SEMINA
Ciências Agrárias

DOI: 10.5433/1679-0359.2024v45n2p537

Comparison between vitrification and slow freezing on the post-thaw development of bovine embryos produced in vitro

Comparação entre vitrificação e congelamento lento sobre o desenvolvimento pós-descongelamento de embriões bovinos produzidos in vitro

Vanessa Rachele Ribeiro Nunes[1]*; Jennifer Teodoro Ferreira Gregianini[1]; Hélton Aparecido Garcia Gregianini[2]; Bárbara Souza Fantin[3]; Bárbara Loureiro[4]; Rafael Augusto Satrapa[5]

Highlights

The development of vitrified and slowly frozen embryos was compared.
The day of cryopreservation was also evaluated.
Vitrified embryos had better re-expansion and embryo hatching rates.
Day of cryopreservation did not affect the rates of re-expansion or embryo hatching.

Abstract

We evaluated the post-thaw development of in vitro-produced (IVP) bovine embryos cryopreserved using vitrification and slow-freezing techniques on different days after in vitro fertilization (IVF). Nine replicates of IVP were performed. Embryos at the expanded blastocyst stage with quality grades 1 and 2 (according to the International Embryo Technology Society (IETS) manual) were selected on days 7, 7.5, and 8 after IVF. Embryos (n = 472) were randomly divided and cryopreserved using slow freezing (n = 257) or vitrification (n = 215). The embryos were organized into six groups according to the cryopreservation technique and the day: 1) Group DT7 (embryos subjected to slow freezing on D7, n = 140); 2) Group DT7.5 (embryos subjected to slow freezing 12 hours after D7, on D7.5; n = 61); 3) Group

[1] Students of the Master's Course of the Postgraduate Program in Health and Sustainable Animal Production in the Western Amazon, Universidade Federal do Acre, UFAC, Rio Branco, AC, Brazil. E-mail: vanessarachele45@gmail.com; jenniferivacre@gmail.com
[2] M.e, Embryo Production Director , Company In vitro Acre, Rio Branco, AC, Brazil. E-mail: heltonivacre@gmail.com
[3] Graduate in Veterinary Medicine, Laboratory Technician, Company In vitro Acre, Rio Branco, AC, Brazil. E-mail:bah_fantin@hotmail.com
[4] Profª Drª, Laboratory of Animal Reproductive Physiology, Universidade de Vila Velha, UVV, Vila Velha, Espírito Santo, Brazil. E-mail: barbara.loureiro@uvv.br
[5] Prof. Dr., Center for Biological and Natural Sciences, Universidade Federal do Acre, UFAC, Rio Branco, Acre, Brazil. E-mail: rafael.satrapa@ufac.br
* Author for correspondence

Received: Nov. 08, 2023 Approved: Apr. 08, 2024

DT8 (embryos subjected to slow freezing on D8, n = 56); 4) Group VIT7 (embryos vitrified on D7, n = 127); 5) Group VIT7.5 (embryos vitrified 12 hours after D7, on D7.5; n = 49); and 6) Group VIT8 (embryos vitrified on D8, n = 39). Data were arcsine-transformed and analyzed using analysis of variance and the GLIMMIX procedure in SAS (SAS 9.2), with P < 0.05. The re-expansion and embryo hatching rates were higher in vitrified embryos than in embryos subjected to slow freezing (P < 0.05). Embryo quality grade did not influence the total developmental rate (P > 0.05). However, grade 1 embryos re-expanded more rapidly (within 24 hours) than grade 2 embryos. Grade 1 embryos showed better results with vitrification than with slow freezing. The experimental groups represented a technique × day interaction and did not differ in terms of re-expansion and hatching rates (P > 0.05).
Key words: Cryosurvival. Direct Transfer. IVEP.

Resumo

Avaliou-se o desenvolvimento pós-descongelamento de embriões bovinos produzidos in vitro (PIV) criopreservados pela técnica de vitrificação e de congelamento lento em diferentes dias após a fertilização in vitro (FIV). Foram realizadas 9 réplicas de PIV. Nos dias 7; 7,5 e 8 após a FIV, foram selecionados embriões no estágio de blastocisto expandido de grau de qualidade 1 e 2 (de acordo com o manual IETS). Os embriões (n=472) foram divididos aleatoriamente para serem criopreservados por congelamento lento (n=257) ou vitrificação (n=215). Organizou-se os embriões em 6 grupos de acordo com a técnica de criopreservação e o dia: 1) Grupo DT7 (embriões submetidos ao congelamento lento no D7, n= 140); 2) Grupo DT7,5 (embriões submetidos ao congelamento lento 12 horas após o D7, no D7,5; n= 61); 3) Grupo DT8 (embriões submetidos ao congelamento lento no D8, n=56); 4) Grupo VIT7 (embriões vitrificados no D7, n= 127); 5) Grupo VIT7,5 (embriões vitrificados 12 horas após o D7, no D7,5; n= 49); 6) Grupo VIT8 (embriões vitrificados no D8, n= 39). Os dados foram transformados para arcoseno e analisados por Análise de Variância utilizando o procedimento GLIMMIX do SAS (SAS 9.2) com P<0,05. A taxa de re-expansão e de eclosão embrionária foi maior para embriões vitrificados que para embriões submetidos ao congelamento lento (P<0,05). O grau de qualidade embrionária não influenciou as taxas de desenvolvimento totais (P>0,05). Porém embriões de grau 1 re-expandiram mais rapidamente (às 24 h) que embriões grau 2. Embriões grau 1 apresentaram melhores resultados com a técnica de vitrificação do que com congelamento lento. Os grupos experimentais representaram a interação técnica*dia e não diferiram entre si quanto as taxas de re-expansão e eclosão (P>0,05).
Palavras-chave: Criossobrevivência. Direct Transfer. PIV.

Introduction

In vitro production (IVP) of embryos is one of the main reproductive biotechnologies used for genetic improvement in Brazil (Gonçalves & Viana, 2019). According to information from the International Embryo Technology Society [IETS] provided by Viana (2018), Brazil has held a prominent position in the global scenario for IVP embryos since 2007. There was a consolidation of in vitro technology in the country in 2017 with a historic milestone in the production of bovine embryos, when the total number

of IVP embryos (992,289) exceeded those generated in vivo (406,287) for the first time (Viana, 2018).

