionais/pecuaria-de-corte

Gardner, D. K., Lane, M., Spitzer, A., & et al. (1994). Enhanced rates of cleavage and development for sheep zygotes cultured to the blastocyst stage in vitro in the absence of serum and somatic cells: Amino acids, vitamins, and culturing embryos in groups stimulate development. Biology of Reproduction, 50, 390-400.

Hadgu, A., & Fesseha, H. (2020). Reproductive biotechnology options for improving livestock production: A review. Advances in Food Technology and Nutritional Sciences - Open Journal, 6(1), 13-20. https://doi.org/10.17140/AFTNSOJ-6-164

Halliwell, B. (1989). Tell me about free radicals, doctor: a review. Journal of the Royal Society of Medicine, 82, 747-753.

Hansen, P. J., & Aréchiga, C. F. (1999). Strategies for managing reproduction in the heat-stressed dairy cow. Journal of Animal Science, 77, 36-50.

Instituto Brasileiro de Geografia e Estatística. (2022). Pesquisa Trimestral de Abate de Animais, Leite, Couro e Ovos. https://ftp.ibge.gov.br/Producao_Pecuaria/Fasciculo_Indicadores_IBGE/abate-leite-couro-ovos_202201caderno.pdf

Lane, L., & Gardner, D. K. (2007). Embryo culture medium: which is the best? Best Practice & Research Clinical Obstetrics & Gynaecology, 21, 83-100.

Leivas, F. G., Brum, D. S., Mezzalira, A., & et al. (2004). Transporte de oócitos bovinos em meio de maturação sem controle de atmosfera gasosa. Ciência Rural, 34(1), 219-224.

Lenz, R. W., Ball, G. D., Leibfried, M. L., & et al. (1983). In vitro maturation and fertilization of bovine oocytes are temperature-dependent processes. Biology of Reproduction, 29, 173-179.

Lim, J. M., Reggio, B. C., Godke, R. A., & et al. (1999). Development of in-vitro-derived bovine embryos cultured in 5% CO2 in air or in 5% O2, 5% CO2 and 90% N2. Human Reproduction, 14, 458-464.

Lin, P., Quamo, S., Ho, K., & Gladding, J. (1991). Hyperthermia enhances the cytotoxic effects of reactive oxygen species to Chinese hamster cells and bovine endothelial cells in vitro. Radiation Research, 126, 43-51.

Loiola, M. V. G., Chaulhoub, M., Rodrigues, A. S., & et al. (2014). Validação de um programa de produção in vitro de embriões bovinos com transporte de oócitos e de embriões por longas distâncias. Ciência Animal Brasileira, 15(1), 93-101.

Miranda, M. S., Carvalho, C. M. F., Cordeiro, M. S., & et al. (2007). Sistemas alternativos de incubação e meios de cultivo para produção in vitro de embrião bovino. Revista Brasileira de Reprodução Animal, 31(2), 218-223.

Miętkiewska, K., Kordowitzki, P., & Pareek, C. S. (2022). Effects of heat stress on bovine oocytes and early embryonic development—An update. Cells, 11(24), 4073. https://doi.org/10.3390/cells11244073

Parrish, J. J., Susko-Parrish, J. L., Leibfried-Rutledge, M. L., & et al. (1986). Bovine in vitro fertilization with frozen-thawed semen. Theriogenology, 25, 591-600.

Ramos, A. A., Polisseni, J., Sá, W. F., & et al. (2006). Efeito do transporte no desenvolvimento de embriões bovinos cultivados in vitro a fresco ou reaquecidos após vitrificação. Revista Brasileira de Zootecnia, 35, 2285-2289.

Rivera, R. M., & Hansen, P. J. (2001). Development of cultured bovine embryos after exposure to high temperatures in the physiological range. Reproduction, 121, 107-115.

Sakatani, M., Kobayashi, S., & Takahashi, M. (2004). Effects of heat shock on in vitro development and intracellular oxidative state of bovine preimplantation embryos. Molecular Reproduction and Development, 67, 77-82.

Seneda, M. M., Esper, C. R., Garcia, J. M., & et al. (2002). Aspectos técnicos e biológicos da obtenção de oócitos bovinos: revisão de literatura. Semina: Ciências Agrárias, 23(1), 101-110.

Takahashi, Y., & First, N. L. (1992). In vitro development of bovine one-cell embryos: Influence of glucose, lactate, pyruvate, amino acids and vitamins. Theriogenology, 37, 963-978.

Takahashi, H., Kuwayama, M., Hamano, S., & et al. (1996). Effect of β-mercaptoethanol on the viability of IVM/IVF/IVC bovine embryos during long-distance transportation in plastic straws. Theriogenology, 46, 1009-1015.

Torres Júnior, J. R. S., Pires, M. F. A., Sá, W. F., & et al. (2008). Effect of maternal heat-stress on follicular growth and oocyte competence in Bos indicus cattle. Theriogenology, 69, 155-166.

Verma, O. P., Kumar, R., Kumar, A., & Chand, S. (2012). Assisted reproductive techniques in farm animals: From artificial insemination to nanobiotechnology. Veterinary World, 5(5), 301-310.

Zanenga, C. A. (2008). Effect of different holding and transport media on conception rates following transfer of in vivo- and IVF-derived bovine embryos. Tecnopec. http://www.tecnopec.com.br/download.php?f=06f29c3a72019efe4de0d9f77b509cb8&countonly=1