Original Research

Animal Reproduction Update
Year 2025, Volume-5, Issue-2 (Jul-Dec)

Vitrification of Bovine Embryos: Can it Replace Slow Freezing?

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ARTICLE INFO

Keywords: Bovine embryo cryopreservation, high-security vitrification (HSV) straws, slow freezing, vitrification

doi:10.48165/aru.2025.5.2.4

ABSTRACT

The ability to effectively cryopreserve embryos plays a critical role in assisted reproductive technologies (ART) by facilitating the global exchange of valuable genetics and minimizing disease transmission risks. However, a key challenge lies in ensuring the post-thaw viability of cryopreserved embryos, particularly in vitro-produced (IVP) embryos, which exhibit lower cryotolerance than their in vivo counterparts. This study assessed the suitability of vitrification as an alternative to the slow freezing method for cryopreservation of bovine IVP embryos. The research compared the effectiveness of vitrification using high-security vitrification (HSV) straws, a closed system, with two different slow-freezing protocols (0.3°C/min and 0.5°C/min). Blastocyst and expanded blastocyst stage embryos produced through ovum pick-up (OPU) and in vitro embryo production were subjected to each cryopreservation method and then thawed and cultured to assess their viability, re-expansion, and hatching rates. The vitrified group demonstrated significantly higher hatching rates (47.7%) than both slow freezing groups (10.6% for 0.5°C/min and 26.4% for 0.3°C/min). While vitrification resulted in higher viability and expansion rates than the 0.5°C/min slow freezing group, it was higher in the 0.3°C/min group in both parameters. Hatching from the zona pellucida is a prerequisite for successful implantation, and the hatching rate was higher in the vitrified embryos, indicating a potential advantage in terms of implantation readiness. These findings suggest that vitrification may offer higher pregnancy rates as indicated by higher hatching rates. The results may be further validated through embryo transfer studies to assess its impact on actual pregnancy success rates.

Introduction

The field of bovine genetics has undergone significant advancements with the development of in vitro embryo production (IVEP), substantially enhancing genetic selection and herd improvement efforts (Gordon, 2003; Sirard et al., 2006; Hansen, 2006; Lonergan et al., 2016). IVEP enables breeders to accelerate genetic gains by producing numerous embryos from genetically superior animals. However, fully exploiting the potential of IVEP depends heavily on efficient and reliable methods for embryo storage and transportation.

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Received: 04.10.2025 Accepted: 02.11.2025

Cryopreservation plays a critical role by facilitating the global exchange of valuable genetics and minimizing disease transmission risks associated with importing live animals (Bielanski and Vajta, 2009). It allows for the long-term storage and cross-border transport of embryos, thereby supporting the strategic use of superior genetic resources (Bielanski and Vajta, 2009).

The ability to cryopreserve embryos offers breeders major advantages, including the creation of biobanks of high-genetic-value embryos, greater flexibility in reproductive scheduling, and a safeguard against loss of elite breeding stock (Pereira and Marques, 2008). Traditionally, slow freezing has been the dominant method for bovine embryo cryopreservation, particularly with the use of ethylene glycol for direct transfer (Pereira and Marques, 2008). While this method provides acceptable pregnancy outcomes, it presents notable limitations-especially for IVP embryos, which display reduced cryotolerance compared to in vivo-derived embryos due to factors such as lipid content and cellular fragility (Seidel, 2006).

To address these issues, vitrification has emerged as a promising alternative. Unlike slow freezing, vitrification rapidly solidifies embryos into a glass-like state, avoiding ice crystal formation and minimizing cryoinjury (Vajta and Nagy, 2006). This technique has shown potential for improving post-thaw survival rates of IVP embryos and is increasingly utilized across species (Bernal-Ulloa et al., 2016). Recent studies have continued to evaluate and optimize vitrification protocols. For instance, Jung et al. (2024) demonstrated enhanced survival outcomes in bovine embryos by refining slow freezing techniques with sucrose pre-equilibration, highlighting the competitive performance of improved slow freezing methods. Despite its promise, vitrification poses practical challenges, especially under field conditions. The technique often requires advanced equipment and operator skill, and its performance varies based on whether an open or closed system is used. Open systems offer superior cooling rates but carry a higher risk of contamination through liquid nitrogen, whereas closed systems, though safer, may reduce cooling efficiency (Yu et al., 2010; Vajta et al., 2015; Caamaño et al., 2015). To mitigate this trade-off, the High Security Vitrification (HSV) straw has been developed. This device combines the biosecurity of closed systems with improved thermal conductivity, making it a preferred choice in both human IVF and animal breeding (Vajta et al., 2015). HSV straws are increasingly being adopted in field settings, including in developing countries, due to their enhanced safety profile and convenience.

Given these developments, this study aimed to assess the efficacy of vitrification using HSV straws by comparing post-thaw viability, re-expansion, and hatching rates of vitrified bovine IVP embryos to those cryopreserved using two conventional slow freezing protocols (0.3°C/min and 0.5°C/min). This comparison is timely and necessary to determine whether vitrification, under practical conditions, can replace slow freezing as the preferred method for embryo cryopreservation in bovine reproductive biotechnology.

Materials and methods

Ethical statement

The results presented in this paper are part of the ongoing commercial bovine embryo production activity conducted at the National Dairy Development Board (NDDB) facility. Considering that OPU in bovine species is a regular, well-standardized, and commercialized process, the activity does not fall under the purview of experimental animal procedures requiring separate ethical approval. Hence, no additional ethical clearance was necessary for this work.

Ovum pick-up and in vitro embryo production (OPU-IVEP)

The research objective was to assess the effectiveness of vitrification compared to the slow freezing of bovine embryos produced through OPU-IVEP. All media, except the slow-freezing media, were procured from IVF Bioscience, UK. The slow freezing media were obtained from IMV Technologies, France.

The OPU-IVEP procedure was performed following already standardized processes in the laboratory (Bharti et al., 2022; Doultani et al., 2022; Patil et al., 2022; Layek et al., 2023). Briefly, donor cows underwent ultrasound-guided transvaginal aspiration under epidural anaesthesia to retrieve oocytes. Ovaries were assessed for the number of follicles and corpus luteum in non-stimulated donors, and follicles measuring 3 mm or larger were aspirated into a 50 ml collection tube containing pre-warmed OPU media. The collection tubes were immediately sealed and transported to the laboratory for oocyte retrieval. A total of 3085 cumulus-oocyte complexes (COCs) were retrieved from 14 donor cows, with an average of 14.7 OPUs per donor (around 12–18 OPUs per donor), with each session yielding an average of 15 ± 3 COCs. This cumulative yield provided a sufficient number of embryos to meet the statistical power requirements and to assign embryos across three cryopreservation protocols.

COCs were retrieved from the aspirated solution and selected based on quality criteria established by the International Embryo Technology Society (IETS, 2022). Oocytes graded 1, 2, and 3 were washed in in vitro maturation (IVM) media before being transferred to pre-equilibrated IVM media drops (100 µl drops under oil overlay). These oocytes were incubated at 5% CO_2 in air at 38.5°C and >90% relative humidity for approximately 22–24 hours in a CO_2 incubator with O_2 control (Forma™ Series 3 Water Jacketed CO_2 Incubator, Thermo Fisher Scientific).

Post-maturation, oocytes were washed with pre-equilibrated in vitro fertilization (IVF) media and transferred to pre-equilibrated IVF media drops (50 µl drops under oil overlay). Frozen semen was prepared using density gradient centrifugation. Initially, frozen semen doses were centrifuged at 2000 × g for 5 minutes using Isolate® gradient (Fujifilm Irvine Scientific, USA), followed by resuspension in pre-equilibrated IVF media and a second centrifugation at 500 × g for 5 minutes. The final concentration of spermatozoa kept in each IVF drop was approximately 2 million motile spermatozoa per mL. The oocytes were incubated with the spermatozoa for 18 hours at 5% CO_2 in air at 38.5°C and >90% relative humidity in a CO_2 incubator with O_2 control (Forma™ Series 3 Water Jacketed CO_2 Incubator, Thermo Fisher Scientific).

Presumptive zygotes were denuded by removing cumulus cells with a denuding pipette, then washed sequentially in wash media and in vitro culture (IVC) media. Thereafter, they were transferred to pre-equilibrated IVC media drops (100 µl drops under oil overlay) and incubated at 5% CO_2, 5% O_2, 90% N_2, 38.5°C, and >90% RH for seven days post-IVF in a mixed gas benchtop incubator (MINC Benchtop Incubator, COOK Medical, Ireland). Cleavage rates were recorded 72 hours post-IVF, and blastocyst rates were determined on Day 7 post-fertilization. The oocytes were in vitro matured, fertilized, and cultured, donor-wise. Embryos that reached the blastocyst and expanded blastocyst stages and were graded as Code 1 according to IETS guidelines (IETS, 2022) were selected for cryopreservation. A subset of embryos was utilized for fresh embryo transfer, while the remaining were cryopreserved.

Cryopreservation, thawing, and in vitro culture of embryos

Viable embryos produced through OPU-IVEP in donor cows were randomly subjected to vitrification or slow freezing on day seven post-fertilization. The embryos produced in one OPU-IVEP cycle were assigned to one method of cryopreservation. Embryos at the blastocyst and expanded blastocyst stages, classified as Code 1 according to IETS guidelines (IETS, 2022), were chosen for cryopreservation. Each group consisted of embryos subjected to different cryopreservation methods, i.e., vitrification in HSVD straws (n=132 embryos), slow freezing with a post-seeding cooling rate of 0.5°C/min (n=501 embryos), and slow freezing with a post-seeding cooling rate of 0.3°C/min (n=91 embryos). Thus, a total of 724 embryos were cryopreserved and included in the study. The number of embryos considered in the group with a post-seeding cooling rate of 0.5°C/min is relatively higher than the other two groups, as we have included the data before starting the vitrification experiment when we were using only slow freezing with a post-seeding cooling rate of 0.5°C/min. After starting the experiment, the embryos were assigned randomly to each cryopreservation method.

Slow freezing was performed using a programmable freezer (Crysalys® PTC-9500, Biogenics Inc., USA) according to a standardized protocol for the cryopreservation of IVEP embryos with Ethylene Glycol medium (Sommerfeld and Niemann, 1999; Leibo and Songsasen, 2002; Jung et al., 2024). For slow freezing, embryos were loaded into 0.25 ml straws containing slow freezing media. The straws were equilibrated in the freezing medium for 10 minutes while they were getting loaded in the 0.25 ml straws. Straws were then placed in the cryochamber of the programmable freezer, which was pre-cooled at -6°C, and manual seeding was performed after 2 minutes of loading the straws in the freezer. The straws were kept at -6°C for 8 min post-seeding, and the cooling rate was then controlled at either 0.5°C/min or 0.3°C/min until reaching -32°C.

Thawing of slow-frozen embryos was performed using a thawing unit (IMV Technologies, France) at 31°C. The embryos were air thawed for 10 secs and immersed in water for 30 sec in the thawing unit. The embryos were then washed in wash media and transferred into pre-equilibrated IVC media drops individually (20 µl drops under oil overlay). They were then incubated in the mixed gas benchtop incubator (at 5% CO_2, 5% O_2, 90% N_2, 38.5°C, >90% RH) for further observation. Embryos were observed at 0 h, 2 h, 24 h, and 48 h post-thawing.

For the vitrification group, embryos were vitrified using the High Security Vitrification (HSVD) straws in BO-VitriCool™ kit media, comprised of three media (IVF Biosciences, UK), in accordance with the manufacturer's guidelines. The vitrification process involved three steps, i.e., embryos were placed in pre-incubation media at room temperature for two minutes, followed by immersion in Cooling 1 media for another two minutes, and finally, the embryos were transferred to Cooling 2 media for 30 seconds and loaded in the HSVD straw. After that, the HSVD straw was sealed using a heat-sealing machine and immediately plunged

into liquid nitrogen (-196°C). Vitrified embryos are stored in liquid nitrogen containers until further analysis.

Warming of vitrified embryos was performed using the Vitriwarm™ kit media, comprised of four media (IVF Biosciences, UK), in accordance with the manufacturer's guidelines. The warming procedure was carried out at 37.5°C to prevent ice crystal formation while warming, which is critical for maintaining embryo viability. Briefly, the cover of the HSVD straw was cut under liquid nitrogen, and the straw was plunged into pre-warmed Warming 1 media, where it was kept for three minutes. Subsequently the embryos were transferred in Warming 2 media (for two minutes), Warming 3 media (for two minutes), and Warming 4 media (for one minute). The embryos were then washed in wash media and transferred into pre-equilibrated IVC media drops (20 µl drops under oil overlay). They were then incubated in the mixed gas benchtop incubator (at 5% CO_2, 5% O_2, 90% N_2, 38.5°C, >90% RH) for further observation. Embryos were observed at 0 h, 2 h, 24 h, and 48 h post-warming, similar to the slow-frozen embryos.

Statistical analysis

Power calculation was performed, and it suggested approximately 64 embryos per group to detect a medium effect size with 80% power at a 0.05 significance level. Average percentages of viable, re-expanded, and hatched embryos were calculated for different cryopreservation groups. The average percentages were compared between groups using Fisher's exact test with a 3x3 contingency table. Pairwise comparisons were made between the groups, with significant differences considered when $p<0.05$. The analysis was performed using RStudio Version 2024.04.1+748.

Results

The present study was conducted to standardize the vitrification procedure of bovine IVP embryos using the High Security Vitrification Device (HSVD) straw. Further, the survival rate of vitrified embryos was compared with slow-frozen embryos frozen at two different rates of post-seeding cooling (0.3°C/min and 0.5°C/min). Upon thawing, embryos were in vitro cultured in 20 µl IVC media drops. The embryos were then observed at 0 h, 2 h, 24 h, and 48 h to assess viability, re-expansion, and hatching. All media utilized in this study were sourced from IVF Biosciences, UK. Oocyte retrieval was performed via ovum pick-up (OPU) from 14 donor cows, yielding an average of 15 ± 3 cumulus-oocyte complexes (COCs) per OPU session. The collected COCs were matured in vitro for 22–24 hours in IVM media. Following IVM, an average of 80 ± 5% of the oocytes matured successfully. After IVF, cleavage was assessed at 48 hours post-insemination, with an average cleavage rate of 75 ± 5%. The embryos were subsequently cultured to the blastocyst stage, with an average blastocyst formation rate of 35 ± 3%.

Post-thaw observations of cryopreserved embryos

This study investigated the impact of various cryopreservation methodologies on post-thaw embryo viability, expansion, and hatching rates. The experiment compared three distinct methods: vitrification and slow freezing at two controlled cooling rates (0.5°C/min and 0.3°C/min).

Embryo viability

The vitrification method achieved a post-thaw viability rate of 78.0 ± 0.44%, significantly higher ($p=0.0001$) than the 0.5°C/min slow freezing protocol (60.3 ± 0.20%). However, the 0.3°C/min slow freezing protocol exhibited a higher viability rate of 82.4 ± 0.58% (Fig. 1 and Table 1), though this difference was not statistically significant ($p=0.4982$).

Embryo Expansion

Vitrification exhibited a significantly higher expansion rate (59.8±0.44%) compared to the 0.5°C/min slow freezing protocol (40.1±0.20%) ($p=0.00007$). In accordance with the viability, the expansion rate was slightly higher in the 0.3°C/min slow freezing protocol (62.6±0.58%) compared to vitrification, though the difference is not statistically significant ($p=0.78$). The data indicates that a slower cooling rate during slow freezing might be beneficial for post-thaw survivability and expansion and warrants further investigation (Fig. 2).

Embryo Hatching

Embryo hatching rate (47.7 ± 0.44%) was significantly higher ($p<0.0001$ and $p=0.0013$, respectively, for 0.5°C/min and 0.3°C/min) in vitrified embryos compared to

both the slow freezing groups. Slow freezing at the rate of 0.3°C/min (26.4 ± 0.58%) exhibited a higher hatching rate than 0.5°C/min (10.6 ± 0.20%), but this difference was significant (p=0.0001) (Fig. 1, Table 1).

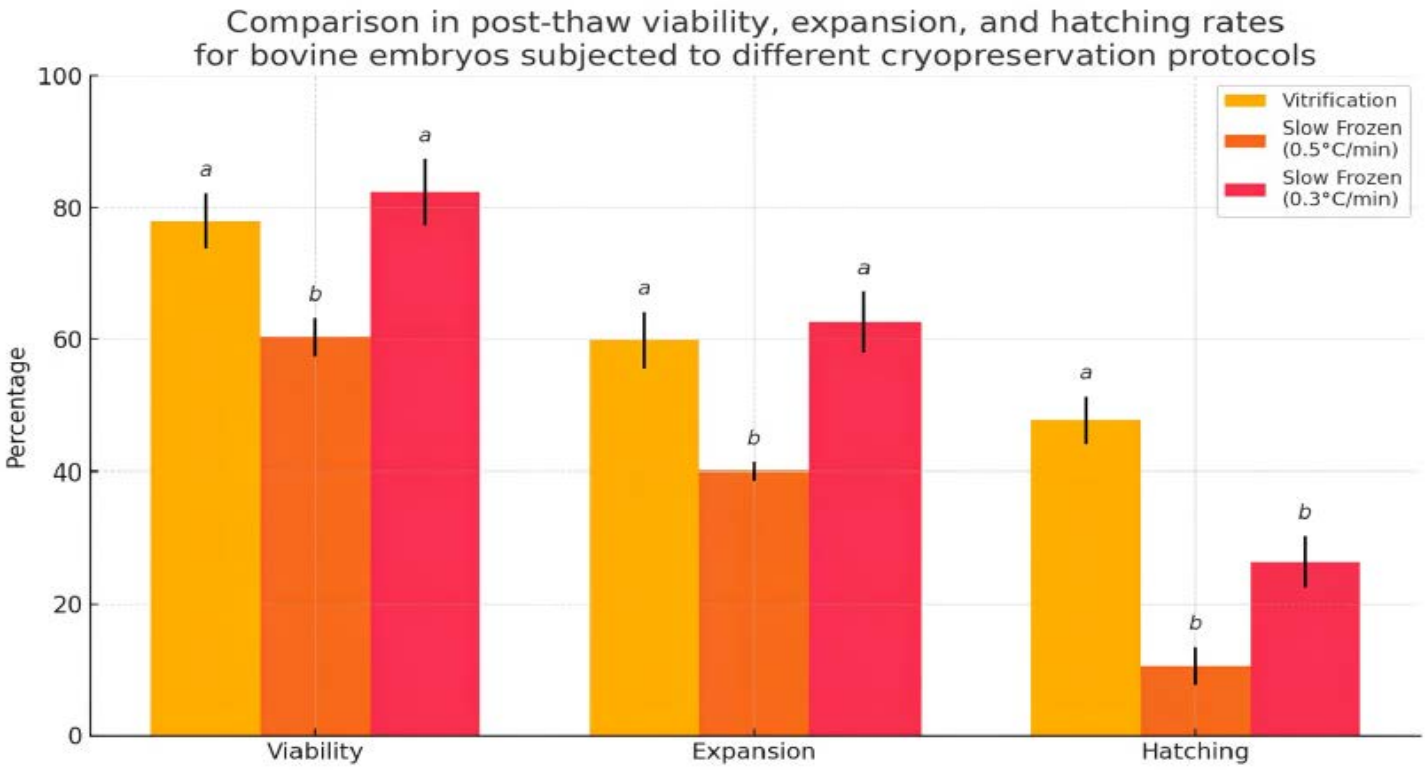

Fig 1. Comparison of post-thaw viability, expansion, and hatching rates for bovine embryos subjected to different cryopreservation protocols. Data are presented as Mean ± SEM. Different superscripts (a, b, c) within parameters indicate statistically significant differences between groups ($p < 0.05$, ANOVA with Tukey's test).

Bovine embryos were cryopreserved using vitrification, slow freezing with a cooling rate of 0.5°C/min, or slow freezing with a cooling rate of 0.3°C/min. Following thawing, embryos were assessed for viability, re-expansion, and hatching rates. Post-thaw comparison of viability, re-expansion, and hatching rates of bovine embryos following vitrification and two slow-freezing protocols (0.5°C/min and 0.3°C/min).

Table 1. Comparison of post-thaw viability, re-expansion, and hatching rates between different cryopreservation methods for bovine embryos

Cryopreservation Method	**Viability Rate** (% ± SEM)	**Expansion Rate** (% ± SEM)	**Hatching Rate** (% ± SEM)
Vitrification (n=132)	78.0 ± 0.44a	59.8 ± 0.44a	47.7 ± 0.44a
Slow Frozen (0.5°C/min) (n=501)	60.3 ± 0.20b	40.1 ± 0.20b	10.6 ± 0.20b
Slow Frozen (0.3°C/min) (n=91)	82.4 ± 0.58a	62.6 ± 0.58a	26.4 ± 0.58b

Within a column means with different superscript letters differ significantly ($p<0.05$).
Viability, expansion, and hatching rate of bovine embryos subjected to different cryopreservation methods *i.e.* vitrification and two different slow freezing protocols (with post-seeding cooling rate of 0.5°C/ min and 0.3°C/ min) were compared.