As highlighted by Viana (2022), the proportion of transferred IVP embryos in 2021 was largely driven by an increase in the utilization of cryopreserved IVP embryos, which represent approximately one-third of all embryo transfers performed globally. Considering that in vitro embryo production is a widely used technique in reproductive and genetic improvement programs for domestic species, cryopreservation is an essential step in the evolution and consolidation of this technique (Gonçalves & Viana, 2019). In addition to enabling the establishment of germplasm banks, cryopreservation aids in optimizing embryo transfer through more efficient utilization of surplus embryos, especially during recipient shortages (Leibo & Mapletoft, 1998). It facilitates the trade of high-quality genetic material (Aksu et al., 2012) and prevents animal welfare issues and disease transmission (Almiñana & Cuello, 2015).

The most common and widely used procedures for embryo cryopreservation are vitrification and slow freezing (Arshad et al., 2021). Vitrification stands out for its ability to achieve ultra-rapid freezing, which prevents the formation of intracellular ice crystals and contributes significantly to the cryogenic survival of embryos (Saragusty & Arav, 2011). However, it is important to note that using high concentrations of cryoprotectants can trigger cytotoxicity, adding complexity to the technique. Additionally, it requires a laboratory setting and technical expertise for the pre-transfer assessment of embryos (Vajta et al., 1998), limiting its viability in large-scale scenarios. The direct application of vitrified embryos, that is, without the need for pre-assessment, has been studied (Caamaño et al., 2015; Inaba et al., 2011), but it still presents limitations, such as the lack of a standardized method and inconsistent results (Ferré et al., 2020; Do et al., 2019).

In contrast, the slow freezing method appears to be more flexible with regard to direct transfer (DT) since the discovery of the use of ethylene glycol as a cryoprotectant, which simplified the post-thaw rehydration step of embryos, eliminating the need for a laboratory setup (Voelkel & Hu, 1992; Oliveira et al., 2020). It has shown consistent results, as found in the study by Sanches et al. (2016), making it a more effective option in commercial contexts. In addition, the use of lower cryoprotectant concentrations is advantageous. However, it is worth noting that ice crystal formation, which may affect the success of cryopreservation, is as a challenge in this method (Ferré et al., 2020). Therefore, choosing between vitrification and slow-freezing techniques should consider the relationships between technical complexity, commercial viability, and effectiveness in preserving bovine embryos.

Morphological quality is one of the most important factors in embryo cryopreservation (Dode et al., 2013). An ideal embryo should be compact and spherical; present blastomeres of similar sizes with uniform color and texture; the cytoplasm should not be granular or vesiculated; the perivitelline space should be clear and free of cellular debris; and the zona pellucida should be uniform without debris on its surface (Bó & Mapletoft, 2013). In this context, the pregnancy rates of cryopreserved IVP

embryos tend to be higher when used at the expanded blastocyst stage (D7; Marinho et al., 2015; Sanches et al., 2016). However, it is important to note that IVP embryos may exhibit a variety of developmental patterns, which may lead to the presence of embryos with exceptional morphological quality at earlier (D6) or more advanced (D8) stages.

Based on these observations, we hypothesized that the vitrification method and slow freezing for the direct transfer (DT) of expanded blastocysts would exhibit similar post-thaw development rates. Additionally, these techniques can be used in embryos cultured for 7 or 8 days in vitro without compromising post-thaw development. Therefore, the aim of the present study was to evaluate the progress of embryonic development after thawing cryopreserved embryos at different times during in vitro culture using vitrification and slow freezing methods.

Materials and Methods

Ethical aspects

All procedures were conducted in accordance with the Ethics Committee on Animal Use in Experimentation (Protocol 23107.001762/2022-54).

Location

The experiment was conducted at the Animal Reproduction Laboratory (LABRA), affiliated with the Center for Biological and Natural Sciences, Federal University of Acre (UFAC; Rio Branco, Acre, Brazil).

Experimental design

Nine replicates of IVP embryos were cryopreserved. The embryos in each of the IVP replicates were subjected to two different cryopreservation techniques: vitrification and slow freezing. Embryos were cryopreserved when they reached the expanded blastocyst stage of grades 1 or 2 (according to the IETS Manual) on days 7, 7.5, and 8 post IVF (D7, D7.5, and D8, respectively).

A total of 472 embryos were cryopreserved and randomly allocated to either the slow freezing (n = 257) or vitrification (n = 215) groups. The embryos were organized into six groups based on the cryopreservation technique and the day of embryonic development (D7, D7.5, and D8; as shown in Figure 1): 1) Group DT7 (embryos subjected to slow freezing on D7, n = 140); 2) Group DT7.5 (embryos subjected to slow freezing 12 hours after D7, on D7.5; n = 61); 3) Group DT8 (embryos subjected to slow freezing on D8, n = 56); 4) Group VIT7 (embryos vitrified on D7, n = 127); 5) Group VIT7.5 (embryos vitrified 12 hours after D7, on D7.5; n = 49); and 6) Group VIT8 (embryos vitrified on D8, n = 39).

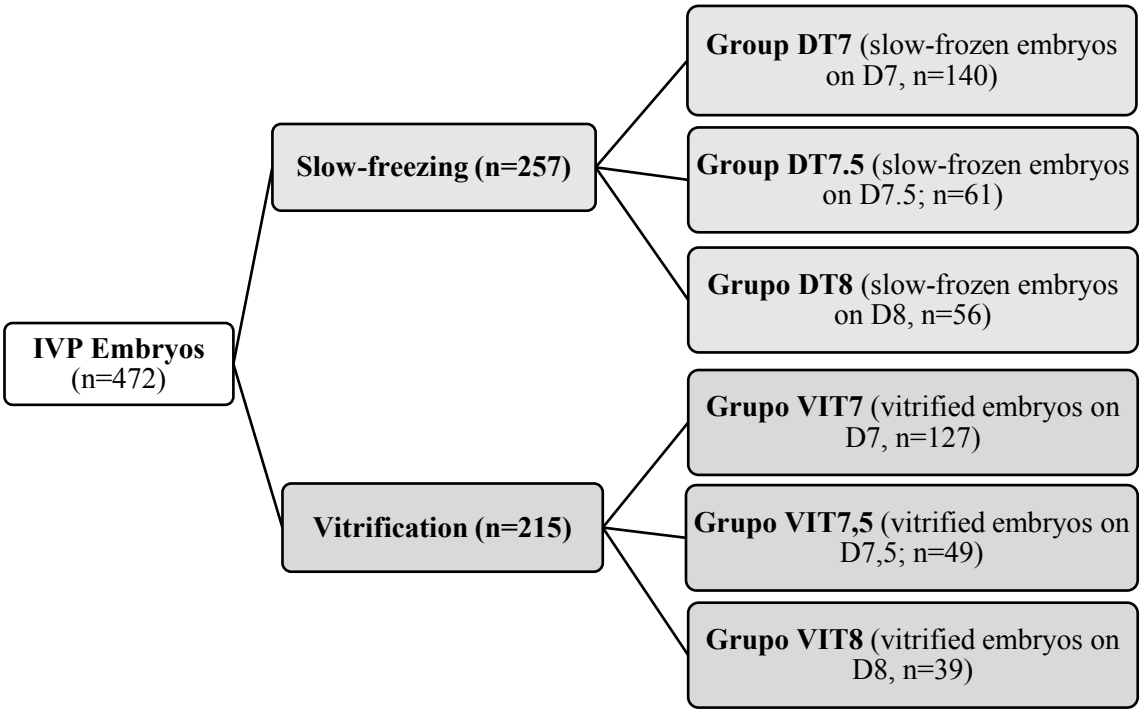

Figure 1. Schematic representation of the experimental design.

