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Can bovine embryos be successfully transferred after 40 years of cryopreservation?

[Os embriões bovinos podem ser transferidos com sucesso após 40 anos de criopreservação?]

J. *Camacho-Ronquillo*[1], E. *Posadas-Manzano*[2], F. *Utrera-Quintana*[1], J. *Hernández-Hernández*[1], J. *Gallegos-Sánchez*[3], J. *Cruz-Aviña*[1], A. *Villa-Mancera*[1*]

[1]Facultad de Medicina Veterinaria y Zootecnia, Benemérita Universidad Autónoma de Puebla, Tecamachalco Puebla, México
[2]Facultad de Medicina Veterinaria y Zootecnia; Universidad Nacional Autónoma de México, CDMX, México
[3]Colegio de Postgraduados, Campus Montecillo, Estado de México, México

ABSTRACT

Global advances in reproductive biotechnology have allowed for the transfer of embryos from donor females with high genetic merit to recipients using the cryopreservation technique, which preserves an embryo of excellent quality and viability, thereby achieving a feasible pregnancy rate. The objective of this study was to evaluate the quality and viability of Holstein embryos that have been cryopreserved for more than 40 years under glycerol freezing. The embryos were transferred to the recipient heifers using a non-surgical method. Two 17-month-old Holstein heifers (360 kg live weights) which were clinically healthy and reproductively active were used as the recipients. Two bovine embryos of Grade 1 quality were thawed and evaluated for their morphology. Of the two embryo transfers, one pregnancy was achieved, resulting in the birth of a calf. Therefore, embryos frozen in liquid nitrogen and glycerol as a cryopreservative for more than 40 years maintained their quality and viability to produce a live calf.

Keywords: bovine, cryopreservation, embryo transfer, pregnancy, embryo survival

RESUMO

Os avanços globais em biotecnologia reprodutiva permitiram a transferência de embriões de fêmeas doadoras com alto mérito genético para receptoras, usando-se a técnica de criopreservação, que preserva um embrião de excelente qualidade e viabilidade, alcançando, assim, uma taxa de gravidez viável. O objetivo deste estudo foi avaliar a qualidade e a viabilidade de embriões Holstein, criopreservados por mais de 40 anos, sob congelamento de glicerol. Os embriões foram transferidos para as novilhas receptoras, usando-se um método não cirúrgico. Duas novilhas Holstein de 17 meses de idade (360kg de peso vivo), que eram clinicamente saudáveis e reprodutivamente ativas, foram utilizadas como receptoras. Dois embriões bovinos de qualidade Grau 1 foram descongelados e avaliados quanto à sua morfologia. Das duas transferências embrionárias, uma gravidez foi obtida, resultando no nascimento de um bezerro. Portanto, os embriões congelados em nitrogênio líquido e glicerol como criopreservante por mais de 40 anos mantiveram sua qualidade e viabilidade para produzir um bezerro vivo.

Palavras-chave: bovinos, criopreservação, transferência embrionária, gravidez, sobrevivência embrionária

INTRODUCTION

Considerable advances in mammalian reproductive biotechnologies occurred in the last half of the 20th century, including *in vitro* production and implementation, embryo sexing, and cloning in some species (Mandawala *et al.*, 2016; Moore and Hasler 2017). Embryo cryopreservation is complemented with *in vitro* fertilization for the maintenance of germplasm banks for research, conservation of valuable

*Corresponding author: abel.villa@gmail.com
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animals, and utilization in practice (Ferre *et al.*, 2020; Morrell and Mayer 2017). Animal breeding biotechnologies, including germplasm cryopreservation, aim to improve species to satisfy market demands (Crowe *et al.*, 2021; Mandawala *et al.*, 2016; Morrell and Mayer 2017). The embryo transfer (ET) method attempts to produce a larger number of animals with strong genetics over a shorter period than those produced under normal management conditions (Crowe *et al.*, 2021; Pulina *et al.*, 2021). Cryoprotectants are chemical components that prevent cryolesions in freezing media, and they significantly improve the structural integrity of cells, although high molar concentrations cause cell toxicity or apoptosis due to stress or osmotic shock (Pereira *et al.*, 2020; Ribeiro *et al.*, 2022).

The rate of thermal descent between certain temperature ranges can produce detrimental alterations to cellular life; therefore, freezing protocols have been formulated which combine different additives and cooling rates (Moore and Hasler 2017; Ribeiro *et al.*, 2022). Penetrant cryoprotectants are organic compounds with low molecular weights (less than 100 kDa) and high amphiphilic properties. These attributes confer permeability across the plasma membrane (Ribeiro *et al.*, 2022; Whaley *et al.*, 2021). The most commonly used penetrant cryopreservatives are glycerol, ethylene glycol, propylene glycol, dimethyl sulfoxide, methanol and butanediol (Elliott *et al.*, 2017; Pereira *et al.*, 2020).

