Slov Vet Res
DOI 10.26873/SVR-2338-2026

Early View

Original Research Article

Comparative Analysis of Post-Thaw Developmental Dynamics in *In Vitro*-Fertilized and Conventionally Produced Bovine Embryos

Key words

embryo transfer; cryopreservation; assisted reproductive technology

Luis Ochoa, John Gibbons*, Jessica Looman

Texas Tech University - School of Veterinary Medicine, Amarillo, TX 79106, USA

***Corresponding author:** jogibbon@ttu.edu

Abstract: *In Vitro* Fertilization (IVF) enables progressive cattle producers to reach genetic, reproductive, and financial goals; however, cryopreservation of IVF embryos, while vital to the industry, remains a challenge. In this study, short-term (48 h) post-thaw *in vitro* culture was used to evaluate the developmental potential of IVF (n = 83) and *in vivo*–derived (IVD; n = 76) embryos, cryopreserved in 1.5 M ethylene glycol (EG). Embryos were thawed for 5 s in air and 27ºC water bath for 30 s, rinsed in commercially available holding media, and placed individually into commercially available *in vitro* culture media (5 µL drops; 5% CO_2, 5% O_2, and 90% N_2, 100% humidity) and evaluated for grade and stage (IETS standards) at thaw, 24, and 48 h (compared with repeated ANOVA within group and ANOVA between groups during 4 replicates; SAS Software Version 9.4). IVF embryos developed ($P<0.05$) from thaw to 24h (6.5±0.06 to 6.8±0.07) and from 24 to 48h (6.8±0.07 to 7.4±0.10) as did the IVD embryos (thaw to 24h = 4.4±0.06 to 6.0±0.13; 24 to 48h = 6.0±0.13 to 7.1±0.14; IETS standards). Although development at 48h was similar between the IVF embryos and IVD embryos, the IVF embryos were cryopreserved at a more ($P<0.05$) advanced stage (6.5±0.06) compared to IVD embryos (4.4±0.06). IVD embryos advanced further ($P<0.05$) during culture (2.7±0.14) compared to IVF embryos (1.0±0.10). The IVD embryos were of higher ($P<0.05$) quality (IETS standards) at 48h compared to IVF embryos (1.7±0.10 vs 2.2±0.13). These data indicated that IVF embryos did not tolerate the freeze/thaw cycle as well as IVD embryos. Embryo quality at thaw for both groups was acceptable; however, some embryos did not advance during the culture period suggesting developmental arrest. These data suggested that culturing IVF (and perhaps IVD) embryos for an additional 24-48 h post-thaw provided an additional selection mechanism and may provide higher quality embryos to be used for embryo transfer. The embryos in this study were collected during commercial embryo transfer activity and donated to the Texas Tech University School of Veterinary Medicine (TTU-SVM) and were from a variety of Holstein donor matings, which is a limitation of this study. Further research is warranted to evaluate the cryosurvivability associated with IVF and IVD embryos.

Received: 12 December 2025
Accepted: 10 June 2026

Introduction

In Vitro Fertilization (IVF) enables progressive livestock producers to reach genetic, reproductive, and financial goals (1, 2, 3); however, cryopreservation of IVF embryos remains a challenge. Most *in vivo* conventional embryos (IVD) are cryopreserved with EG to allow for direct transfer post-thaw; however, this limits post-thaw embryo evaluation. A statistical information committee report from the American Embryo Transfer Association indicates that of 96 US Certified Embryo Transfer Businesses, 87.5% produce IVD embryos, while 37.5% produce IVF embryos from oocytes aspirated via a transvaginal ultrasound guided approach (4). Further, the number of IVF embryos produced in the US and Worldwide has supplanted the number of embryos produced *in vivo*, especially in the dairy industry (Figure 1; (4)). Most frozen

embryos are cryopreserved using EG. The polar nature of EG allows it to penetrate cells effectively during cryopreservation and enables the direct transfer of embryos to recipients after thawing as rehydration is rapid, efficient, occurs in utero, and is associated with acceptable pregnancy success rates following transfer (5). However, the full effects of EG post-thaw are not well understood, as EG can be oxidized to oxalic acid, which interferes with mitochondrial function (6). This could affect embryo viability if embryos are exposed to EG for prolonged periods or at elevated temperatures, post-thaw. In practice, embryos are not evaluated post-thawing, rather they are introduced directly into the recipient so the true effects of freezing and thawing on embryo morphology and viability are unknown. In this study, the developmental dynamics of IVF and IVD embryos were evaluated for 48h post-thaw using an *in vitro* culture system. Results suggested that culturing IVF (and perhaps IVD) embryos for an additional 24-48 h post-thaw provided an additional selection mechanism and may allow for higher quality embryos to be used for embryo transfer, thus improving pregnancy rates and calf production.

