RESEARCH ARTICLE

Evaluation of Different Vitrification Methods and Cryoprotectants on Viability of *In Vitro* Matured Buffalo Oocytes

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Abstract

The developmental competence of buffalo oocytes is greatly influenced by the vitrification process and the cryoprotectants used during cryopreservation. The present study was undertaken to evaluate the effect of different cryoprotectants on the developmental potential of vitrified buffalo oocytes in terms of cleavage, and blastocyst formation. A total of 201 (18.3±0.8/session) buffalo ovaries were collected from the slaughterhouse in 11 sessions, and 447 (40.6±3.5/session) cumulus-oocyte complexes (COCs) were recovered from 663 aspirated follicles. Following *in vitro* maturation (IVM), 397 (36.1±3.5/session; 87.83%) oocytes attained maturity and were allocated to four experimental groups: Group I (fresh, non-vitrified control; n=198), Group II (Vitrogen vitrification; n=64), Group III (DMSO + EG vitrification; n=74), and Group IV (DMSO + EG + Sucrose vitrification; n=61). After warming and *in vitro* fertilisation (IVF), a total of 318 presumptive zygotes were cultured *in vitro* (IVC). Fresh oocytes exhibited superior developmental competence compared with vitrified oocytes. The cleavage rate was highest in the fresh control group, with 130 out of 198 presumptive zygotes (11.8±1.8; 63.23%) undergoing cleavage, whereas vitrified groups (Groups II, III, and IV) showed markedly lower cleavage rates of 11.61% (0.7±0.3), 11.97% (0.6±0.2), and 11.61% (0.5±0.2), respectively. Similarly, blastocyst formation was highest (n=15 BLs) in the fresh control group (8.09%; 1.4±0.3), in comparison to Groups II, III, and IV, 2.44% (0.2±0.1), 0.91% (0.09±0.8), and 1.52% (0.1±0.08), respectively. Although minor variations were observed among the results of different vitrification protocols, all vitrified groups demonstrated substantially reduced embryonic development compared with fresh oocytes.

Key words: Blastocyst, Buffalo, Cleavage rate, Cryoprotectants, In vitro fertlization, Oocyte vitrification.

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Introduction

The success of *in vitro* embryo production (IVEP) largely depends on the availability of high-quality, developmentally competent oocytes. However, buffalo ovaries contain comparatively fewer primordial follicles and exhibit a high incidence of follicular atresia, resulting in a limited pool of viable oocytes available for embryo production. Furthermore, cryopreservation of oocytes and embryos is a supplementary technique for *in vivo* and *in vitro* embryo production. In the last decade, the technique has been improved, but did not achieve a satisfactory commercial level (Kumar *et al*., 2022). These constraints adversely affect the success of conventional breeding programs and slow down the rate of genetic improvement (Suthar and Dhami, 2010). Although assisted reproductive technologies (ART), including IVEP and cryopreservation, have been adopted to overcome these limitations and accelerate genetic gain (Misra, 1993; Suthar and Shah, 2009).

Oocyte cryopreservation has emerged as a crucial tool in modern reproductive biotechnology. The ability to preserve oocytes for extended periods provides greater flexibility in the application of ART by allowing long-term storage of female genetic resources, facilitating the establishment of germplasm banks, supporting breeding programs, and enabling the production of embryos without the immediate availability of donor animals. The cryopreservation of mammalian embryos has become an integral part of methods to control animal reproduction. It is of practical importance for the development of reproductive technologies such as nuclear transfer, cryobanking of animal species and the routine or commercial application of breed improvement, such as embryo transfer (Mahmoud

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and El-Sokary, 2013). Several cryopreservation methods, such as conventional (slow), equilibrium rapid freezing (vitrification), and ultra rapid freezing, have been used to preserve embryos and oocytes of many animal species, resulting in the birth of live offspring.

Vitrification, which is a relatively recent approach in buffalo oocyte freezing, is defined as a physical process by which a highly concentrated solution of cryoprotectants solidifies during cooling, without formation of ice crystals (Niemann, 1991). According to Parnpai *et al.* (2016), vitrification is the solidification of a solution brought about not by crystallization but by extreme elevation in viscosity during cooling. Although successful oocyte vitrification has been achieved in several animal species, subsequent progress is still limited, especially in buffalo (Mahmoud and El-Sokary, 2013; Patel *et al*., 2023). Many compounds act as cryoprotectants and are used for the protection of cells against freezing. There are two types of cryoprotectants: (a) membrane-permeating, which can freely diffuse through the membrane, such as glycerol (G), ethylene glycol (EG) and dimethyl sulfoxide (DMSO); (b) non-membrane-permeating, which cannot permeate the cell membrane, such as sugars (Pedro *et al*., 2005). The primary problem with cooling or cryopreservation of oocytes is the low percentage of oocytes retaining the ability to undergo normal maturation and fertilization. This may be attributed to the exposure to the cryoprotectant and the cooling process (Le Gal, 1996). To improve the effectiveness of vitrification of buffalo oocytes, the present study evaluated the comparative effect of cryoprotectants (glycerol, dimethyl sulfoxide and sucrose) on the developmental capacity of vitrified-thawed mature buffalo oocytes.