Oocyte collection and selection

Ovaries from crossbred cows (Bos indicus × Bos taurus) slaughtered at a local abattoir (Rio Branco, Acre, Brazil) were collected and transported to the LABRA in 0.9% saline solution at 37°C for follicular aspiration. Cumulus-oocyte complexes (COCs) were aspirated from antral follicles measuring 3-8 mm using hypodermic needles with a gauge of 21 (30 × 8 mm) attached to 20 mL syringes.

Following aspiration, the COCs were filtered using phosphate-buffered saline solution (DPBS) through a mini oocyte filter for selection and washed in Tissue Culture Medium-199 (TCM-199) buffered with HEPES, supplemented with 10% Fetal Bovine Serum (FBS; Sigma-Aldrich, St. Louis, MO, USA), 2 µg/mL pyruvate, and 75 µg/mL gentamicin (Instituto Bioquímico, Rio de Janeiro, Brazil). Only COCs surrounded by at least one layer of cumulus cells and a homogeneous cytoplasm were selected and classified.

In vitro maturation (IVM)

After selection and classification, 25 to 30 COCs were added to 100 µL drops of maturation medium consisting of TCM-199 supplemented with bicarbonate and 10% FBS (Sigma-Aldrich), 2 µg/mL pyruvate, 75 µg/mL gentamicin (Instituto Bioquímico, Rio de Janeiro, Brazil), 20 µg/mL follicle-stimulating hormone (FSH; Ceva Veterinária S.A., São Paulo, Brazil), and 10 IU/

mL luteinizing hormone (LH; UCB Biopharma, São Paulo, Brazil). This was immersed in 4 mL of mineral oil in a Petri dish and maintained at 5% atmospheric CO2 and saturated humidity at 38.7°C for 22-26 hours from the time of follicular aspiration.

In vitro fertilization (IVF)

In vitro fertilization (IVF) was performed using the semen from a single Aberdeen Angus bull (Bos Taurus). The semen was thawed (37°C for 30 seconds) and washed twice using centrifugation (1st at 6700 ×g for 5 minutes and 2nd at 1100 × g for 3 minutes) in 1 mL of Tyrode's albumin lactate pyruvate (TALP; Sigma-Aldrich) supplemented with 2.2 mg of pyruvate and 83.4 g/µL of amikacin, buffered with 2.3 g/ µL of HEPES. An inseminating dose of 10 µL (1 × 106 sperm/mL) was added to each 50 µL drop of TALP-FIV (TALP; Sigma-Aldrich) supplemented with 10 mg/µL heparin and 160 µL of penicillamine, hypotaurine, and epinephrine solution) and covered with mineral oil. Each drop contained 25-30 oocytes. The spermatozoa and oocytes were incubated for 18-22 hours in an incubator at 38.7°C, 5% CO2, and saturated humidity. The day of fertilization was considered day zero (D0).

In vitro culture (IVC)

The potential zygotes resulting from fertilization were subjected to removal of cumulus cells through successive pipetting and cultured in 100 µL drops of modified synthetic oviductal fluid (mSOF) containing amino acids (BME amino acid solution, 45 mL/mL and MEM non-essential amino acid solution, 3.3 mL/mL), citrate (0.1 mg/mL), myo-inositol (0.5 mg/mL), and BSA (6 mg/ mL) with 0.1% (v/v) FBS (Sigma-Aldrich). The in vitro culture was conducted at 38.7°C, 5% CO2, 5% O2, 90% N2, and saturated humidity. All structures were cultured in mSOF with 0.5 mg/mL polyvinyl alcohol PVA (HSOF) under 200 µL mineral oil on day 6 and finally sealed with silicone stoppers, containing a maximum of 35 structures/tube. These tubes remained in a controlled environment within the incubator solely to maintain the temperature (38.7°C).

Cryopreservation

Embryos were evaluated for their morphological quality by a single trained technician prior to cryopreservation procedures on days 7, 7.5, and 8 of in vitro culture. Embryos at the expanded blastocyst stage of grade 1 or 2 (according to the IETS manual) were considered suitable for cryopreservation and randomly and proportionally divided to undergo vitrification or slow freezing.

Vitrification, warming, and recultivation

Embryos were exposed to an equilibrium solution containing 10% ethylene glycol (EG) + 10% dimethyl sulfoxide (DMSO) in TCM-HEPES medium (25 mM HEPES) + 10% FBS for 1 minute and then for 20 seconds in the vitrification solution (VS ¼ 20% EG + 20% DMSO in TCM-HEPES with 0.5 M sucrose(Sigma-Aldrich) for vitrification. Up to five embryos were placed in hemi-straws (Liebermann & Tucker, 2002) during the 20

seconds of exposure to VS and immediately placed in liquid nitrogen (−196°C).

The rewarming/devitrification process involved removing the VS through two washes with 0.3 M sucrose and 0.15 M sucrose for 5 minutes each, before applying the maintenance medium TCM-HEPES. Once rewarmed, the embryos were washed in a drop of HSOF and transferred to tubes with 400 µL of HSOF maintenance medium covered by 200 µL of mineral oil, sealed with silicone stoppers.

After thawing, the embryos were separated into tubes according to the experimental groups and embryo quality grade to evaluate their initial development, mainly regarding the blastocyst re-expansion rate and embryo hatching rate at 24, 48, and 72 hours.