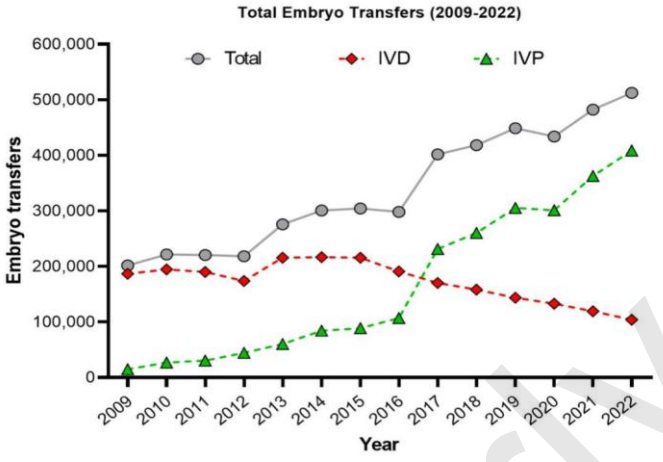

Figure 1: Embryo transfer activity in the US duri

Materials and methods

IVF and Embryo production

All embryos in this report were donated from Sunshine Genetics, Inc. (Whitewater, WI USA) under their Animal Use Protocols (no IACUC required for TTU-SVM as there was no animal work) and were either produced *in vitro* or recovered from superovulated donors. The Holstein sires were the same for all IVF embryos and IVD embryos, although there were a small pool (3-6 depending upon replicate) of oocyte or embryo Holstein donors. Briefly, the IVF embryos were produced by aspirating oocytes from donors using a trans-vaginal ultrasound-guided approach. Oocytes were then placed into maturation for 18-22 h and then co-incubated with sperm cells for an additional 18-20 h, rinsed and placed into *in vitro* culture for 6-7 d. Resulting embryos were evaluated for grade and stage (7) rinsed and cryopreserved. IVD embryos were recovered from hyper-stimulated donors using a trans-cervical uterine lavage 7 d following artificial insemination. Embryos were evaluated (7), rinsed, and cryopreserved.

Cryopreservation

All embryos (IVF and IVD) were cryopreserved 7 d following *in vitro* or *in vivo* exposure to sperm cells in a phosphate buffered saline solution with 0.4% bovine serum albumin and 1.5 M EG. The embryos were placed individually in ¼ cc plastic straws, allowed to equilibrate for 6-8 m and then cooled from -6 °C to –32 °C at 0.5 °C per m. Straws were then plunged into liquid nitrogen and packaged into goblets and placed on canes for storage.

Post-thaw Embryo Evaluation

Eighty-three (n=83) frozen donated IVF embryos and seventy-six (n=76) IVD embryos cryopreserved in EG and stored in liquid nitrogen were thawed and cultured at TTU-SVM. First, they were removed and exposed to air for 5 s, then warmed in a 27°C water bath for 30 s. Embryos were rinsed in commercially available holding media (ABT 360, Canton, TX USA) and placed individually into commercially available *in vitro* culture media (Stroebech, Inc, Denmark). All embryos were cultured in 5 µL drops at 38.5°C in a fully humidified atmosphere of 5% CO_2, 5% O_2, and 90% N_2. Embryos were evaluated at thaw, 24, and 48 h post-thaw for stage of development and quality grade, based on IETS standards (7). The numeric code for stage of development for the embryos in this report was 4 = morula, 5 = early blastocyst, 6 = full blastocyst, 7 = expanded blastocyst, 8 = hatching blastocyst, while embryo quality grade ranged from 1 = excellent, 2 = fair, 3 = poor, and 4 = degenerate (7). Data were analyzed by repeated measures ANOVA (SAS Version 9.4) to compare embryo evaluations within a group, one-way ANOVA to compare embryo evaluations between groups during 4 replicates. The developmental slope was based upon the trendline for development between thaw and 48 h of culture. A Chi-square analysis (SAS Version 9.4) was used to compare binomial data either associated with embryo development between thaw and 24 h of culture and 24 and 48 h of culture (individual embryos either developed during a time period or did not) or hatching rates (embryo hatched or did not by 48 h of culture). The developmental slope was calculated by comparing the developmental stage at thaw to the developmental stage at 48 h of culture individually for each embryo, and compared using linear regression in SAS (Version 9.4)

Results

The stage of IVF embryo development increased (P<0.05) from thaw to 24 h (6.5±0.06 to 6.8±0.07) and from 24 to 48 h (6.8±0.07 to 7.4±0.10) as did the IVD embryos (thaw to 24 h = 4.4±0.06 to 6.0±0.13; 24 to 48 h = 6.0±0.13 to 7.1±0.14; Figure 2B). The stage of development at 48 h was similar between the IVF embryos and IVD embryos. However, in commercial embryo transfer business that perform IVF, as in this study, the IVF embryos were cryopreserved at a more (P<0.05) advanced stage (6.5±0.06; this study) compared to IVD embryos (4.4±0.06; Figure 2B; this study). The IVD embryos advanced further (P<0.05) during the 48 h *in vitro* culture period (2.7±0.14) compared to

IVF embryos (1.0±0.10) and the developmental slope was steeper (Table 1). Most (P<0.05) of the development for the IVD embryos was during the thaw to 24 h period (57.9±0.05%), while most (P<0.05) of the development for the IVF embryos was during the 24 to 48 h period (61.5±0.04%). Further, the IVD embryos were of higher (P<0.05) quality at 48 h compared to the IVF embryos (1.7±0.10 vs 2.2±0.13; Figure 2A). Although post-thaw development was limited for the IVF embryos, there was no difference in the hatching rates (Table 1) between IVF (55.4±0.05%) and IVD (53.9±0.06%) embryos.