The cryoprotective properties of glycerol in sperm were discovered in 1949 by Christopher Polge and Audrey Smith, and ET was first successfully practiced in a cow in 1951 (Estudillo *et al.*, 2021; Hansen 2020). The American Embryo Transfer Association (AETA) has reported an average transfer (1997-2017) of 470,000 *in vitro*-produced embryos worldwide and greater than 16 million transfers in the last 20 years (Ferre *et al.*, 2020; Viana 2018). Frozen embryos do not transmit disease and they can remove sanitary barriers, be thawed and transferred any time of the year in any region, and be used to schedule births according to the management of the production unit (Ferre *et al.*, 2020; Molina-Coto *et al.*, 2020; Schnuelle 2021). The pregnancy success of dairy cows using frozen embryos has been reported at 47.3%, which is lower than that obtained with fresh embryos of 55.3% (Hansen 2020).

The use of frozen embryos allows for the efficient use of donors and recipients through the incorporation of genetic progress at a low cost, considering the expense of the embryo and its transport compared to that of standing animals, as well as the ability to transfer embryos while maintaining the remainder while production records of the offspring are analyzed (Alminana and Cuello 2018; Ferre *et al.*, 2020). In addition, this method can ensure the control of exotic diseases, replace the importation of standing animals with unrelated frozen embryos, and create embryo banks of high genetic value (Alminana and Cuello 2018; Colazo and Mapletoff 2007; Crowe *et al.*, 2021; Vries and Kaniyamattam 2020). Few studies have described the effect of long-term cryopreserved bovine embryos, although 13-year cryopreserved embryos have been transferred in sheep (Fogarty *et al.*, 2000). The purpose of the present study was to evaluate the quality and viability of bovine embryos cryopreserved in glycerol for more than 40 years and to transfer these embryos to recipient heifers to achieve gestation.

MATERIAL AND METHODS

The experiment was conducted at the Posta Zootécnica El Salado of the FMVZ-BUAP, located in Tecamachalco Puebla, Mexico (18° 52' N and 97° 43' W), which has an altitude of 2055 m above sea level, semi-dry temperate climate with rainfall in the summer, average annual rainfall of 700 mm and an average annual temperature of 18 °C (INEGI 2017).

Two embryos were frozen in liquid nitrogen in 1978 with glycerol as a cryoprotectant. The embryos were identified, classified according to their morphological features using a stereoscopic microscope with a magnification of 10× to 50×, and graded using the quality scales of Córdova-Isquierdo *et al*., 2015, as follows:

Grade 1, excellent quality: Spherical embryo, zona pellucida intact, perfectly structured cell cluster with cells of the same size, color, and texture.

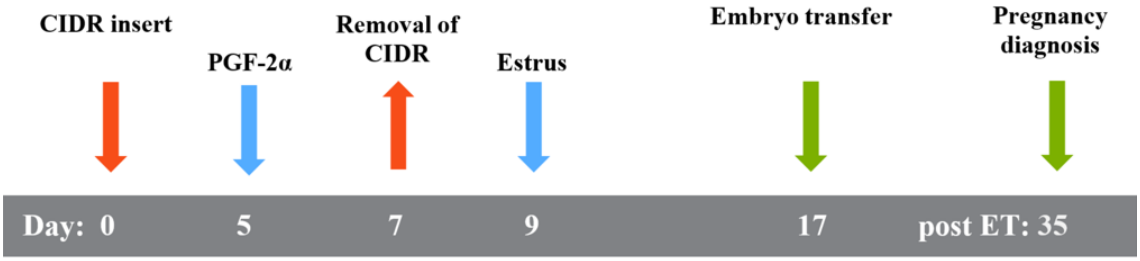
Grade 2, good quality: Spherical or slightly ellipsoidal embryo, intact zona pellucida, some loose blastomeres and perfect cell cluster.

Grade 3, fair quality: Intact or damaged zona pellucida, blastomeres of different sizes, cell cluster with 30% to 60% intact blastomeres, slight developmental delay (32-cell stage).

Grade 4, poor quality: intact or damaged zona pellucida, degenerate and loose blastomeres in considerable number, loose cell cluster with less than 30% intact blastomeres, delayed development and embryonic stages with large blastomeres (16 and 32 cells).

Thawing was performed by exposing the straws to room temperature for 10 seconds, after which they were placed in a water bath at 30-35 °C for 20-30 seconds. Cryoprotectant was removed from the embryos in a stepwise manner using 0.5 M and 0.25 M sucrose and the embryo was maintained for 5 minutes in each step until a final concentration of phosphate-buffered saline (PBS) without cryoprotectant was achieved.

