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Assessment of morphological integrity and *in vitro* developmental competence of vitrified buffalo oocytes

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Abstract

In the present study, a total of 399 ovaries collected from slaughtered buffaloes were aspirated for the recovery of cumulus-oocyte complexes (COCs). The recovered COCs were subjected to *in vitro* maturation (IVM) to assess their survivability following vitrification and subsequent thawing (Devitrification). After maturation, oocytes were partially denuded and vitrified using the Vitrogen vitrification kit with different exposure times, followed by storage in liquid nitrogen (LN₂). Devitrification was performed using the Vitrogen devitrification kit according to the standard protocol. Morphological evaluation of devitrified COCs revealed a significantly higher proportion of normal COCs in the control group (87.37%) compared to vitrified Group I (70.79%) and Group II (65.66%). Correspondingly, the proportions of damaged COCs were 12.63%, 29.21%, and 34.34% in the control, Group I, and Group II, respectively. Among the damaged COCs, the percentages of zona crack, cytoplasmic leakage, shrunken ooplasm, presence of fewer than five vacuoles, and more than five cytoplasmic vacuoles were 0.00%, 0.00%, 50.00%, 16.67%, and 33.33% in the control group; 69.23%, 19.23%, 38.46%, 53.85%, and 11.54% in Group I; and 52.94%, 14.71%, 47.06%, 50.00%, and 20.59% in Group II, respectively. Statistical analysis indicated a significant difference ($p < 0.05$) among groups in the proportion of COCs exhibiting zona cracks, shrunken ooplasm, and fewer than five cytoplasmic vacuoles.

The cleavage rates were 27.40% (24/89), 22.92% (23/99), and 62.80% (292/465), while blastocyst formation rates were 3.37% (3/89), 2.02% (2/99), and 16.34% (76/465) in Group I, Group II, and the control group, respectively. Although cleavage and blastocyst rates did not differ significantly between Group I and Group II, both groups showed significantly lower rates ($p < 0.01$) compared to the control. Viability assessment using Hoechst 33342 staining revealed that out of 112 devitrified and 63 fresh oocytes examined under a fluorescence-equipped inverted microscope, 50.89% (57/112) and 96.83% (61/63) oocytes were viable, respectively. Overall, vitrification of buffalo COCs using the Vitrogen system significantly compromised post-thaw morphological integrity, viability, and developmental competence compared to fresh controls, with prolonged exposure offering no additional benefit.

Keywords: *In vitro* maturation, buffalo, vitrification, oocytes

Introduction

The Asian water buffalo (*Bubalus bubalis*) was domesticated approximately 5,000 years ago in the Indian subcontinent and continues to play a pivotal role in the agrarian economy. India is currently the highest milk-producing country in the world, contributing nearly 22% of global milk production. Within the Indian dairy sector, buffaloes account for more than 50% of total milk output. In the Asia-Pacific region, annual buffalo milk production exceeds 45 million tonnes, of which approximately 30 million tonnes are produced in India. Together, India and Pakistan contribute nearly 90% of the world's buffalo milk production (FAOSTAT, 2012, 2013) [16]. The average daily milk yield of indigenous buffaloes during 2018-2019 was reported to be 5.15 kg in Gujarat and 5.62 kg at the national level (BAHS, 2019) [2]. In addition to higher milk fat content compared to cattle, buffaloes are valued as a multipurpose livestock species for milk, meat, and draft power. Their adaptability to harsh environmental conditions and superior feed conversion efficiency contribute to reduced management and veterinary costs for farmers.

Despite these advantages, buffalo reproduction is constrained by several inherent limitations, including delayed puberty, silent or weak estrus expression, seasonal breeding behavior, low

conception rates, poor embryonic survival, prolonged inter-calving intervals, and a high incidence of postpartum anestrus (Barile, 2005) [3]. These reproductive inefficiencies significantly hamper genetic improvement and productivity enhancement in buffalo populations.

To overcome such limitations and to increase the number of offspring from genetically superior dams and sires, various Assisted Reproductive Technologies (ARTs) have been developed and refined. Artificial insemination (AI) remains the most widely adopted and successful ART; however, it primarily facilitates genetic improvement through sire selection alone. Embryo Transfer Technology (ETT), although highly effective in cattle, exhibits lower efficiency in buffaloes. This reduced efficiency is attributed to the smaller pool of recruitable follicles in buffalo ovaries; for example, Danell (1987) [8] reported approximately 12,000 primary follicles in buffaloes compared to 133,000 in cattle. Furthermore, superovulation using follicle-stimulating hormone (FSH) is less predictable and often yields inconsistent responses in buffaloes (Manik *et al.*, 2002) [29]. The availability of suitable recipient buffaloes for embryo transfer is also limited due to lower synchronization efficiency compared to cattle (Presicce, 2007) [32].

Cryopreservation has emerged as a critical component of ARTs, enabling long-term preservation of genetic material. The cryoprotective properties of glycerol were first discovered by Polge *et al.* (1949) [31], leading to the development of modern cryobiology. Cryopreservation allows the production of multiple offspring from valuable genetic resources even after decades of storage. Importantly, oocytes from subfertile, pregnant, or prepubertal animals can also be preserved, facilitating the establishment of oocyte banks and contributing to genetic conservation programs. However, compared to oocytes of mice and rabbits, bovine oocytes are particularly sensitive to low temperatures (Martino *et al.*, 1996) [30].

Oocyte and embryo cryopreservation was initially achieved through slow-freezing techniques; however, these have largely been replaced by vitrification, a rapid cooling method involving direct immersion into liquid nitrogen. Vitrification prevents ice crystal formation by solidifying highly concentrated cryoprotectant solutions into a glass-like state. Embryo vitrification was first reported by Rall and Fahy (1985) [34], and since then, vitrification has become a preferred method due to its simplicity, speed, and elimination of the need for expensive programmable freezing equipment.

In buffaloes, vitrification has shown superior outcomes compared to slow freezing, particularly for immature and *in vitro* matured oocytes. Gautam *et al.* (2008) [18] demonstrated that slow freezing is unsuitable for immature buffalo oocytes, while vitrification significantly improves post-thaw survival and developmental competence. Nevertheless, achieving consistently high success rates with oocyte vitrification remains challenging. The inherent sensitivity of oocytes to cryoinjury manifested as chromosomal abnormalities, microtubule depolymerization, spindle disorganization, and disruption of the actin cytoskeleton limits developmental potential following warming (Saunders and Parks, 1999) [37]. Moreover, species-specific differences in oocyte physiology, cryoprotectant type and concentration, and culture conditions contribute substantially to variability in survival and developmental outcomes (Dujčková *et al.*, 2021) [11]. Hence the objective

of this manuscript was to evaluate the effect of different vitrification exposure times using the Vitrogen system on post-thaw morphological integrity, viability, and *in vitro* developmental competence of *in vitro* matured buffalo cumulus-oocyte complexes.

Materials and Methods

Collection of ovaries and recovery of cumulus-oocyte complexes (COCs)

Buffalo ovaries were collected from slaughtered animals at a slaughterhouse managed by the Ahmedabad Municipal Corporation (AMC), Ahmedabad, Gujarat, India. Immediately after collection, ovaries were washed thoroughly in pre-warmed normal saline solution (NSS) supplemented with 1% (w/v) antibiotic solution containing penicillin (10,000 IU/ml), streptomycin (10,000 µg/ml), and amphotericin B (25 µg/ml) (Vassena *et al.*, 2003) [41]. The ovaries were transported to the laboratory in a thermos containing warm NSS maintained at 34-36 °C.