Table 1: Embryo hatching rates at 48 hours of in vitro culture and the developmental slope based upon the data trendlines for IVD and IVF frozen/thawed bovine embryos

Embryo Type	Hatched (48h)	Developmental slope *
IVD	41/76; 53.9%	1.36 ± 0.07
IVF	46/83; 55.4%	0.48 ± 0.05

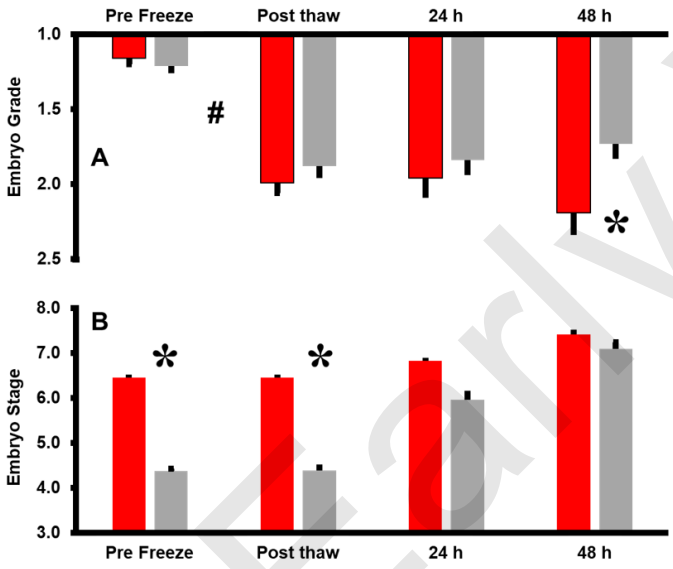

Figure 2: Mean (± SEM) grade (Panel A; 1 = excellent, 2 = fair, 3 = poor, 4 = degenerated) and stage (Panel B; 4 = morula, 5 = early blastocyst, 6 = full blastocyst, 7 = expanded blastocyst, 8 = hatching blastocyst) of IVF ( ) and IVD embryos ( ), frozen in 1.5 M EG, thawed and cultured individually for 48 h in vitro; between * = P<0.05, and within groups # = P<0.05.

Discussion

These data indicated that IVF embryos do not tolerate the freeze/thaw cycle as well as IVD embryos, and apparently the damage is manifested in poor development during the first 24 h post-thaw. Embryo quality at thaw for both groups was acceptable; however, some embryos did not advance during the culture period suggesting developmental arrest. The IVF embryos were cryopreserved at a more advanced stage of development compared to the IVD embryos suggesting that perhaps more advanced embryos (from whatever source) do not respond well to the in and outflux of cryoprotectants and the freezing process (5, 6). Embryos produced via IVF when evaluated at ultrastructural level had fewer desmosomal junctions and microvilli and higher than average number of lipid droplets and increased debris in the perivitelline space (8) which may impair cryotolerance, but does not appear to be stage specific. They were also characterized by an increase in large intercellular cavities when compared to IVF counterparts, which may affect their ability to withstand cryopreservation (8).

Although logistically challenging, perhaps embryos should be cultured *in vitro* post-thaw to determine which embryos develop (9) and are perhaps more likely to produce pregnancies. This may be especially helpful for IVF embryos as most of their development was during the 24-48 h period in this study, suggesting that frozen-thawed IVF embryos experienced a post-thaw delay in redevelopment in culture. Although there are many examples of communication among embryos in culture, there does not seem to be a beneficial effect of group *in vitro* culture of frozen-thawed bovine embryos (10), which may facilitate the use of post thaw culture as a tool to increase pregnancy rates, de-pending upon the genetic goals.

In vitro embryo production provides a critical opportunity to facilitate reaching genetic, reproductive, and financial goals and enables other assisted reproductive technologies (11). Although pregnancy rates per embryo transferred may be lower with this technology compared to conventional embryo production (12), more embryos (and thus more pregnancies) can be produced per unit of time (12) thus enabling specific goals. Further research is warranted to evaluate the freeze/thaw damages associated with IVF embryos and the modest *in vitro* development of IVF embryos post-thaw. However, a post-thaw culture approach may allow for an additional selection mechanism, which may produce higher quality IVF (and IVD) embryos for transfer on Day 8 or 9.

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L. Ochoa, J. Gibbons, J. Looman

Izvleček:

Ključne besede: