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Optimizing Hepes Levels for Short Term Hypothermic Storage of *In Vitro*-Produced Bovine Embryos

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Abstract

Hypothermic storage at 4°C offers a practical and low-cost approach for short-term handling of bovine *in vitro*-produced (IVP) embryos; however, optimal buffering conditions and storage limits for preserving embryo viability remain insufficiently defined. This study evaluated how varying HEPES concentrations and storage durations influence post-chilling survival and developmental competence of Day 7 IVP blastocysts. Across treatments, TCM199 supplemented with 50% FBS supported the highest embryo recovery, and the response to HEPES showed a clear concentration-dependent pattern. Moderate buffering markedly improved embryo stability, with embryos stored in 25-mM HEPES exhibiting the highest post-storage survival and hatching rates (approximately 63% and 37%, respectively). In contrast, insufficient (0mM) or excessive supplementation (≥ 50mM) resulted in substantially reduced developmental performance, confirming that an intermediate buffering range provides the greatest protection during chilling. Storage duration further shaped embryo outcomes. Embryos maintained relatively high viability between 24-48h of hypothermic exposure (survival ~54-62%), whereas prolonged storage led to a sharp decline in developmental potential, with hatching becoming rare or absent beyond 72h. The total cell number also decreased markedly after extended storage, indicating progressive structural compromise. These findings identify an optimal HEPES range and a practical storage window that together enhance the success of hypothermic preservation of bovine IVP embryos. The study establishes biologically validated parameters that can support the development of reliable, low-cost protocols for embryo storage and transport in research and field applications.

Keywords

Bovine embryos, *in vitro* production (IVP), hypothermic storage, HEPES buffer

Introduction

Beef and dairy cattle production has been rapidly expanding toward industrialization in many countries worldwide. Advanced reproductive technologies such as artificial insemination (AI) and embryo transfer (ET) have been widely applied to improve reproductive efficiency and economic outcomes. However, conception rates following AI remain low during periods of heat stress in dairy herds, as elevated ambient temperatures and humidity negatively affect reproductive performance (Nishisozu *et al*., 2023; Baccouri *et al*., 2025). Elevated thermal stress also impairs oocyte quality, early embryonic development, and the uterine environment, contributing to early embryonic loss and lowered pregnancy outcomes (Al-Katanani *et al.*, 2002; Wolfenson & Roth, 2019; Miętkiewska *et al*., 2022).

The introduction of embryo transfer (ET) technology has been shown to improve pregnancy outcomes under heat stress conditions by bypassing the most heat-sensitive period of early development, with several studies reporting higher conception and pregnancy rates after ET compared with artificial insemination during periods of elevated ambient temperatures (Drost *et al.*, 1999; Hansen, 2019). With continuous advancements in embryo production and transfer technologies, the global number of bovine embryos produced and transferred has steadily increased each year. Notably, the use of *in vitro*-produced (IVP) embryos and cryopreserved embryos has become increasingly popular due to their high efficiency and broad applicability (IETS, 2024).

IVP has made it possible to produce large numbers of embryos, increasing the need for efficient storage and transport strategies. For embryo storage, cryopreservation in liquid nitrogen has become a standard practice in assisted reproduction. However, pregnancy rates following the transfer of frozen–thawed animal embryos remain variable and are often lower than those achieved with fresh embryo transfers, possibly due to cryoinjury induced by the freezing–thawing process (Hansen, 2020). Moreover, embryo transport in liquid nitrogen poses logistical and safety challenges. Therefore, developing low-temperature storage media that can maintain embryo viability and developmental potential during short-term preservation is of practical importance.

Low-temperature embryo storage was first explored in the 1940s (Chang, 1948). Phosphate-buffered saline (PBS) containing 10% (v/v) fetal bovine serum (FBS) could maintain chilled bovine embryos for 1-3 days, but survival rates were below 50% (Lindner & Ellis, 1985). Ideta *et al.* (2013) successfully preserved in vivo-derived bovine embryos at 4°C in TCM199 supplemented with 50% FBS for up to 72 hours, achieving approximately 90% viability. However, limited studies have examined low-temperature storage of *in vitro*-produced bovine embryos.

HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid) is a widely used buffering agent in cell-culture and embryo-handling media for maintaining physiological pH stability. Due to its CO_2-independent buffering capacity, HEPES is particularly useful during embryo handling procedures performed outside a controlled CO_2 incubator, where maintaining a stable pH is critical for embryo development (Will *et al*., 2011). It has been shown to improve cell viability and reduce oxidative stress under *in vitro* conditions (Hlas *et al*., 2024). However, inappropriate concentrations or environmental conditions may lead to adverse oxidative effects (Liu *et al*., 2023). Owing to its pH-stabilizing function and widespread use in embryo-handling media, HEPES may enhance the viability of embryos during low-temperature storage. Therefore, this study aimed to evaluate the effects of HEPES-supplemented storage medium on the viability and quality of *in vitro*-produced bovine embryos stored at 4°C, and to determine the optimal concentration and preservation duration under these conditions.

Materials and Methods

Materials

Bovine cumulus–oocyte complexes (COCs) and embryos were produced *in vitro* using dairy cow ovaries (Holstein-Friesian) collected from a local abattoir. Ovaries were transported to the laboratory within 2-3h in ovary-holding solution (PBS supplemented with 100 IU mL^{-1} penicillin and 0.1 mg mL^{-1} streptomycin) maintained at 30-35°C. All culture media and reagents were purchased from Sigma-Aldrich (USA). Cell culture dishes were obtained from Corning (USA), and 4-well plates were from Nunc (Thermo Fisher Scientific, USA).

Experimental Procedures

In vitro embryo production

Follicles 3-8mm in diameter were aspirated using a 5-mL syringe fitted with an 18G needle containing 0.5mL of collection medium (HEPES–TALP supplemented with 3% fetal bovine serum (FBS), 100 IU mL^{-1} penicillin, and 0.1 mg mL^{-1} streptomycin). Aspirated follicular fluid was transferred to 90-mm Petri dishes and allowed to settle at 37°C for 5-10min. COCs were recovered under a stereomicroscope and graded according to Goodhand *et al.* (2000); only grade A and B oocytes with a compact cumulus and homogeneous cytoplasm were used. Selected COCs were cultured for 20-22h in TCM199 (25 mM HEPES) supplemented with 2.5 µg mL^{-1} taurine, 0.02 IU mL^{-1} FSH, 5% FBS, 20 µg mL^{-1} EGF, and 50 µg mL^{-1} gentamicin. Maturation was carried out in 4-well plates at 38.5°C under 5% CO_2 and saturated humidity. Mature COCs were washed and transferred into fertilization medium containing: 90mM NaCl, 12mM KCl, 25mM $NaHCO_3$, 0.5mM NaH_2PO_4, 0.5mM $MgSO_4$, 10mM lactate, 10mM HEPES, 8mM $CaCl_2$, 2mM sodium pyruvate, 2mM caffeine, and 5 mg mL^{-1} BSA. Frozen–thawed spermatozoa from a single bull (Holstein-Friesian) were washed by centrifugation in TCM199 (pH 7.8). Approximately 10µL of thawed semen was diluted in 90µL fertilization medium containing 20 matured oocytes to achieve 5×10^5 sperm mL^{-1}. Gametes were co-incubated for 5h at 38.5°C under 5% CO_2, 5% O_2, and saturated humidity. Presumptive zygotes were denuded and transferred to TCM199 supplemented with 5% FBS, 50 µg/mL gentamicin, and 0.5 µg mL^{-1} insulin. Embryos were cultured at 38.5°C under 5% CO_2 and 5% O_2. Cleavage was assessed at 48h post-fertilization, and morulae and blastocysts were collected on day 7.

Hypothermic storage of in vitro-produced embryos at 4°C

(i) Effect of storage medium (PBS vs. TCM199)

Day 7 IVP bovine blastocysts were randomly allocated to PBS or TCM199 supplemented with 50% FBS and stored at 4°C for 24h. After storage, embryos were washed and cultured in CR1aa medium supplemented with 5% FBS for 48h. Embryo survival and hatching rates were evaluated at the end of the recovery culture period. This experiment was repeated three times, with 15–16 embryos per replicate per treatment group.

(ii) Effect of HEPES concentration

Day 7 IVP embryos (morula to blastocyst stages) were randomly assigned to TCM199 supplemented with 50% FBS and 0, 12.5, 25, or 50 mM HEPES, and stored at 4°C for 24h. Following storage, embryos were cultured in CR1aa + 5% FBS for 48h and evaluated for survival and hatching to determine the optimal HEPES concentration. This experiment was repeated three times, with 8-9 embryos per replicate per treatment group.

(iii) Effect of storage duration

Based on the optimal storage conditions identified above, Day 7 IVP blastocysts were stored in TCM199 supplemented with 50% FBS and 25mM HEPES at 4°C for 24, 48, 72, 96, or 120h. After storage, embryos were cultured in CR1aa medium supplemented with 5% FBS for 48h, and survival and hatching rates were assessed. This experiment was repeated three times, with 8-9 embryos per replicate per treatment group.

The viability and hatching rates of embryos were evaluated at the end of the recovery culture period. Embryos were considered non-viable (degenerated) when they showed severe morphological damage, including darkened cytoplasm, shrinkage, extensive fragmentation, or loss of cellular integrity. In contrast, embryos maintaining normal morphology and showing blastocoel re-expansion were classified as viable.

Blastocysts exhibiting a clear rupture of the zona pellucida caused by trophectodermal cell expansion were recorded as hatching blastocysts.

(iv) Hoechst 33342 staining

Embryos were stained to determine the total cell number. Hoechst stock solution was prepared at 250 µg mL^{-1} in ethanol; the working solution contained 50µL stock diluted in 450µL ethanol. Embryos were washed in PBS + 0.3% PVP and incubated in staining solution for 2-3h. Subsequently, embryos were sequentially rinsed in ethanol and glycerol, mounted individually on slides, and examined under a fluorescence microscope.

Statistical analyses

The rates of embryo survival (%) and hatching (%) after storage and the average cell number per blastocyst were used as the main outcome variables. Percentage data were subjected to arcsine square-root transformations to stabilize variance and analyzed by one-way ANOVA or two-way ANOVA (as applicable) using StatView (Abacus Concepts, Berkeley, CA, USA). When ANOVA showed significance, means were compared using Fisher's protected least significant difference (PLSD) test. Differences were considered significant at $P < 0.05$.

Results

Effect of storage media on the viability of in vitro-produced bovine embryos at 4°C

A total of 143 bovine ovaries were collected from the abattoir, yielding 705 grade A and B oocytes for *in vitro* embryo production. From these, 93 Day 7 *in vitro*-produced (IVP) blastocysts were obtained and used to evaluate the effects of TCM199 and PBS supplemented with 50% FBS on embryo survival (**Figure 1**) and hatching (**Figure 2**) after 24h of storage at 4°C. As shown in T**able 1**, embryos stored in TCM199 exhibited significantly higher survival and hatching rates than those stored in PBS (45.7% vs. 23.5% and 15.2% vs. 8.5%, respectively; $P < 0.05$), indicating that TCM199 provides a more suitable environment for short-term hypothermic storage of IVP bovine embryos.

Effect of HEPES concentration on the viability of IVP embryos stored at 4 °C

One hundred and three Day 7 IVP bovine blastocysts, derived from 760 grade A and B oocytes collected from 152 abattoir ovaries, were allocated to TCM199 supplemented with 50% FBS and varying concentrations of HEPES (0, 12.5, 25, or 50mM) to evaluate the effect of HEPES on embryo survival and hatching after 24h of storage at 4°C. As shown in **Table 2**, embryos stored in medium containing 25 mM HEPES exhibited significantly higher survival (63.0%) and hatching rates (37.0%) compared with the other groups ($P < 0.05$), indicating that 25mM is the optimal concentration for short-term hypothermic storage of IVP bovine embryos under these conditions.

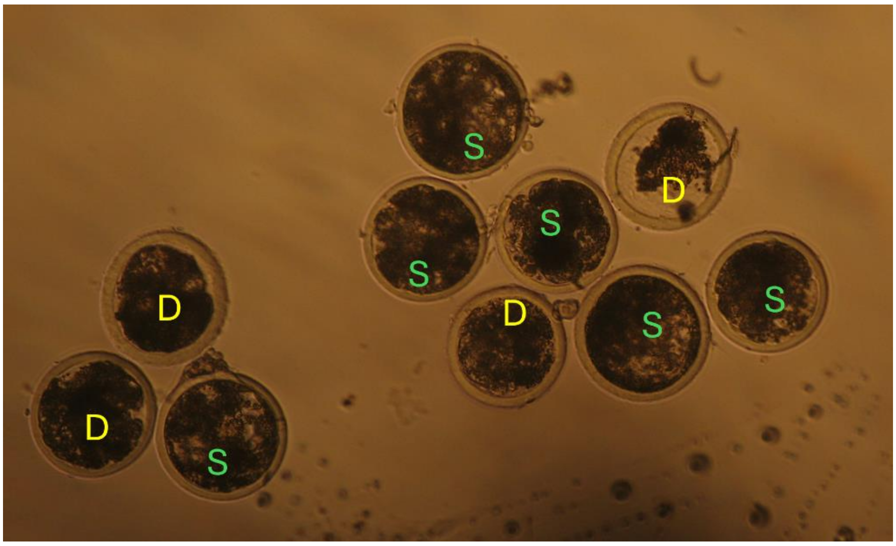
Effect of storage duration on the viability of IVP embryos stored at 4°C

From the 175 bovine ovaries collected at the abattoir, 805 grade A and B oocytes were retrieved and used to produce 132 Day 7 IVP blastocysts for evaluating the effect of storage duration on embryo survival and development at 4°C. The blastocysts were stored in TCM199 supplemented with 50% FBS and 25 mM HEPES for 24, 48, 72, 96, or 120h (**Table 3**).

Table 1. Effect of PBS and TCM199 as storage media on the survival of *in vitro*-produced bovine embryos at 4°C

Storage media	No. of embryos		
	Evaluated	Survival after 24h (%)	Hatching after 24h (%)
PBS	47	11 (23.5 ± 2.4)^{a}	4 (8.5 ± 1.8)^{a}
TCM199	46	21 (45.7 ± 1.0)^{b}	7 (15.2 ± 2.1)^{b}

Note: Data in parentheses are expressed as mean ± SE. *Values within the same column with different superscript letters differ significantly (P <0.05).*



Note: Embryos labeled S indicate surviving embryos showing normal morphology and blastocoel re-expansion after recovery culture, whereas embryos labeled D indicate degenerated embryos characterized by shrinkage, darkened cytoplasm, and loss of structural integrity.

Figure 1. Representative images of in vitro-produced bovine blastocysts at 24h after hypothermic storage at 4°C.

Figure 2. Representative *in vitro*-produced bovine blastocyst hatching after storage at 4°C

Table 2. Effect of HEPES concentration on the survival and hatching of *in vitro*-produced bovine embryos at 4°C

HEPES concentration (mM)	No. of embryos evaluated	Survival after 24h (%)	Hatching after 24h (%)
0	25	11 (44.0 ± 3.6)^{b}	4 (16.2 ± 4.4)^{b}
12.5	26	12 (45.8 ± 5.3)^{ab}	6 (23.1 ± 0.9)^{b}
25	27	17 (63.0 ± 1.4)^{a}	10 (37.0 ± 4.9)^{a}
50	25	12 (48.6 ± 8.5)^{ab}	5 (20.4 ± 4.6)^{b}

Note: Data in parentheses are presented as mean ± SE. Values within the same column with different superscript letters differ significantly (P <0.05).

Embryo survival and hatching rates declined progressively with increasing storage duration. The highest survival was observed at 24h (61.5%), followed by 48h (53.7%), with no significant difference between these two time points (*P* >0.05). Beyond 48h, both the survival and hatching rates decreased sharply, and embryos stored for ≥ 96h exhibited minimal or no hatching ability. Similarly, the total cell number per blastocyst (**Figure 3**) decreased significantly with prolonged storage. Embryos stored for 24-48h maintained an average of > 60 cells per blastocyst, whereas those stored for ≥ 72h contained fewer than 40 cells. These results indicate that IVP bovine blastocysts can be stored at 4°C for 24-48h without substantial loss of viability or developmental potential.

Discussion

This study provides the first systematic evaluation of low-temperature (4°C) storage conditions for *in vitro*-produced (IVP) bovine embryos under the conditions tested. As IVP embryo production continues to expand globally, practical methods for short-term storage are increasingly important, particularly in regions with limited access to liquid nitrogen or when embryos must be transported over moderate distances (Drost *et al.*, 1999). Short-term hypothermic storage provides a cost-effective

Table 3. Effect of storage duration on the survival, hatching, and total cell number of *in vitro*-produced bovine embryos at 4°C

Storage duration (h)	No. of embryos evaluated	Survival (%)	Hatching (%)	Total cell number per blastocyst
24	26	16 (61.5 ± 1.4)[a]	10 (38.5 ± 4.6)[a]	69 ± 0.9[a]
48	27	14 (53.7 ± 1.9)[a]	7 (26.8 ± 3.3)[a]	66 ± 2.1[a]
72	26	10 (38.4 ± 3.2)[b]	4 (15.3 ± 3.5)[b]	45 ± 1.3[b]
96	27	3 (11.1 ± 6.4)[c]	0	39 ± 1.6[b]
120	26	1 (3.8 ± 4.3)[c]	0	30[b]

Note: Data in parentheses are presented as mean ± SE. Values within the same column with different superscript letters differ significantly (P <0.05).

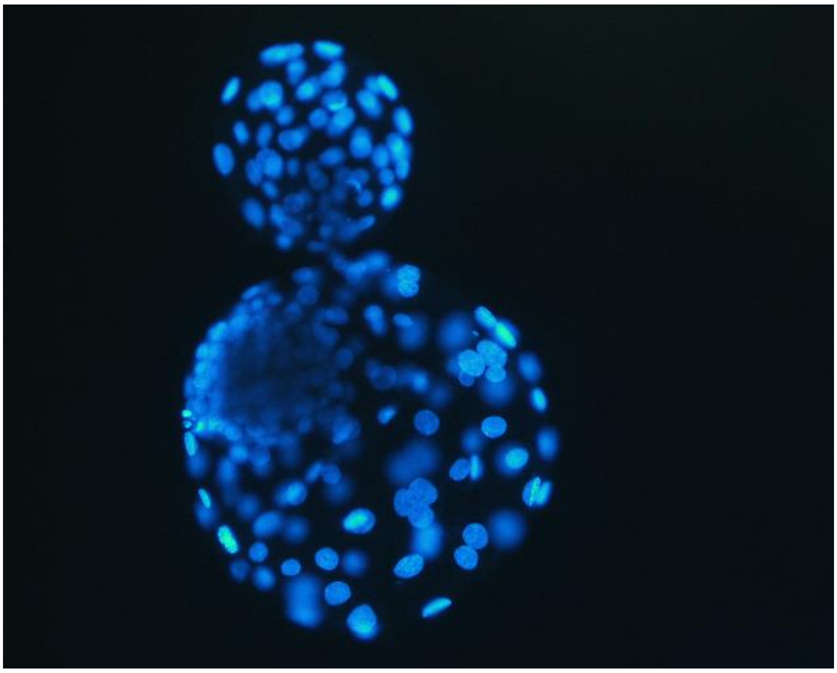


Figure 3. Representative *in vitro*-produced bovine blastocyst undergoing hatching after cold storage and stained with Hoechst 33342 to determine the average total cell number per blastocyst. Image was captured under a fluorescence microscope.

alternative to cryopreservation by reducing logistical complexity and avoiding cryoinjury associated with ice crystal formation and frozen–thaw stress (Hansen, 2020). Similar non-freezing low-temperature preservation approaches have been applied in various biological systems to maintain viability for hours to days (Robinson & McGann, 2024).

Effect of storage media

Our results demonstrated that TCM199 supplemented with 50% fetal bovine serum (FBS) significantly outperformed phosphate-buffered saline (PBS) in supporting IVP embryo survival and hatching at 4°C. Specifically, the survival and hatching rates in TCM199 were 45.7% and 15.2%, respectively, compared with 23.5% and 8.5% in PBS (P <0.05). These findings align with Ideta *et al.* (2013), who reported superior maintenance of in vivo-derived bovine embryos in TCM199 + 50% FBS compared with PBS or L15 medium under hypothermic conditions. PBS, while commonly used for routine embryo handling, lacks sufficient buffering capacity and essential nutrients, making it inadequate for maintaining IVP embryo viability under cold stress (Chang, 1948). In contrast, TCM199, initially developed for avian fibroblast culture, contains amino acids, vitamins, and bicarbonate buffering that preserve membrane integrity and mitigate cold-induced stress (Morgan *et al.*, 1950; Hafez, 1971; Costa *et al.*, 2005). Supplementation with serum further stabilizes cell membranes and osmotic balance, thereby reducing chilling injury. These results emphasize that choosing an appropriate storage medium is critical, especially for IVP embryos, which are generally more sensitive than their in vivo-derived counterparts.

Effect of HEPES supplementation

Adding 25mM HEPES to TCM199 + 50% FBS significantly improved survival (63.0%) and hatching (37.0%) after 24 hours at 4°C. HEPES maintains extracellular pH independently of CO_2, which is particularly important under hypothermic conditions where CO_2 solubility increases and metabolic CO_2 production decreases (Ideta *et al.*, 2013). Because chilled embryo storage is performed outside a controlled CO_2 incubator, CO_2-dependent bicarbonate buffering may be unstable, and pH drift can occur during handling and storage. In this context, HEPES may provide more stable CO_2-independent buffering, thereby maintaining a more physiological extracellular pH environment (Will *et al.*, 2011). Maintaining pH stability is essential because small deviations in extracellular pH can disrupt intracellular pH regulation, enzyme activity, and mitochondrial metabolism, ultimately affecting embryo viability and developmental competence. The stabilization of pH protects embryos from intracellular acidosis and oxidative stress. However, excessive HEPES or exposure to visible light in the presence of riboflavin can generate reactive oxygen species (ROS), requiring careful optimization (Liu *et al.*, 2023). Thus, the reduced performance observed in the 50mM HEPES group may reflect a concentration-dependent negative effect, potentially related to increased oxidative stress or altered cellular homeostasis. The discrepancy between our findings and Ideta *et al.* (2013), who observed no difference in survival among in vivo embryos stored with different HEPES concentrations, likely reflects differences in embryo origin. IVP embryos derived from slaughterhouse oocytes generally have lower developmental competence and higher intracellular lipid content, making them more susceptible to chilling injury (Sudano *et al.*, 2011; Janati *et al.*, 2021).

HEPES has also been reported to support oocyte maturation (Downs & Mastropolo, 1997), fertilization (Hagen *et al.*, 1991), and embryo development (Ozawa *et al.*, 2006). In pigs, HEPES preserves sperm membrane integrity during cold storage (Will *et al.*, 2011), suggesting a conserved protective effect on cellular membranes under hypothermic conditions. Ideta *et al.* (2013) confirmed that HEPES supplementation in cold storage media improves the plasma membrane integrity and survival in bovine in vivo-derived blastocysts. Our findings indicate that 25mM HEPES is optimal for supporting survival and hatching of IVP bovine embryos at 4°C, consistent with previous recommendations (Ideta *et al.*, 2013).

In addition to buffering capacity, HEPES may influence embryo responses to cold stress by indirectly supporting membrane stability and osmotic balance. During chilling, reduced membrane fluidity and altered ion transport may increase susceptibility to cellular swelling, membrane leakage, and mitochondrial dysfunction. Stable extracellular pH and buffering conditions may help maintain ionic equilibrium and reduce membrane destabilization during storage. Although these mechanisms were not directly evaluated in the present study, they may partly explain the improved survival and hatching rates observed at the optimal HEPES concentration.

Effect of storage duration

Embryo survival and hatching progressively declined with prolonged storage. IVP bovine blastocysts retained acceptable viability at 24 and 48 hours at 4°C (61.5% and 53.7% for survival and 38.5% and 26.8% for hatching, respectively), while the total cell numbers per blastocyst decreased from over 60 cells at 24 and 48 hours to below 40 cells after 72 hours. These results indicate that 24-48 hours is the optimal window for IVP embryo storage under these conditions. In contrast, Ideta *et al.* (2013; 2015) reported successful storage of in vivo-derived embryos at 4°C for 7-10 days. The lower tolerance of IVP embryos likely reflects two major factors: the higher intracellular lipid content, which alters membrane fluidity and compromises mitochondrial function (Sudano *et al.*, 2011; Janati *et al.*, 2021), and the reduced developmental competence of oocytes obtained from slaughterhouse ovaries (BonDurant, 1982; Ideta *et al.*, 2013). These intrinsic differences explain the shorter safe storage window for IVP embryos.

Furthermore, prolonged hypothermic exposure may increase oxidative damage due to impaired mitochondrial activity and reduced antioxidant defense, resulting in progressive cellular degeneration and reduced blastocyst cell numbers. This is consistent with the marked decline in survival and hatching observed after 72h, suggesting that chilling stress accumulates over time and exceeds the recovery capacity of IVP embryos. Embryo cell number is a key indicator of blastocyst quality and is closely associated with hatching and implantation potential. In vivo-derived bovine blastocysts generally exhibit higher total cell numbers and more stable developmental competence than IVP embryos (Thompson, 1997; Ushijima *et al*., 2008), which may partly explain their greater tolerance to prolonged hypothermic storage. Therefore, the significant reduction in cell number observed after 72h in our study likely contributed to the reduced hatching ability of IVP embryos.

Taken together, our findings suggest that supplementation with 25mM HEPES may support short-term hypothermic storage by improving CO_2-independent pH stability, whereas prolonged storage or excessive HEPES concentration may exacerbate stress-related damage. Further studies evaluating ROS levels and membrane integrity are required to clarify these mechanisms and optimize the storage conditions for IVP embryos.

Conclusions

Short-term hypothermic storage at 4°C demonstrated clear potential for preserving the viability and post-storage developmental capacity of IVP bovine blastocysts for up to 48h. These results support the feasibility of using optimized HEPES-supplemented media as a practical, low-cost alternative to cryopreservation for short-distance transport. Further studies, including embryo transfer trials and refinement of storage formulations, are needed to confirm developmental competence and strengthen the applicability of this approach in both research and commercial contexts.

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