Slow freezing, thawing, and reculturing

The direct transfer (DT) of embryos was used for the slow-freezing technique. The embryos were loaded into 0.25 mL IMV straws (HUMECO, Huesca, Spain) with a filling pattern as described by Sanches et al. (2016), placed in a central column of 1.5 M ethylene glycol solution, surrounded by four columns of thawing solution (0.75 M EG diluted in HSOF medium), and separated from each other by air columns. After a 10-minute equilibration, the straws were placed in a freezing machine (TK 1000, TK Tecnologia) previously stabilized at −6ºC. Crystallization ("seeding") occurred in the columns immediately above and below the embryo column 2 minutes after the straws were placed in the machine. The embryos remained at −6 ºC for 10 minutes, after which the freezing curve began, lowering the temperature by −0.5 °C per minute until reaching −32 °C. After the end of the freezing curve, the straws containing the embryos were immersed in liquid nitrogen (−196°C) and stored in canisters until thawing.

Straws containing the embryo was removed from the liquid nitrogen were removed one at a time and held in the air at room temperature for 5 seconds, then immersed in water at 30°C for 30 seconds for thawing. After this period, the embryo was removed from the straw using an inoculation mandrel by pushing the plunger of the straw to expel its contents into a Petri dish. The embryos were washed in a drop of HSOF maintenance medium before being transferred to tubes with 400 µL of HSOF maintenance medium covered by 200 µL of mineral oil and sealed with silicone stoppers.

Statistical analysis

The data were arcsine-transformed and analyzed using analysis of variance and the GLIMMIX procedure in SAS (SAS 9.2, North Carolina, SAS Institute Inc.). Replicates were considered for the analysis (N = 9). The effects of cryopreservation method (vitrification or slow freezing for direct transfer [DT]), culture days (D7, D7.5, and D8), embryo quality (Grades 1 and 2), and their interactions on the variables of embryo re-expansion at 24 hours, embryo re-expansion at 48 hours, total embryo re-expansion, embryo hatching at 24 hours, embryo hatching at 48 hours, and total embryo hatching were evaluated. Data are presented as least-squares means and standard errors of the mean. Differences were adjusted using the Tukey-Kramer test when individual differences were compared. Statistical significance was set at $P < 0.05$.

Results and Discussion

Considering that the rates of re-expansion and hatching of bovine blastocysts are criteria used to estimate the viability and developmental potential of the embryo post-cryopreservation (Aksu et al., 2012), we selected these parameters to test the central hypothesis of this study, which was to obtain similar developmental rates with vitrification and slow freezing techniques. Embryonic development after cryopreservation/thawing was evaluated 24-72 hours after thawing. However, re-expansion and hatching of embryos occurred only for up to 48 hours.

The total re-expansion rate was higher in embryos subjected to vitrification than in those subjected to slow freezing (DT), with values of 95% and 86%, respectively (P = 0.0117; Figure 2). In the context of the vitrification technique, 90% of the embryos resumed expansion within the first 24 hours of in vitro culture, whereas 5% resumed expansion between 24 and 48 hours of in vitro culture. In the case of DT embryos, a re-expansion rate of 81% was recorded after 24 hours, which was lower than that of vitrified embryos, which showed a rate of 90% (P = 0.0508); additionally, 5% of DT embryos resumed expansion after 48 hours, showing similarity to the re-expansion rate of vitrified embryos (P > 0.05).

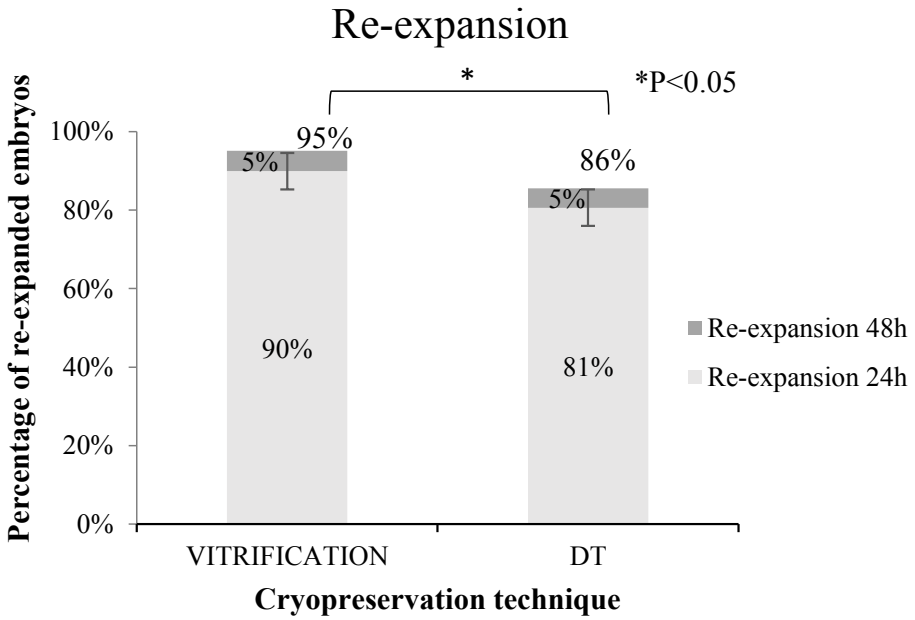

Figure 2. Re-expansion rates (at 24 hours, 48 hours, and total), according to the cryopreservation technique used.
*P < 0.05 indicates statistical significance.

The overall hatching rate was also higher in embryos subjected to vitrification than in those subjected to slow freezing, with values of 89% and 78%, respectively (P = 0.0346; Figure 3). In the vitrified embryo group, 71% hatched within the first 24 hours of in vitro culture, whereas 18% hatched between 24 and 48 hours. A hatching rate of 61% was observed after 24 hours and 17% between 24 and 48 hours (P > 0.05) in the group of embryos subjected to slow freezing. Although differences in hatching rates between vitrified embryos and those cryopreserved using slow freezing have not been identified (Barceló-Fimbres & Seidel, 2007; Sanches et al., 2016), most studies have reported superior results with vitrification of PIV bovine embryos (Liebermann & Tucker, 2006; Mucci et al., 2006; Inaba et al., 2011; Caamaño et al., 2015; Do et al., 2017), corroborating our results.