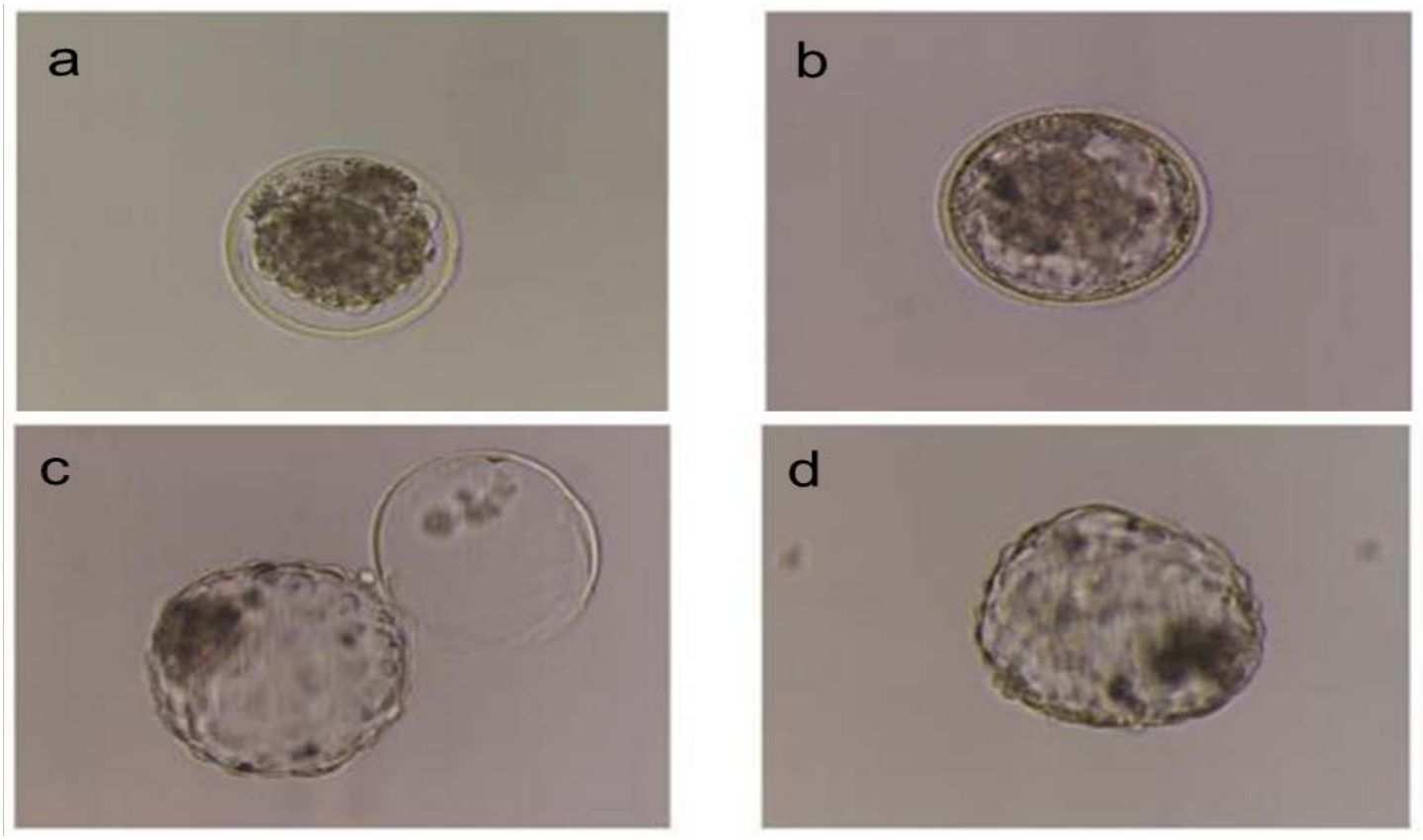

Fig 2. Post-thaw re-expansion and hatching of a vitrified embryo (a) Bovine expanded blastocyst observed under an inverted microscope (100x magnification) immediately after warming showed shrunken inner cell mass and blastocoel (b) Re-expanded embryo after 2 h of in vitro culture, which is rehydrated and viable with more than 50% blastocoel cavity (c) after 24 h of in vitro culture embryo showing hatching (d) after 48 h of in vitro culture fully hatched and viable embryo.

Discussion

With the concern over the spread of exotic diseases through germplasm and issues of global pandemics, the industry is slowly inclining towards closed carrier systems (Bielanski et al., 2000). The present study evaluated the efficacy of High Security Vitrification Device (HSVD) straws for cryopreserving bovine embryos. The HSVD straws have been developed to improve post-thaw survival rates while minimizing the risk of disease transmission and storing them in liquid nitrogen. By enhancing thermal conductivity and allowing direct thawing, HSVD straws mitigate the issues of a slow cooling rate.

However, the closed carrier systems are not preferred due to slow cooling rate and lower post-thaw survivability (Vajta and Nagy, 2006; Morató et al., 2008; Vajta et al., 2015; Caamaño et al., 2015). However, in the present study using HSVD straws, the results obtained are at par with other open carriers of vitrification (Steponkus et al., 1990; Vajta et al., 1999; Hamawaki et al., 1999; Lane et al., 1999; Vanderzwalmen et al., 2002; Chian et al., 2009; Almodin et al., 2010; Matsunari et al., 2012; Villamil et al., 2012; Gutnisky et al., 2013). The superiority of the HSVD straw over another closed carrier may be attributable to the material used in the straw, which allows higher heat transfer, and the embryos are directly thawed in vitrification media like open vitrification systems.

The results obtained are also in accordance with previous experiences with HSVD devices in other species (Abdel Hafez et al., 2011; Lopes et al., 2015; Reed et al., 2002; Bartolac et al., 2015; Stoop et al., 2014; Bernal-Ulloa et al., 2016). Further survival rates obtained in vitrification of bovine embryos with other vitrification carriers are either lower or comparable to the present study (Ishimori et al., 1993; Vajta et al., 1997; Vajta et al., 1999; Matsumoto et al., 2001; Mtango et al., 2003; Huang et al., 2005; Yu et al., 2010; Kim et al., 2012; Gutnisky et al., 2013; Sirisha et al., 2013; Caamaño et al., 2015; Bernal-Ulloa et al., 2016).

While our study supports the efficacy of HSVD straws, the observed outcomes may also be influenced by differences in breed of donors, embryo quality, donor variability,

and laboratory conditions. Lower post-thaw viability, re-expansion, and hatching rate obtained by us in slow freezing compared to reports of previous studies (Sommerfeld and Niemann, 1999; Jung et al., 2024) may be attributed to differences in breed and culture conditions. We are observing similar results across India with IVEP embryos (mostly working on similar breeds and producing embryos using the same commercial media); thus, the majority of the laboratories are shifting towards vitrification.

Dehydration of embryos takes place with the increase in concentration of CPA. Upon thawing and culturing or transfer of embryos in the reproductive tract of animals, water enters into the blastocoelic cavity via tight junctions, either diffusing passively or being pumped actively (Vajta and Nagy, 2006). As a result of diffusion of permeable cryoprotectants, while cooling, embryos shrink, and warming embryos re-expand as they are placed in culture. Cryopreserved-warmed blastocysts undergo several morphological changes and collapse during cryopreservation. The size of the blastocoelic cavity is the primary factor in examining the expansion rate. Within 24 hours, the rehydration of the blastocoel is expected. The rehydration degree of the blastocoel is the most important morphological predictor for transfer and live birth rates. Multiple factors influence the dehydration and rehydration rates of embryos, such as composition and the permeability characteristics of the cell membrane, surface-to-volume ratio of the cell, cooling rate, difference in osmotic pressure between the intracellular and extracellular environment, etc. (Mazur, 1984; Rall and Fahy, 1985; Matsumoto et al., 2001; Liebermann, 2012; Fuller and Paynter, 2004; Kasai and Mukaida, 2004; Stachecki and Cohen, 2004; Vajta and Nagy, 2006; Yavin and Arav, 2007; Saragusty and Arav, 2011; Arav, 2014). The present study observes a higher expansion rate in the vitrified embryos compared to the slow-frozen ones with a post-seeding cooling rate of 0.5°C/min, which signifies the suitability of HSVD vitrification for maintaining the viability and integrity of embryos.

In a normal IVEP cycle, embryos hatch on day eight post-fertilization and, in a few cases, on day 7. After the thawing procedure, embryos are likely to hatch within 24 to 48 h. The hatching rate can be determined by the zona pellucida (ZP) breakage, which leads the embryo to come out of the ZP. Hatching in culture conditions indirectly indicates implantation rates after transfer to a vitrified/frozen culture. Thus, in the present study, the higher hatching rate obtained in the vitrified group indicates a higher implantation potential of the embryos vitrified using HSVD straws.

Conclusion

The findings of this study underscore the efficacy of vitrification using High-Security Vitrification (HSVD) straws as a reliable cryopreservation method for bovine in vitro produced (IVP) embryos. Our results demonstrate significant improvements in rehydration and hatching rates for vitrified embryos compared to those cryopreserved using slow freezing with a post-seeding cooling rate of 0.5°C/min. In addition, the use of closed carrier systems like HSVD straws offers the added benefit of minimizing the risk of disease transmission during cryo-storage.

Importantly, embryos cryopreserved using a slower cooling rate of 0.3°C/min via the slow freezing method showed comparable outcomes to vitrification in terms of viability and re-expansion, though hatching rates were still lower. This indicates that optimized slow freezing protocols can serve as a viable alternative to vitrification under certain conditions.

Hatching from the zona pellucida is a critical prerequisite for embryo implantation in the uterus, and thus the elevated hatching rate in the vitrified embryos suggests an enhanced potential for implantation readiness. Furthermore, vitrification not only improved hatching outcomes but also resulted in higher viability and expansion rates when compared with the 0.5°C/min slow freezing group. Although the 0.3°C/min group showed better viability and expansion than the 0.5°C/min group, vitrification still outperformed both in hatching rate. However, to confirm these advantages, further validation through embryo transfer studies and subsequent pregnancy assessments is recommended, which would provide more direct evidence of the implementational efficacy of vitrification in bovine embryo transfer. Further, HSVD straws provide additional biosecurity through avoidance of contact between the embryos while stored in liquid nitrogen.

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