Two 17-month-old Holstein heifers (360 kg each) which were clinically healthy and reproductively active were used as recipients. On the first day of the study (Day 0), the recipient females were fitted with an intravaginal controlled internal drug release (CIDR, Zoetis, Mexico) device containing 1.9 g of progesterone. On Day 5, 25 mg of prostaglandin F2-alpha (PGF-2α, Zoetis, Mexico) was applied, and on Day 7 the device was removed. Estrus detection was performed twice daily, and the heifer was considered in estrus when she allowed mounting to occur. The synchronization protocol of the recipient females is presented in Fig. 1. This study was approved by the Ethics and Animal Care Committee of the Benemerita Autonomous University of Puebla, and all procedures complied with the National Legislation on Animal Health Research.



CIDR: controlled internal drug release; PGF-2α: prostaglandin F2-alpha; ET: embryo transfer

Figure 1. Estrus synchronization protocol of recipient heifers and transfer of bovine embryos after 40 years of cryopreservation.

After eight days of estrus detection, a bovine embryo was transferred using the recto-vaginal technique. The transfer was performed using an artificial insemination applicator. Each embryo, along with a small volume of EPS solution, was aspirated into a 0.5mL French straw, with a small air bubble on each side to facilitate identification. An epidural block was applied with 2mL of 2% lidocaine hydrochloride (Pisacaina, Pisa, Mexico), and the ovaries were gently palpated to determine which ovary contained the corpus luteum, as the embryo was to be deposited in the uterine horn adjacent to the corpus luteum (Molina-Coto *et al.*, 2020).

The perivulvar region was adequately cleaned to avoid contamination. The applicator was inserted through the vagina, passed over the cervix and carefully advanced along the horn adjacent to the corpus luteum. The plunger of the applicator was compressed to expel the embryo and then carefully removed. The diagnosis of pregnancy by rectal palpation was performed 35 days after embryo transfer. Blood samples were obtained from the cystic vein to quantify progesterone using a commercial indirect ELISA kit (IDEXX Laboratories Inc., USA), according to the manufacturer's instructions.

RESULTS AND DISCUSSION

The cryopreservation of bovine embryos is widely used in breeding programs to ensure donors have high genetic merit. In this study, embryos from donor cows with excellent classification grades (viability) and good family histories and productive records were used. Embryo cryopreservation has been shown to be affected by factors such as the composition of the cryopreservation medium, temperature of incubation and method of cryoprotectant addition or deletion (Moore and Hasler 2017; Ribeiro *et al.,* 2022).

Two bovine embryos that were frozen in liquid nitrogen in 1978 using glycerol as cryoprotectant were transferred to recipient heifers. Cryoprotectants are chemical components that allow for the recovery of a high percentage of embryos after thawing by preventing injury during cryopreservation (Ribeiro *et al.,* 2022; Whaley *et al.,* 2021).

The bovine embryos that had been cryopreserved for more than 40 years were thawed for evaluation and two with Grade 1 quality were selected for transfer to donor heifers. Morphological evaluation is the main method of embryo evaluation and selection. However, the classification of embryos according to their morphology, cell number and apoptosis is insufficient to reflect their quality and involves a level of subjectivity by the embryologist (Demetrio *et al.,* 2020; Ramos-Ibeas *et al.,* 2019). Several researchers have studied the possible damage to embryos after cryopreservation, including effects on the functionality of the cell and its structures, physical damage to cell membranes caused by the transition from water to ice, osmotic changes from cell dehydration and effects relating to cryoprotectant exposure (Jones 2021; Ribeiro *et al.,* 2022).

A positive result was obtained for the first heifer subjected to Grade 1 quality embryo transfer, in terms of a pregnancy diagnoses 35 days after embryo transfer followed by gestation, which resulted in the birth of a live and clinically healthy calf. In ewes, two embryos which were cryopreserved with glycerol for 13 years were transferred into each recipient, and a 31.4% calving rate was achieved (Fogarty *et al.,* 2000). However, no experiments have been identified that use viable bovine embryos cryopreserved for more than 40 years to produce a full-term gestation. Embryos exposed to *in vivo* and *in vitro* stress conditions associated with supplementation with fetal bovine serum as a culture medium have been shown to develop large offspring syndrome (LOS), which is characterized by a large birth size, increased birth weight and organ abnormalities (Ramos-Ibeas *et al.,* 2019).

The second recipient heifer that received the Grade 1 quality viable bovine embryo transfer returned to estrus 14 days after the transfer; therefore, since only two embryos were used in this study, 50% gestation was obtained. Several factors contribute to embryo quality, developmental capacity and cryotolerance, such as embryo metabolism, cytoplasmic lipid content, cell division rate, morula-compact stage, and gene expression (Ferre *et al.,* 2020).

CONCLUSIONS

The production of bovine embryos is a powerful method of providing high-quality animals using genetic improvements. An embryo frozen for greater than 40 years that was cryopreserved with glycerol was transferred to a donor heifer and developed into a viable embryo, resulting in a healthy offspring.

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