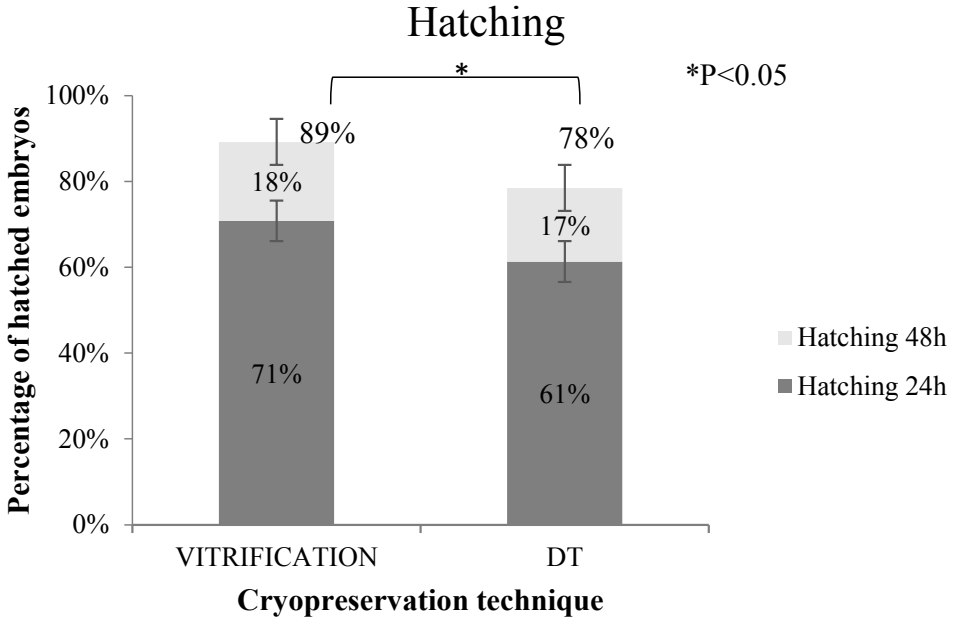

Figure 3. Hatching rates (at 24 hours, 48 hours, and total), according to the cryopreservation technique used.
*P < 0.05 indicates statistical significance.

Additionally, Amstislavsky et al. (2019) reported that vitrification is a better choice for cryopreservation than slow freezing for embryos rich in lipid content, such as cattle, sheep, and pigs. This is mainly associated with a reduction in cryogenic damage caused by vitrification during the early stages of embryonic development (Arshad et al., 2021).

However, despite observing inferior results with the slow freezing technique in our study, the total rates of re-expansion (86%) and hatching (78%) were good indices of the technique. Other studies using expanded day 7 blastocysts that were cryopreserved via slow freezing showed similar or lower rates. For example, Zolini et al. (2019) reported re-expansion and hatching rates of 78.1%, 81.4%, and 79.1%, and 27%, 53.8%, and 61.8%, respectively, at 24, 48, and 72 hours. Similarly, Gómez et al. (2020) obtained lower survival and hatching rates than those found in our study using slow freezing (65.6% survival and 41.2% hatching); however, the pregnancy rates in their study matched those obtained using vitrification and fresh transfer. Such evidence was also observed in a meta-analysis of Bos taurus embryo cryopreservation conducted by Arshad et al. (2021), who reported that both vitrification and slow freezing presented consistently successful pregnancy rates, indicating that satisfactory results can also be achieved with the slow freezing technique.

The second hypothesis examined in this study was whether embryos could be cryopreserved on 7 and 8 days after IVF without compromising their development after thawing. Many embryos reached the expanded blastocyst stage between these two days, leading to the inclusion of D7.5 in the analyses. The specific timing at which the embryos were subjected to cryopreservation (D7, D7.5, and D8) did not have a significant effect on the rate of post-cryopreservation/thawing embryonic development ($P > 0.05$).

However, these results contradict several studies demonstrating that embryos that develop more rapidly have better quality (Saha et al., 1996; George et al., 2008), show higher hatching rates after cryopreservation (Morató et al., 2010; Gómez et al., 2020; Martínez-Rodero et al., 2021), and have higher pregnancy rates (Hasler et al., 1995; Lonergan et al., 1999; Florentino et al., 2013).

Additionally, the interaction between vitrification and slow freezing (DT) and the day of cryopreservation (D7, D7.5, and D8) did not influence variables related to post-cryopreservation/de-thawing embryonic development ($P > 0.05$). The overall re-expansion rate showed statistically significant differences between the two groups ($P = 0.039$; Table 1), with a higher rate observed in embryos vitrified on day 7 (VIT7 group, 98%) than in embryos subjected to DT and frozen on day 8 (DT8 group, 78%). However, no significant difference was observed in the total hatching rate ($P > 0.05$; Table 1).

Table 1
Embryonic development rate according to the experimental groups

Groups*	Embryonic development rate	
	Total re-expansion (%)	Total hatching (%)
DT7	90 ± 0.04 ^{AB}	84 ± 0.05^{a}
DT7.5	88 ± 0.04 ^{AB}	77 ± 0.05^{a}
DT8	78 ± 0.04 ^{B}	74 ± 0.05^{a}
VIT7	98 ± 0.04 ^{A}	93 ± 0.05^{a}
VIT7.5	91 ± 0.04 ^{AB}	89 ± 0.06^{a}
VIT8	96 ± 0.05 ^{AB}	87 ± 0.06^{a}

% of re-expansion/hatching: Sum of blastocysts re-expanded/hatched within 24 hours and between 24 to 48 hours divided by the number of embryos cultured after thawing;
DT7 (embryos subjected to slow freezing on day 7); DT7.5 (embryos subjected to slow freezing 12 hours after day 7 on day 7.5); DT8 (embryos subjected to slow freezing on day 8); VIT7 (embryos vitrified on day 7); VIT7.5 (embryos vitrified 12 hours after day 7 on day 7.5); and VIT8 (embryos vitrified on day 8).
A, B, and different superscript letters in the same column indicate statistically significant differences ($P < 0.05$).

In commercial laboratories, surplus embryos from a fresh transfer on day 7, which show later development, are often discarded on day 8 because of concerns regarding the potential for unsuccessful outcomes using embryos with slower development, as reported in previous studies (Morató et al., 2010; Gómez et al., 2020; Martínez-Rodero et al., 2021). This highlights the importance of the data obtained in our study, as they provide support for greater utilization of surplus embryos, with the possibility of cryopreserving expanded blastocysts on day 8, using vitrification or slow-freezing techniques.

Embryo quality grade (Grades 1 and 2, according to IETS) only affected the developmental rates at 24 and 48 hours ($P < 0.05$). However, the overall rates of re-expansion and hatching did not significantly differ ($P > 0.05$; Figure 4). Specifically, the re-expansion rate was higher in Grade 1 than in Grade 2 embryos (91% and 79%, respectively; $P = 0.0183$) after 24 hours. Conversely, the re-expansion rate was higher in Grade 2 embryos than that in Grade 1 embryos (19% and 9%, respectively; $P = 0.0168$) at 48 hours. Similarly, hatching rates at 24 hours were higher in Grade 1 than in Grade 2 embryos (76% and 55%, respectively; $P = 0.0188$), whereas hatching rates at 48 hours were higher in Grade 2 than in Grade 1 embryos (24% and 12%, respectively; $P = 0.0181$).

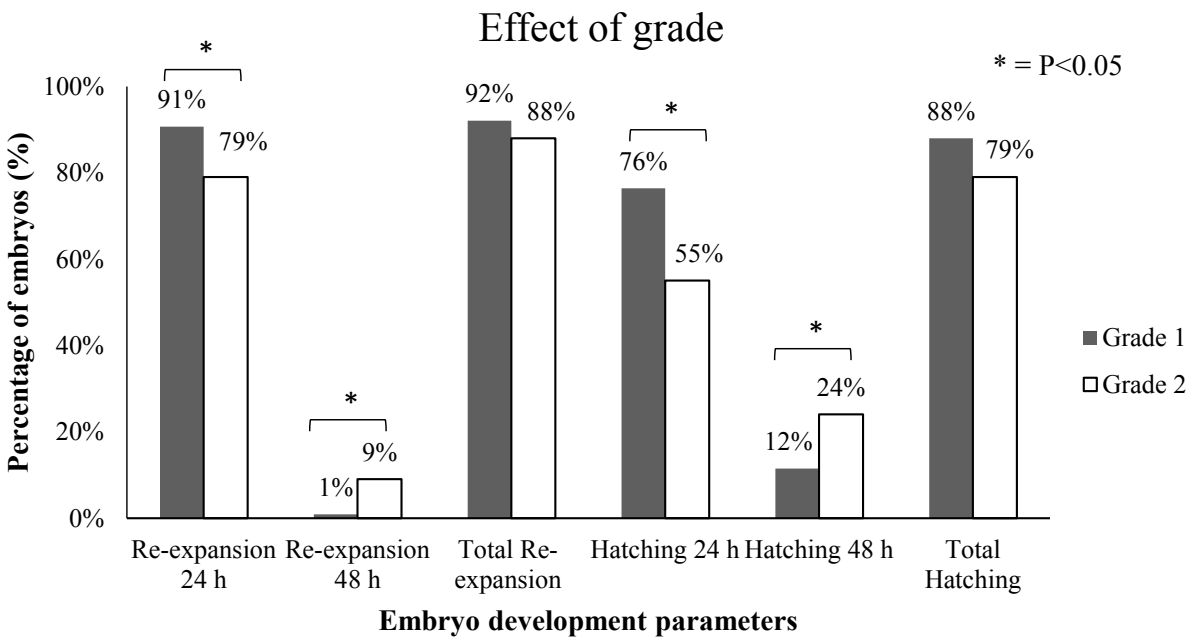

Figure 4. Re-expansion and hatching rates according to the time of re-expansion or hatching and the embryo quality grade.
*P < 0.05 indicates statistical significance.

These findings suggested that grade 1 embryos have a better development potential after thawing. This conclusion is supported by a study by Shu et al. (2008), which indicated that embryos with faster re-expansion after thawing have higher pregnancy rates.

Bó and Mapletoft (2013) stated that Grade 1 embryos have good survival rates after the freezing/thawing procedure, while Grade 2 and 3 embryos are more suitable for fresh transfer to appropriate recipients. Furthermore, Erdem et al. (2020) observed that Grade 1 embryos were associated with higher pregnancy rates during fresh embryo transfer than were Grade 2 embryos in heifers.

When considering the interaction between cryopreservation technique and embryo quality grade, an effect on the total rates of re-expansion and hatching was observed. Vitrified embryos with Grade 1 quality showed a higher total re-expansion rate than DT embryos of the same grade (97% and 87%, respectively; P = 0.027). Additionally, Grade 1 vitrified embryos tended to exhibit higher hatching rates than Grade 1 DT embryos (95% and 85%, respectively; P = 0.079; Figure 5), suggesting that vitrification may be particularly beneficial for high-quality embryos, contributing to a more positive post-cryopreservation/thawing embryonic development rate.

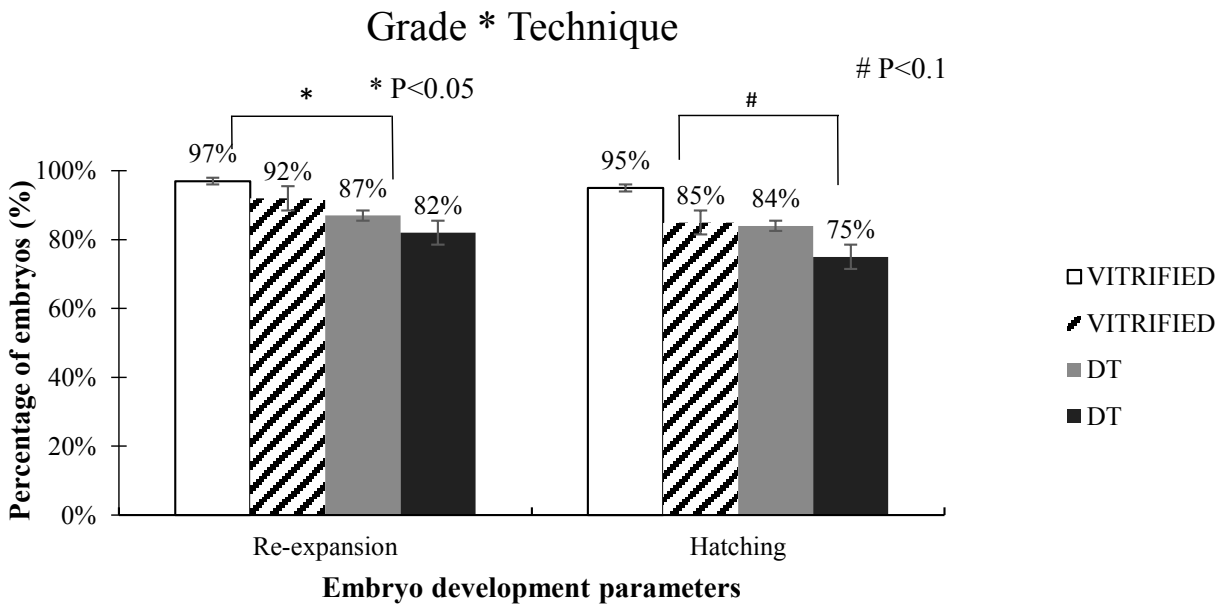

Figure 5. Effect of the interaction between embryo quality grade and cryopreservation technique on the re-expansion and hatching rate of embryos.
*P < 0.05 indicates statistical significance; #P = 0.0796 indicates a trend to be statistically different.

Conclusion

In vitro-produced embryos subjected to vitrification exhibited higher rates of post-thaw re-expansion and hatching than embryos cryopreserved using slow freezing. Additionally, the day of cryopreservation (D7, D7.5, and D8 post-IVF) did not influence the subsequent post-thaw development of the embryos cryopreserved by either vitrification or slow freezing. Quality Grades 1 and 2 of the cryopreserved embryos did not affect the total rates of re-expansion and hatching. However, Grade 1 embryos showed faster post-thaw development, especially when vitrification was used.

Acknowledgments

The authors thank the Coordination for the Improvement of the Higher Education Personnel (CAPES) for the scholarship grant. This study was supported by the Foundation for Support of Research in the State of Acre (FAPAC) for the funding provided. We thank In Vitro Acre Company for their assistance in conducting this project.

References

Aksu, D. A., Agca, C., Aksu, S., Bagis, H., Akkoc, T., Caputcu, A. T., Arat, S., Taskin, A. C., Kizil, S. H., & Karasahin, T. (2012). Gene expression profiles of vitrified in vitro and in vivo-derived bovine blastocysts. *Molecular Reproduction and Development, 79*(9), 613-625. doi: 10.1002/mrd.22068

Almiñana, C., & Cuello, C. (2015). What is new in the cryopreservation of embryos? *Animal Reproduction, 12*(3), 418-427. https://www.animal-reproduction.org/article/5b5a6031f7783717068b460b/pdf/animreprod-12-3-418.pdf

Amstislavsky, S., Mokrousova, V., Brusentsev, E., Okotrub, K., & Comizzoli, P. (2019). Influence of cellular lipids on cryopreservation of mammalian oocytes and preimplantation embryos: a review. *Biopreservation and Biobanking, 17*(1), 76-83. doi: 10.1089/bio.2018.0039

Arshad, U., Sagheer, M., Gonzalez-Silvestry, F. B., Hassan, M., & Sosa, F. (2021). Vitrification improves in-vitro embryonic survival in Bos taurus embryos without increasing pregnancy rate post embryo transfer when compared to slow-freezing: a systematic meta-analysis. *Cryobiology, 101*(8), 1-11. doi: 10.1016/j.cryobiol.2021.06.007

Barceló-Fimbres, M., & Seidel, Jr., G. E. (2007). Effects of fetal calf serum, phenazine ethosulfate and either glucose or fructose during in vitro culture of bovine embryos on embryonic development after cryopreservation. *Molecular Reproduction and Development, 74*(11), 1395-1405. doi: 10.1002/mrd.20699

Bó, G. A., & Mapletoft, R. J. (2013). Evaluation and classification of bovine embryos. *Animal Reproduction, 10*(3), 344-348. https://www.animal-reproduction.org/article/5b5a604cf7783717068b46a2

Caamaño, J., Gómez, E., Trigal, B., Muñoz, M., Carrocera, S., Martín, D., & Díez, C. (2015). Survival of vitrified in vitro-produced bovine embryos after a one-step warming instraw cryoprotectant dilution procedure. *Theriogenology, 83*(5), 881-890. doi: 10.1016/j.theriogenology.2014.11.021

Do, V. H., Catt, S., Kinder, J. E., Walton, S., & Taylor-Robinson, A. W. (2019). Vitrification of in vitro-derived bovine embryos: targeting enhancement of quality by refining technology and standardising procedures. *Reproduction, Fertility and Development, 31*(5), 837-846. doi: 10.1071/RD18352

Do, V. H., Walton, S., Catt, S., & Taylor-Robinson, A. W. (2017). A comparative analysis of the efficacy of three cryopreservation protocols on the survival of in vitro-derived cattle embryos at pronuclear and blastocyst stages. *Cryobiology, 77*(8), 58-63. doi: 10.1016/j.cryobiol.2017.05.007

Dode, M. A. N., Leme, L., & Spricigo, J. V. W. (2013). Criopreservação de embriões bovinos produzidos in vitro. *Revista Brasileira de Reprodução Animal, 37*(2), 145-150. http://cbra.org.br/pages/publicacoes/rbra/v37n2/pag145-150%20(RB453).pdf

Erdem, H., Karasahin, T., Alkan, H., Dursun, S., Satilmis, F., & Guler, M. (2020). Effect of embryo quality and developmental

stages on pregnancy rate during fresh embryo transfer in beef heifers. *Tropical Animal Health and Production, 52*(5), 2541-2547. doi: 10.1007/s11250-020-02287-6

Ferré, L., Kjelland, M., Strobech, L., Hyttel, P., Mermillod, P., & Ross, P. (2020). Recent advances in bovine in vitro embryo production: reproductive biotechnology history and methods. *Animals, 14*(5), 991-1004. doi: 10.1017/S1751731119002775

Florentino, C., Mariani, A., Souza, J., Dias, F., Santos, H., Arrivabene, M., Neves, W., Souza, J., Cavalcante, T., & Wischral, A. (2013). Pregnancy rates bovine recipients inovulated with in vitro produced IVP embryos in the Legal Amazon. *Journal of Animal Science Advances, 3*(4), 193-202. doi: 10.5455/jasa. 20130522044741

George, F., Daniaux, C., Genicot, G., Verhaeghe, B., Lambert, P., & Donnay, I. (2008). Set up of a serum-free culture system for bovine embryos: Embryo development and quality before and after transient transfer. *Theriogenology, 69*(5), 612-623. doi: 10.1016/j.theriogenology.2007.11.008

Gómez, E., Carrocera, S., Martín, D., Pérez-janez, J. J., Prendes, J., Prendes, J. M., Vázquez, A., Murillo, A., Gimeno, I., & Muñoz, M. (2020). Efficient one-step direct transfer to recipients of thawed bovine embryos cultured in vitro and frozen in chemically defined medium. *Theriogenology, 146*(1), 39-47. doi: 10.1016/j.theriogenology.2020.01.056

Gonçalves, R. L. R., & Viana, J. H. M. (2019). Situação atual da produção de embriões bovinos no Brasil e no mundo. *Revista Brasileira de Reprodução Animal, 43*(2), 156-159. http://cbra.org.br/portal/downloads/publicacoes/rbra/v43/n2/p156-159%20(RB785).pdf

Hasler, J. F., Henderson, W. B., Hurtgen, P. J., Jin, Z. Q., Mccauley, A. D., Mower, S. A., & Trimmer, S. A. (1995). Production, freezing and transfer of bovine IVF embryos and subsequent calving results. *Theriogenology, 43*(1), 141-152. doi: 10.1016/0093-691X(94)00020-U

Inaba, Y., Aikawa, Y., Hirai, T., Hashiyada, Y., Yamanouchi, T., Misumi, K., Ohtake, M., Somfai, T., Kobayashi, S., & Saito, N. (2011). In-straw cryoprotectant dilution for bovine embryos vitrified using Cryotop. *Journal of Reproduction and Development, 11*(4), 04010362. doi: 10.1262/jrd.10-154m

Leibo, S. P., & Mapletoft, R. J. (1998). Direct transfer of cryopreserved cattle embryos in North America. *Proceedings of the Annual Conven Tion of American Embryo Transfer Association,* San Antonio, Misiones, Argentina, 17.