Materials and Methods

During the May 2024 to August 2024, a total of 201 buffalo ovaries were collected over 11 sessions and transported to the GBRC, Gandhinagar Lab from the abattoir managed by Ahmedabad Municipal Corporation, Ahmedabad, in an isothermal container (34-37°C) containing pre-warmed normal saline with 1% antibiotic (Strepto-penicillin, Amphotericin-B) and processed within 4 h for oocyte retrieval. Institutional Animal Ethics Committee approval was not required, as the study utilized slaughterhouse-derived buffalo ovaries, and no live animal experimentation was involved.

Cumulus-oocyte complexes (COCs) were recovered by aspirating follicular fluid from ovarian follicles using an 18G needle attached to a 10 mL syringe containing 2 mL pre-warmed ovum pick-up (OPU) media (IMV, France). The aspirated fluid was transferred into a 90 mm Petri dish, and COCs were searched under a stereo zoom microscope (50x magnification). COCs were graded into four categories (Grade I-IV) according to the criteria described by Das *et al*. (1996) based on the number and compactness of cumulus cell layers and the homogeneity of ooplasm. Grade I, II and, Grade III as they are suitable for IVEP, were transferred into pre-equilibrated *in vitro* maturation (IVM) media (Vitrogen, Brazil). Incubation was carried out in a benchtop incubator at 38.5°C for 18-22 h, and maturation was assessed following criteria of Das *et al*. (1996).

After maturation, COCs of I to III grades were randomly allotted to four experimental groups. Group I consisted of non-vitrified (fresh) COCs, while groups II, III, and IV were subjected to vitrification using three different cryoprotectant treatments. The COCs were washed 3 times with pre-warmed washing media. Expanded cumulus mass was partially removed from the COCs using a 10 µL micropipette fixed with the microtip.

Vitrification of Matured COCs for Group II

The vitrification of COCs using the commercially available vitrification media (Vitrification Kit, Vitrogen, Brazil) was performed. The COCs were vitrified using the Vitrification Kit (Vitrogen, Brazil) at room temperature (approximately 25°C), following the manufacturer's guidelines. After exposure to vitrification media, the COCs were immediately loaded onto the vitrification device (VitriFit, ref. 42802001A, Cooper Surgical). The excess media was carefully removed using a micropipette (10 µL), leaving approximately 0.3 µL of media on the device tip. The devices were then rapidly submerged in liquid nitrogen and sealed with a pre-cooled lid. Finally, the vitrification devices were transferred into labelled pencil goblets and stored in a liquid nitrogen tank until the devitrification process.

Vitrification of Matured COCs for Group III and IV

Following partial denudation, mature COCs of Group III and IV were randomly selected for vitrification using a solution containing 20% DMSO and 20% EG, and 20% DMSO, 20% EG, and 0.5 M sucrose, respectively, in a holding medium (DPBS supplemented with 0.4% BSA). Manipulations were performed on a heated stage set to 39 °C within a 25 °C room temperature. For vitrification, groups of 2-4 COCs were initially kept in a solution of respective holding medium for 30 seconds. They were then transferred in a 1-2 µL droplet into a 20 µL droplet of above respective holding medium for an additional 25 seconds. The COCs were loaded onto the vitrification device by briefly touching 1-2 µL droplet to the narrow end of the device, allowing capillary loading. Excess media was removed with a 10 µL micropipette, and then immediately immersed in liquid nitrogen, following the protocol outlined by Vajta *et al*. (1998) for group III and by Attanasio *et al*. (2010) for group IV.

Devitrification of Vitrified Group II COCs

The vitrified oocytes were devitrified using the de-vitrification media (D1, D2, and D3; Vitrogen, Brazil) as per the manufacturer's instructions. Once the preparations of devitrification media were completed, the vitrification device containing the COCs was opened in liquid nitrogen (LN_2) and immediately transferred into media as per the manufacturer's guidelines. The devitrified oocytes were then incubated for 2 h in pre-equilibrated IVF media drops.

Devitrification of Vitrified Group III and IV COCs

Warming of vitrified matured COCs of Group III was performed according to the protocol described by Vajta *et al*. (1998) with slight modifications. Briefly, the tip of the vitrification straw containing the COCs was immediately immersed in 1.2 mL of holding medium supplemented with 0.25 M sucrose and maintained at 37°C for 1 min. Subsequently, the COCs were transferred to 1.2 mL of holding medium containing 0.15 M sucrose for 5 min to facilitate the gradual removal of cryoprotectants and osmotic re-equilibration. For Group IV COCs, warming was performed by immersing the end of the straw containing the COCs in 1.25 M Sucrose for 1 min, then 0.62 M, 0.42 M, and 0.31 M for 30 secs each (Attanasio *et al*., 2010).

Morphological Evaluation of Devitrified COCs

The thawed COCs were examined under an inverted microscope (Olympus, UCMAD3, Japan) to assess their post-thaw morphology. The criteria applied for the morphological evaluation of all three groups, as described by Dhali *et al*. (2000) were: (1) Normal COCs exhibiting a symmetrical spherical shape without signs of lysis, vacuolization, swelling, zona damage, degeneration, or cytoplasmic leakage, (2) COCs displaying a cracked zona pellucida, (3) COCs with leaked cytoplasmic content, (4) COCs with shrunken cytoplasm and, (5) COCs showing vacuolization in the cytoplasm.

Following completion of vitrification-warming procedures, the devitrified COCs from the three cryoprotectant treatment groups (Group II, III, and IV), along with the non-vitrified COCs (Group I), were processed for IVF. Briefly, COCs of each group were transferred into pre-equilibrated *in vitro* fertilization (IVF) media (Vitrogen, Brazil). Frozen semen straws were thawed at 37°C for 30 sec, transferred to 600 µL pre-equilibrated semen preparation media (BO-Semen-prep, Bioscience, UK), and centrifuged at 330 g for 5 min. The supernatant was discarded, and the process was repeated. The final suspension (100 µL) was gently mixed and incubated in a CO_2 incubator for 5 min. Sperm concentration was adjusted to 2×10^6 sperm/mL using a Neubauer chamber and added to IVF media containing oocytes of each group. The IVF drops were incubated in a benchtop incubator at 38.5°C for 18-22 h. Presumptive zygotes were washed, denuded using a 10 µL micropipette, and cultured for 6 days in the multi-gas benchtop incubator (Planner, BT37 system, Cooper Surgical Pvt Ltd, USA). Cleavage of each group (Group I–IV) was assessed at 72 h post-IVF, and blastocyst formation of each group was evaluated on Day 6 under a stereo zoom microscope (50x magnification).

Statistical Analysis

All observations were recorded in an Excel sheet (MS Office 2016) and analyzed using SPSS 26.0 software (IBM SPSS software Bengaluru, India). The descriptive analysis was performed using a descriptive function in the analysis drop-down menu of SPSS software. The mean difference among cryo-preservative group-wise maturation, and developmental competence of oocytes was assessed using the ANOVA. Pairwise multiple comparisons were evaluated using post hoc Fisher's Least Significant Difference (LSD) as a post hoc test.

Results and Discussion

The present study was carried out to evaluate the effect of different cryoprotectants on the cleavage and blastocyst rate of vitrified matured COCs.

Collection and Maturation of Oocytes

A total of 201 (18.3±0.8/session, range 16-24) ovaries were collected from 11 sessions for this study, and 447 (40.6±3.5/session, range 25-63) COCs were retrieved from 663 (60.3±4.8/session, range 30-88) aspirated ovarian follicles. The average oocytes retrieved per ovary was 2.27±0.18 (range 1.7-3.3). Following retrieval, 397 (36.1±3.5, 87.83%) COCs attained maturity. Subsequently, these matured COCs were randomly divided into distinct experimental groups: Group I comprised 198 (18±2.0) non-vitrified or fresh oocytes, while Groups II, III, and IV comprised vitrification consisting of 64 (5.8±0.7), 74 (6.7±1.0), and 61 (5.5±0.7) COCs, using Vitrogen, EG+DMSO, and EG+DMSO+Sucrose protocols, respectively (Table 1; Fig. 1).

Cleavage of Vitrified Oocytes

After devitrification (Table 2) and IVF, a total of 318 presumptive zygotes were kept in IVC. From that, groups I, II, III, and IV included 198 (18±2.0), 40 (3.6±0.8), 40 (3.6±0.7), and 40 (3.6±0.5) presumptive zygotes, respectively (Table 3; Fig. 2). Out of 318 zygotes, 130 (11.8±1.8; 63.23%), 8 (0.7±0.3; 11.61%), 7 (0.6±0.2, 11.97%), and 6 (0.5±0.2, 11.61%) were cleaved in Groups I, II, III, and IV, respectively. A statistically significant difference was observed in cleavage among the four groups. The significantly higher cleavage was noted in Group I (11.8±1.8) than in the other groups (p=0.0001). No

Table 1: Session wise *in vitro* matured COCs and distribution of COCs into different groups

Sessions	COCs for IVM	Matured COCs (%)	Group I (Non-Vitrified)	Group II (Vitrogen)	Group III (E+D)	Group IV (E+D+S)
1	46	43(93.48)	20	5	7	11
2	40	36(90.00)	14	7	9	6
3	52	44(84.62)	26	6	8	4
4	47	44(93.62)	24	10	4	6
5	34	32(94.12)	9	8	9	6
6	25	20(80.00)	10	4	3	3
7	52	48(92.31)	24	6	12	6
8	30	24(80.00)	15	5	2	2
9	30	23(76.67)	12	3	4	4
10	28	25(89.29)	14	2	4	5
11	63	58(92.06)	30	8	12	8
Total	**447**	**397(87.83)**	**198**	**64**	**74**	**61**
Mean ±SE	**40.6±3.5**	**36.1±3.5**	**18±2.0**	**5.8±0.7**	**6.7±1.0**	**5.5±0.7**

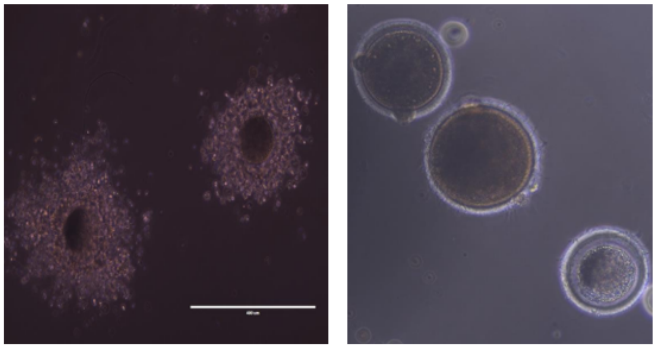


Fig. 1: Comparative pictures of mature cumulus oocyte complexes before (a) and after (b) vitrification recovered from the slaughter origin buffalo ovaries

statistically significant difference was observed between Groups II, III, and IV (p>0.05). In comparison to our results, Vajta *et al*. (1998), Checura and Seidel (2007), and Morato *et al*. (2008) using OPS; Prentice-Biensch *et al*. (2012), Yashiro *et al*. (2015), and Punyawai *et al*. (2015) using Cryotop; Dutta *et al*. (2013) using French straw, and Arav *et al*. (2018) using Sarah® found higher cleavage rate than the present study using different concentrations of CPA. While Chaves *et al*. (2017) using OPS and Patel *et al.* (2026) using VitriFit as a vitrification carrier found similar results to the present study.

Blastocyst Production of Vitrified Oocytes

A total of 318 presumptive zygotes were kept in IVC. Out of which, 15 (1.4±0.3; 8.09%), 2 (0.2±0.1, 2.44%), 1 (0.09±0.08; 0.91%), and 1 (0.1±0.08; 1.52%) blastocysts were formed in Groups I, II, III, and IV, respectively. The cleavage and blastocyst rates differed distinctly between sessions and groups (Table 3; Fig. 3). The statistical analysis revealed a highly significant difference across all four groups. A statistically higher number of BLs was recorded in Group I (1.4±0.3) than in other groups (p=0.0001). No statistically significant difference was observed among groups II, III, and IV (p>0.05). In comparison to our results, Gasparrini *et al*. (2007), Attanasio *et al*. (2010), and Liang *et al.* (2011) found higher blastocyst rates after vitrification of oocytes. While Morato *et al*. (2008), Gautam *et al*. (2008) and Chaves *et al*. (2017) used OPS and Patel *et al.* (2026) using VitriFit found a similar blastocyst rate to the present study. The developmental competence of cumulus-oocyte complexes (COCs) was negatively impacted by cryopreservation processes, resulting in significantly lower maturation and blastocyst rates compared to controls, which was in accordance with Liang *et al*. (2012) and Patel *et al*. (2024, 2026).

Conclusion

Vitrification reduces both cleavage and blastocyst rates of mature oocytes, indicating an adverse effect of cryopreservation on oocyte developmental competence. No significant differences were observed among the cryoprotectant treatments, suggesting that the vitrification procedure had a greater influence on embryonic development than the type of cryoprotectant used.

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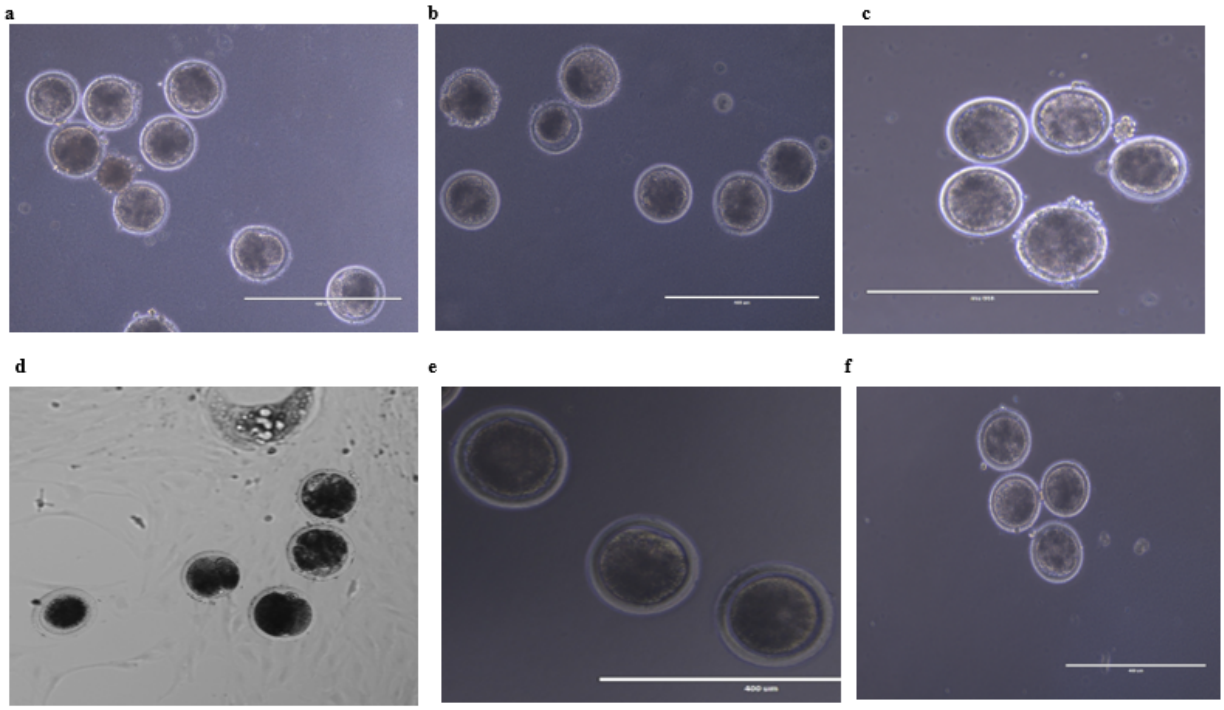


Fig. 2: Representative pictures of cleavage stages (2-32 cells) achieved at 72 hrs of *in vitro* culture of non-vitrified (Group I; a, b, c) and after vitrification-warming procedure (Group II-IV; d, e, f)

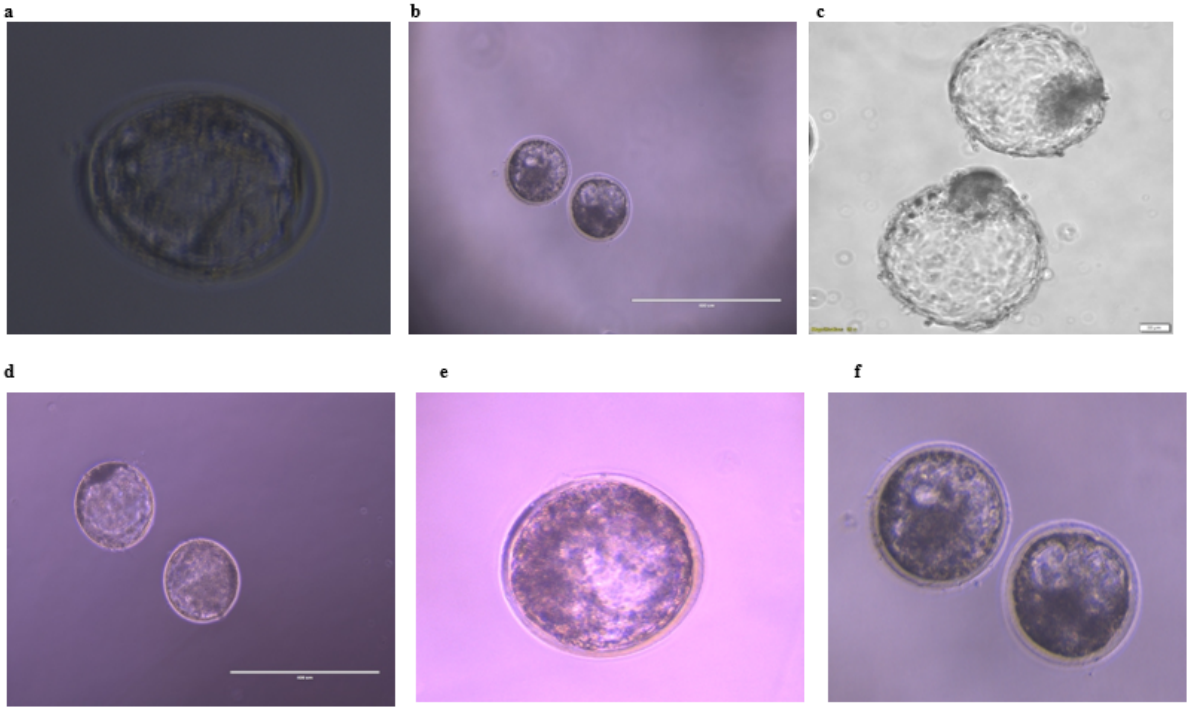


Fig. 3: Representative pictures of different buffalo blastocyst stages on day 6 of *in vitro* culture of fresh (Group I; a,b,c) and vitrified COCs (Group II-IV; d, e, f)

Table 2: Devitrification of Matured COCs of Different Groups (I-IV)

Session	Matured COCs	Non-Vitrified	Devitrification		
		Group I (Non-Vitrified)	Group II (Vitrogen)	Group III (E+D)	Group IV (E+D+S)
1	43 (93.48)	0	3	1	4
2	36 (90.00)	0	0	5	0
3	44 (84.62)	0	2	6	3
4	44 (93.62)	0	8	3	3
5	32 (94.12)	0	6	4	6
6	20 (80.00)	0	4	3	2
7	48 (92.31)	0	5	0	5
8	24 (80.00)	0	2	1	2
9	23 (76.67)	0	3	4	4
10	25 (89.29)	0	0	3	5
11	58 (92.06)	0	7	10	6
Total	**397 (87.83)**	**0**	**40**	**40**	**40**
Mean ±SE	**36.1±3.5**	0	**3.6±0.8**	**3.6±0.7**	**3.6±0.5**

Group I: Non-vitrified; Group II: Vitrogen; Group III: 20% DMSO + 20% EG; Group IV: 20% DMSO +20% EG +0.5M Sucrose

Table 3: Session- and group- (I to IV) wise presumptive zygotes observed after 72 h of *in vitro* culture and blastocysts produced on day 6 of *in vitro* culture

Session	Presumptive Zygotes for IVC in Groups				No. of ≥4 cell Embryos (%) in Groups				No. of Blastocysts on 6th day (%) in Groups			
	I	II	III	IV	I	II	III	IV	I	II	III	IV
1	20	3	1	4	15 (75.00)	0	0	0	0	0	0	0
2	14	0	5	0	6 (42.86)	0	0	0	0	0	0	0
3	26	2	6	3	19 (73.08)	0	2 (33.33)	0	1 (3.85)	0	0	0
4	24	8	3	3	18 (75.00)	3 (37.50)	1 (33.33)	1 (33.33)	2 (8.33)	1 (12.50)	0	0
5	9	6	4	6	6 (66.67)	1 (16.67)	1 (25.00)	1 (16.67)	2 (22.22)	0	0	0
6	10	4	3	2	6 (60.00)	1 (25.00)	0	0	1 (10.00)	0	0	0
7	24	5	0	5	15 (62.50)	1 (20.00)	0	0	3 (12.50)	0	0	0
8	15	2	1	2	6 (40.00)	0	0	0	1 (6.67)	0	0	0
9	12	3	4	4	8 (66.67)	0	0	1 (25.00)	1 (8.33)	0	0	0
10	14	0	3	5	8 (57.14)	0	0	1 (20.00)	1 (7.14)	0	0	0
11	30	7	10	6	23 (76.67)	2 (28.57)	2 (20.00)	2 (33.33)	3 (10.00)	1 (14.29)	1 (10.00)	1 (16.67)
Total	**198**	**40**	**40**	**40**	**130 (63.23)**	**8 (11.61)**	**7 (11.97)**	**6 (11.61)**	**15 (8.09)**	**2 (2.44)**	**1 (0.91)**	**1 (1.52)**
Mean ±SE	**18±2**	**3.6 ±0.8**	**3.6 ±0.7**	**3.6 ±0.5**	**11.8 ±1.8^{a}**	**0.7 ±0.3^{b}**	**0.6 ±0.2^{c}**	**0.5 ±0.2^{d}**	**1.4 ±0.3^{a}**	**0.2 ±0.1^{b}**	**0.09 ±0.08^{c}**	**0.1 ±0.08^{d}**

Group I: Non-vitrified; Group II: Vitrogen; Group III: 20% DMSO + 20% EG; Group IV: 20% DMSO +20% EG +0.5M Sucrose. Values with superscripts a, b, c and d within the raw differed highly significantly at 1% level.

References

Arav, A., Natan, Y., Kalo, D., Komsky-Elbaz, A., Roth, Z., Levi-Setti, P. E., Leong, M., & Patrizio, P. (2018). A new, simple, automatic vitrification device: Preliminary results with murine and bovine oocytes and embryos. *Journal of Assisted Reproduction and Genetics, 35*(7), 1161–1168.

Attanasio, L., Boccia, L., Vajta, G., Kuwayama, M., Campanile, G., Zicarelli, L., & Gasparrini, B. (2010). Cryotop vitrification of buffalo (*Bubalus bubalis*) *in vitro* matured oocytes: Effects of cryoprotectant concentrations and warming procedures. *Reproduction in Domestic Animals, 45*(6), 997–1002.

Chaves, D. F., Corbin, E., Alminana, C., Locatelli, Y., Souza-Fabjan, J. M. G., Bhat, M. H., Campello, C. C., Moreno, J. F., & Mermillod, P. (2017). Vitrification of immature and *in vitro* matured bovine

cumulus-oocyte complexes: Effects on oocyte structure and embryo development. *Livestock Science, 199*, 50–56.

Checura, C. M., & Seidel, G. E., Jr. (2007). Effect of macromolecules in solutions for vitrification of mature bovine oocytes. *Theriogenology, 67*(5), 919–930.

Das, G. K., Jain, G. C., Solanki, V. S., & Tripathi, V. N. (1996). Efficacy of various collection methods for oocyte retrieval in buffalo. *Theriogenology, 46*(8), 1403–1411.

Dhali, A., Manik, R. S., Das, S. K., Singla, S. K., & Palta, P. (2000). Post-vitrification survival and *in vitro* maturation rate of buffalo (*Bubalus bubalis*) oocytes: Effect of ethylene glycol concentration and exposure time. *Animal Reproduction Science, 63*(3–4), 159–165.

Dutta, D. J., Dev, H., & Raj, H. (2013). *In vitro* blastocyst development of post-thaw vitrified bovine oocytes. *Veterinary World, 6*(10), 730–733.

Gasparrini, B., Attanasio, L., De Rosa, A., Monaco, E., Di Palo, R., & Campanile, G. (2007). Cryopreservation of *in vitro* matured buffalo (*Bubalus bubalis*) oocytes by minimum volumes vitrification methods. *Animal Reproduction Science, 98*(3–4), 335–342.

Gautam, S. K., Verma, V., Palta, P., Chauhan, M. S., & Manik, R. S. (2008). Effect of type of cryoprotectant on morphology and developmental competence of *in vitro*-matured buffalo (*Bubalus bubalis*) oocytes subjected to slow freezing or vitrification. *Reproduction, Fertility and Development, 20*(4), 490–496.

Kumar, S., Chaves, M. S., Silva, A. F. B., Vale, W. G., Negreiros, N. A. B., Arcce, I. M. L., & de Figueiredo Freitas, V. J. (2022). Factors affecting the cryopreservation of oocytes and embryos in buffalo (*Bubalus bubalis*): A review. *Research, Society and Development, 11*(4), 1–12.

Le Gal, F. (1996). *In vitro* maturation and fertilization of goat oocytes frozen at the germinal vesicle stage. *Theriogenology, 45*(6), 1177–1185.

Liang, Y., Rakwongrit, D., Phermthai, T., Somfai, T., Nagai, T., & Parnpai, R. (2012). Cryopreservation of immature buffalo oocytes: Effects of cytochalasin B pretreatment on the efficiency of cryotop and solid surface vitrification methods. *Animal Science Journal, 83*(9), 630–638.

Liang, Y. Y., Phermthai, T., Nagai, T., Somfai, T., & Parnpai, R. (2011). *In vitro* development of vitrified buffalo oocytes following parthenogenetic activation and intracytoplasmic sperm injection. *Theriogenology, 75*(9), 1652–1660.

Mahmoud, K., & El-Sokary, M. M. (2013). Improvement of the efficacy of buffalo oocytes vitrification. *Global Veterinarian, 11*(4), 420–431.

Misra, A. K. (1993). Superovulation and embryo transfer in buffaloes: Progress, problems and future prospects in India. *Buffalo Journal, 9*, 1–13.

Morato, R., Izquierdo, D., Paramio, M. T., & Mogas, T. (2008). Cryotops versus open-pulled straws (OPS) as carriers for the cryopreservation of bovine oocytes: Effects on spindle and chromosome configuration and embryo development. *Cryobiology, 57*(2), 137–141.

Niemann, H. (1991). Cryopreservation of ova and embryos from livestock: Current status and research needs. *Theriogenology, 35*(1), 109–124.

Parnpai, R., Liang, Y., Ketudat-Cairns, M., Somfai, T., & Nagai, T. (2016). Vitrification of buffalo oocytes and embryos. *Theriogenology, 86*(1), 214–220.

Patel, P. S., Suthar, V. S., Suthar, B. N., Patil, D. B., & Joshi, C. G. (2023). Efficiency evaluation of two media for *in vitro* maturation of buffalo oocytes. *Indian Journal of Veterinary Sciences and Biotechnology, 19*(3), 17–21.

Patel, R., Prajapati, J., Suthar, V. S., Mansuri, M. M., Suthar, B. N., Jhala, D., Samova, S., Joshi, M., Joshi, C. G., & Nakhashi, H. C. (2024). Assessing factors influencing *in vitro* maturation and success of vitrification in buffalo oocytes. *International Journal of Veterinary Sciences and Animal Husbandry, 9*(2), 615–621.

Patel, R., Prajapati, J., Suthar, V. S., Suthar, B. N., Jhala, D., Joshi, M., Joshi, C. G., & Nakhashi, H. C. (2026). Assessment of morphological integrity and *in vitro* developmental competence of vitrified buffalo oocytes. *International Journal of Veterinary Sciences and Animal Husbandry, 10*(2), 24–32.

Pedro, P. B., Yokoyama, E., Zhu, S. E., Yoshida, N., Valdez, D. M., Jr., Tanaka, M., & Kasai, M. (2005). Permeability of mouse oocytes and embryos at various developmental stages to five cryoprotectants. *Journal of Reproduction and Development, 51*(2), 235–246.

Prentice-Biensch, J. R., Singh, J., Mapletoft, R. J., & Anzar, M. (2012). Vitrification of immature bovine cumulus-oocyte complexes: Effects of cryoprotectants, the vitrification procedure and warming time on cleavage and embryo development. *Reproductive Biology and Endocrinology, 10*, 1–7.

Punyawai, K., Anakkul, N., Srirattana, K., Aikawa, Y., Sangsritavong, S., Nagai, T., & Parnpai, R. (2015). Comparison of cryotop and micro-volume air cooling methods for cryopreservation of bovine matured oocytes and blastocysts. *Journal of Reproduction and Development, 61*(5), 431–437.

Suthar, V. S., & Dhami, A. J. (2010). Estrus detection methods in buffalo. *Veterinary World, 3*(2), 94–96.

Suthar, V. S., & Shah, R. G. (2009). Bovine *in vitro* embryo production: An overview. *Veterinary World, 2*(12), 478–479.

Vajta, G., Holm, P., Kuwayama, M., Booth, P. J., Jacobsen, H., Greve, T., & Callesen, H. (1998). Open pulled straw (OPS) vitrification: A new way to reduce cryoinjuries of bovine ova and embryos. *Molecular Reproduction and Development: Incorporating Gamete Research, 51*(1), 53–58.

Yashiro, I., Tagiri, M., Ogawa, H., Tashima, K., Takashima, S., Hara, H., & Hochi, S. (2015). High revivability of vitrified-warmed bovine mature oocytes after recovery culture with alpha-tocopherol. *Doctoral dissertation*. Shinshu University, Matsumoto, Nagano, Japan.