Liebermann, J., & Tucker, J. (2002). Blastocyst development after vitrification of multipronuclear zygotes using the flexipet denuding pipette. *Reproduction BioMedecine Online, 4*(2), 146-150. doi: 10.1016/s1472-6483(10)61932-3

Liebermann, J., & Tucker, M. J. (2006). Comparison of vitrification and conventional cryopreservation of day 5 and day 6 blastocysts during clinical application. *Fertility and Sterility, 86*(1) 20-26. doi: 10.1016/j.fertnstert.2006.01.029

Lonergan, P., Khatir, H., Piumi, F., Rieger, D., Humblot, P., & Boland, M. P. (1999). Effect of time interval from insemination to first cleavage on the developmental characteristics, sex ratio and pregnancy rate after transfer of bovine embryos. *Journal of Reproduction and Fertility, 117*(1), 159-167. doi: 10.1530/jrf.0.1170159

Marinho, L. S. R., Sanches, B. V., Rosa, C. O., Tannura, J. H., Rigo, A. G., Basso, A. C., & Seneda, M. M. (2015). Pregnancy rates to fixed embryo transfer of vitrified IVP Bos indicus, Bos taurus or Bos indicus × Bos taurus embryos. *Reproduction in Domestic Animals, 50*(5), 807-811. doi: 10.1111/rda.12591

Martínez-Rodero, I., García-Martínez, T., Ordóñez-Léon, E. A., Vendrell-Flotats, M., Olegario Hidalgo, C., Esmoris, J., Mendibil, X., Azcarate, S., López-Béjar, M., Yeste, M., & Mogas, T. (2021). A shorter equilibration period improves post-warming outcomes after vitrification and in straw dilution of in vitro-produced bovine embryos. *Biology, 10*(2), 1-15. doi: 10.3390/biology10020142

Morató, R., Izquierdo, D., Paramio, M. T., & Mogas, T. (2010). Survial and apoptosis rates after vitrification in cryotop devices of in vitro-produced calf and cow blastocysts ar diferente developmental stages. *Reproduction, Fertility and Development, 22*(7), 1141-1147. doi: 10.1071/RD10013

Mucci, N., Aller, J., kaiser, G. G., Hozbor, F., Cabodevila, J., & Alberio, R. H. (2006). Effect of estrous cow serum during bovine embryo culture on blastocyst development and cryotolerance after slow freezing or vitrification. *Theriogenology, 65*(8), 1551-1562. doi: 10.1016/j.theriogenology.2005.08.020

Oliveira, C. S., Feuchard, V. L. S., Freitas, C., Rosa, P. M. S., Camargo, A. J. R., & Saraiva, N. Z. (2020). In-straw warming protocol improves survival of vitrified embryos and allows direct transfer in cattle. *Criobiology, 97*(12), 222-225. doi: 10.1016/j.cryobiol.2020.02.007

Saha, S., Rajamahendran, R., Boediono, A., Sumantril, C., & Suzuki, T. (1996). Viability of bovine blastocysts obtained after 7, 8 or 9 days of culture in vitro following vitrification and one-step rehydration. *Theriogenology, 46*(2), 331-343. doi: 10.1016/0093-691x(96)00189-6

Sanches, B. V., Lunardelli, P. A., Tannura, J. H., Cardoso, B. L., Pereira, M. H. C., Gaitkoski, D., Basso, A. D., Arnold, D. R., & Seneda, M. M. (2016). A new direct transfer protocol for cryopreserved IVF embryos. *Theriogenology, 85*(6), 1147-1151. doi: 10.1016/j.theriogenology.2015.11.029

Saragusty, J., & Arav, A. (2011). Current progress in oocyte and embryo cryopreservation by slow freezing and vitrification. *Reproduction, 141*(1), 1-19. doi: 10.1530/REP-10-0236

Shu, Y., Watt, J., Gebhardt, J., Zhao, Q., Milki, A., & Behr, B. (2008). Elective single-embryo transfer in frozen blastocyst transfers cycles. *Fertility and Sterility, 90*(1), S205. doi: 10.1016/j.fertnstert.2008.07.484

Vajta, G., Holm, P., Kuwayama, M., Booth, P. J., Jacobsen, H., Greve, T. & Callense, H. (1998). Open pulled straw (OPS) vitrification: a new way to reduce cryoinjuries of bovine ova and embryos.

Molecular Reproduction Develepment, 51(1), 51-53. doi:10.1002/(SICI)1098-2795(199809)511%3C53AID-MRD6%3E3.0.CO;2-V

Viana, J. H. M. (2018). 2017 Statistics of embryo production and transfer in domestic farm animals. *Embryo Technology Newsletter, 36*(1), 8-25. https://www.iets.org/Portals/0/Documents/Public/Committees/DRC/IETS_Data_Retrieval_Report_2017.pdf

Viana, J. H. M. (2022). 2021 Statistics of embryo production and transfer in domestic farm animals. A new milestone has been reached: transfer of IVP embryos were over over one million worldwide. *Embryo Transfer Newsletter, 40*(4), 22-40. https://www.iets.org/Portals/0/Documents/Public/News/December_ 2021.pdf

Voelkel, S. A., & Hu, Y. X. (1992). Direct transfer of frozen-thawed bovine embryos. *Theriogenology, 27*(1), 23-30. https://doi.org/10.1016/0093-691X(92)90245-M

Zolini, A. M., Carrascal-Triana, E., King, A. R., Hansen, P. J., Torres, C. A. A., & Block, J. (2019). Effect of addition of L-carnitine to media for oocyte maturation and embryo culture on development and cryotolerance of bovine embryos produced in vitro. *Theriogenology, 133*(15), 135-143. doi: 10.1016/j.theriogenology.2019.05.005