Upon arrival, ovaries were washed again with NSS and maintained at 36 °C until aspiration. Cumulus-oocyte complexes (COCs) were aspirated from visible follicles using a sterile 18-gauge needle attached to a 5 ml syringe containing pre-warmed ovum pick-up (OPU) fluid (IMV, France). The aspirated follicular fluid was examined under a stereo zoom microscope, and COCs were recovered and transferred into a 35 mm Petri dish containing 3 ml of pre-warmed OPU/wash medium.

Grading of COCs

Recovered COCs were morphologically evaluated and graded into four categories (Grade I-IV) based on cumulus cell investment, ooplasm uniformity, and nuclear integrity, as described by Das *et al.* (1996) [9] with slight modifications. Grade I, II, and III COCs were selected for further experimentation, whereas Grade IV COCs were discarded.

In vitro maturation (IVM) of COCs

Selected Grade I, II, and III COCs were washed three times in pre-equilibrated *in vitro* maturation (IVM) medium. Groups of up to 20 COCs were cultured in 90 µl IVM medium drops covered with mineral oil in 35 mm Petri dishes. COCs aspirated from ovaries with and without corpus luteum were cultured separately.

The COCs were incubated for 22 hours at 38.5 °C in a humidified benchtop incubator with a controlled gas atmosphere of 5% O₂, 5% CO₂, and 90% N₂. Maturation was assessed based on the degree of cumulus cell expansion.

Evaluation of *in vitro* maturation

The degree of cumulus cell expansion (CCE) was evaluated after 22 hours of IVM and graded according to Kobayashi *et al.* (1994) [24]. COCs showing partial or complete cumulus expansion (Degree 1 and Degree 2; Table 2) were considered mature and selected for subsequent vitrification or use as fresh controls.

Partial denudation of matured COCs

Matured COCs selected for vitrification were washed three times in pre-warmed washing medium. Partial removal of expanded cumulus cells was performed using a denuding pipette attached to a denuding handle, leaving a thin layer of cumulus cells around the oocyte.

Vitrification of matured COCs

Vitrification was carried out using a commercially available vitrification kit (Vitrogen®, Brazil) at room temperature (~25 °C) following the manufacturer's instructions.

Matured COCs were randomly assigned to two vitrification exposure groups:

- **Group I:** Exposure to equilibration solution (V1) for 4 minutes
- **Group II:** Exposure to equilibration solution (V1) for 6 minutes

After equilibration, COCs from both groups were transferred to vitrification solution (V2) for 40 seconds and then loaded onto VitriFit vitrification devices (Cooper Surgical, USA). Excess medium was removed, leaving approximately 0.3 µl surrounding the COCs. The devices were immediately plunged into liquid nitrogen and stored until devitrification.

Devitrification of vitrified COCs

Devitrification was performed using warming solutions (D1, D2, and D3; Vitrogen®, Brazil) as per the manufacturer's protocol. The vitrification device was opened under liquid nitrogen and immediately transferred to pre-warmed D1 solution (37 °C) for 1 minute. Subsequently, COCs were transferred to D2 for 3 minutes and D3 for 5 minutes at room temperature (25 °C). Following warming, COCs were washed three times in pre-warmed washing medium and incubated for 2 hours in pre-equilibrated IVF medium prior to further evaluation.

Morphological Evaluation of Devitrified COCs

Post-thaw morphology of devitrified and fresh (control) COCs was assessed under an inverted microscope (Olympus, Japan). COCs were classified based on zona pellucida integrity, cytoplasmic appearance, presence of vacuolization, and cytoplasmic leakage as described by Dhali *et al.* (2000)^[10].

In vitro fertilization (IVF)

Following IVM or devitrification, COCs were washed in pre-equilibrated IVF medium and transferred into IVF drops. Frozen-thawed buffalo semen was prepared to achieve a final concentration of 2×10^6 sperm/ml. A volume of 4 µl of sperm suspension was added to each IVF drop containing COCs. Gametes were co-incubated for 18-22 hours at 38.5 °C in a benchtop incubator under 5% O₂, 5% CO₂, and 90% N₂.

In vitro culture (IVC)

After IVF, presumptive zygotes were washed and denuded to remove cumulus cells using a denuding pipette. Zygotes were washed in pre-equilibrated wash medium followed by *in vitro* culture (IVC) medium and cultured for 6 days under standard incubation conditions. Cleavage was assessed at 72 hours post-IVF, and blastocyst formation was evaluated on day 6 of culture.

Evaluation of viability by Hoechst 33342 staining

Viability of fresh and devitrified COCs was evaluated using Hoechst 33342 staining. Matured or devitrified COCs were denuded using trypsin treatment followed by gentle pipetting. Completely denuded oocytes were incubated in Hoechst 33342 solution (10 µg/ml) for 15 minutes at 38.5

°C in the dark. Oocytes were examined under an inverted fluorescence microscope. Oocytes exhibiting nuclear fluorescence were considered viable.

Experimental design

The study was designed to assess the effect of vitrification exposure duration on post-thaw survival, developmental competence, and viability of *in vitro* matured buffalo oocytes. Matured COCs were randomly allocated into three experimental groups: 1. Fresh non-vitrified control group, 2. Vitrification Group I (4-minute equilibration exposure), 3. Vitrification Group II (6-minute equilibration exposure).

Post-devitrification, oocytes were evaluated for morphological integrity, viability, fertilization potential, cleavage rate, and blastocyst development. Each experiment was replicated independently to ensure reliability of results.

Statistical analysis

All data were expressed as mean ± standard error (SE). Percentages of maturation, post-thaw survival, cleavage, blastocyst development, and viability were calculated based on the total number of oocytes or embryos evaluated. Statistical differences among experimental groups were analyzed using one-way analysis of variance (ANOVA). When significant differences were detected, Tukey's post hoc multiple comparison test was applied. Categorical data were analyzed using the Chi-square test where appropriate. Statistical analyses were performed using SPSS software (IBM SPSS Statistics, Version 26.0), and differences were considered statistically significant at $p < 0.05$.

Results and Discussion

Vitrification of COCs

Vitrification of selected *in vitro* matured buffalo cumulus-oocyte complexes (COCs) was performed using a commercially available vitrification kit (Vitrogen, Brazil). Oocytes obtained from two experimental replicates were randomly allocated into two vitrification groups based on the duration of exposure to equilibration solution (V1): Group I (4 min) and Group II (6 min). Fresh, non-vitrified COCs served as the control group.

Morphological evaluation of devitrified COCs

In vitro matured COCs subjected to vitrification were evaluated post-devitrification for morphological integrity and compared with fresh control COCs. A total of 89 and 99 devitrified COCs were evaluated in Group I and Group II, respectively, while 95 fresh COCs were assessed in the control group.

Among devitrified oocytes, 63/89 (70.79%) in Group I and 65/99 (65.66%) in Group II were morphologically normal, whereas a significantly higher proportion of normal COCs (83/95; 87.37%) was observed in the fresh control group (Table 3-5; Fig. 4). Correspondingly, the proportion of damaged COCs was 29.21%, 34.34%, and 12.63% in Group I, Group II, and control groups, respectively.

Analysis of the nature of morphological damage revealed that, among damaged COCs, the incidence of zona pellucida cracking, cytoplasmic leakage, and shrunken cytoplasm in Group I was 69.23% (18/26), 19.23% (5/26), and 38.46% (10/26), respectively, while in Group II these values were 52.94% (18/34), 14.71% (5/34), and 47.06% (16/34). Notably, no zona cracking or cytoplasmic leakage was observed in the control group; however, 50.00% (6/12) of

damaged control COCs exhibited shrunken cytoplasm. The proportion of COCs showing < 5 vacuoles and > 5 vacuoles in the cytoplasm among damaged oocytes was 53.85% and 11.54% in Group I, 50.00% and 20.59% in Group II, and 16.67% and 33.33% in the control group, respectively (Table 3-4; Fig. 4). Statistical analysis revealed no significant difference between Group I and Group II, whereas both vitrified groups differed significantly ($p < 0.05$) from the fresh control group. Furthermore, significant differences ($p < 0.05$) were observed among all three groups with respect to zona cracking, shrunken cytoplasm, and the presence of fewer than five cytoplasmic vacuoles. Several studies have considered post-thaw morphological normality as an indirect indicator of oocyte viability. The proportion of morphologically normal oocytes observed in the present study is comparable to earlier reports by Mahmoud *et al.* (2010a) [27] and El-Shahat and Hammam (2018), although higher survival rates have been reported by Mahmoud *et al.* (2010b) [28] and El-Sokary *et al.* (2013) [15]. The relatively higher incidence of shrunken cytoplasm observed in the present study may be attributed to osmotic stress and cryoprotectant toxicity during vitrification and warming. Species-specific lipid content of oocytes plays a critical role in cryotolerance. Buffalo oocytes contain a high concentration of cytoplasmic lipid droplets, which increases chilling sensitivity and predisposes them to structural damage during cryopreservation (Boonkusol *et al.*, 2007) [5]. Damage to lipid droplets during vitrification has been associated with altered membrane integrity and cytoskeletal disruption, thereby reducing post-thaw survival.

Cleavage and blastocyst development of fresh and devitrified COCs

A total of 653 presumptive zygotes derived from fresh and vitrified oocytes were subjected to *in vitro* culture. Cleavage assessment at 72 h post-fertilization revealed a significantly higher cleavage rate in the fresh control group (62.80%; 292/465) compared to vitrified Group I (27.40%; 24/89) and Group II (22.92%; 23/99) (Table 6; Fig. 5). Similarly, the blastocyst development rate was significantly higher ($p < 0.01$) in fresh COCs (16.34%; 76/465) than in vitrified groups, with 3.37% (3/89) in Group I and 2.02% (2/99) in Group II. Although cleavage and blastocyst rates were numerically higher in Group I compared to Group II, the difference between the two vitrified groups was not statistically significant.

The cleavage and blastocyst rates observed in fresh COCs are consistent with previous studies in buffalo (Abdoon *et al.*, 2001; Wani and Wani, 2003; Gasparrini *et al.*, 2007; Yang *et al.*, 2021) [1, 44, 17, 45]. Reduced developmental competence following vitrification agrees with reports by Wani *et al.* (2004) [43], Gautam *et al.* (2008) [18], Bhat *et al.* (2014) [4], and Gutnisky *et al.* (2020) [21]. Lower embryonic development in vitrified oocytes has been attributed to zona hardening, cytoskeletal damage, and impaired sperm penetration (Gook *et al.*, 1993) [19-20].

Evaluation of viability of COCs by Hoechst 33342 staining

Viability assessment using Hoechst 33342 staining revealed a marked reduction in live oocytes following vitrification. Of 112 devitrified COCs, only 57 (50.89%) were viable, whereas 61 out of 63 fresh COCs (96.83%) were viable

(Table 7; Fig. 6). The difference in viability between fresh and vitrified oocytes was highly significant ($p < 0.01$).

Hoechst 33342 is a membrane-permeable DNA-binding dye that selectively stains live cells by binding to adenine-thymine-rich regions of nuclear DNA (Chazotte, 2011) [7]. Reduced viability following vitrification observed in the present study corroborates findings using other viability assays such as trypan blue (El-Sokary *et al.*, 2013) [15] and MTT staining (López *et al.*, 2021) [25], indicating that vitrification induces substantial cellular stress and compromises oocyte membrane integrity.

In conclusion, vitrification significantly compromises the morphological integrity, developmental competence, and viability of *in vitro* matured buffalo oocytes compared to fresh controls. Although no significant differences were observed between 4- and 6-minute equilibration exposure times, the shorter exposure showed marginally better outcomes. The high lipid content and chilling sensitivity of buffalo oocytes remain major challenges for successful cryopreservation. Optimization of vitrification protocols aimed at minimizing cryo-injury may enhance post-thaw survival and embryonic development in buffalo ART programs.

Table 1: Grading of buffalo cumulus oocyte complexes

Grade of oocytes	Characteristics
Grade I	- The COCs with ≥ 5 layers of cumulus cells. - Uniform granulation of ooplasm. - No nuclear irregularities of the COCs.
Grade II	- The COCs with 3 to 5 layers of cumulus cells - Uniform granulation of ooplasm. - No nuclear irregularities of the COCs.
Grade III	- The oocytes with 1 or 2 layers of cumulus cells - Uniform granulation of ooplasm. - Fragmented oocyte nucleus.
Grade IV	- The oocytes with no layers of cumulus cells. - Denuded oocytes, uneven cytoplasm and misshapen. - Partially absent or vacuolated cytoplasm and nucleus.

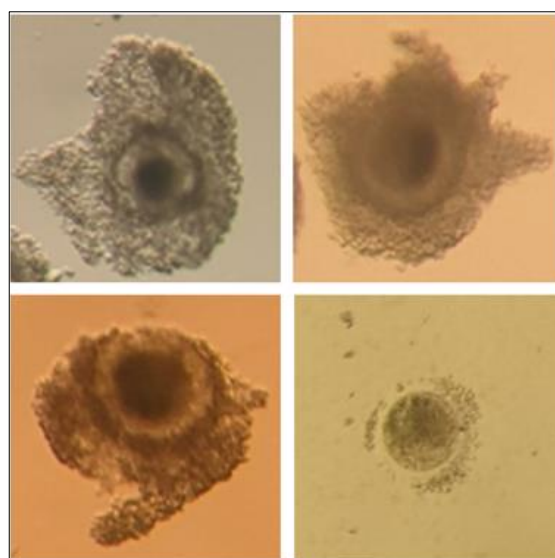
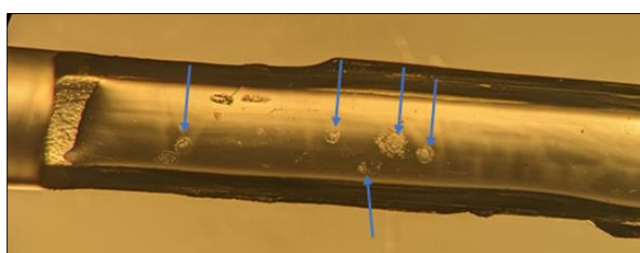


Fig 1: Different grades of aspirated immature COCs

Table 2: Degree of cumulus expansion after 22 hours *in vitro* maturation

Degree 0	Minor or no expansion (Cumulus mass remain adhered to zona pellucida)
Degree 1	Partial cumulus expansion (Non-homogenously spread of cumulus mass with the presence of clusters of cells)
Degree 2	Complete cumulus expansion (Homogenously spread of all cumulus cells, no cell clusters).

**Fig 2:** COCs with partially denuded cumulus mass for vitrification**Fig 3:** Vitrification device with loaded COCs (Arrows indicate COCs)**Table 3:** Replicate wise morphological changes of frozen-thawed COCs at 4 mins exposure time in V1 vitrification media

Replicates	Devitrified COCs	Normal oocytes (%)	Damaged oocytes (%)	Zona crack (%)	Leaked zona (%)	Shrunk cytoplasm (%)	No. of vacuoles	
							<5 (%)	>5 (%)
1	25	20(80)	5(20)	3(60)	1(20)	3(60)	2(40)	0(0.00)
2	25	15(60)	10(40)	7(70)	3(30)	3(30)	5(50)	2(20)
3	25	18(72)	7(28)	4(57.14)	1(14.26)	2(28.57)	4(57.14)	1(14.29)
4	14	10(71.43)	4(28.57)	4(100)	0(0.0)	2(50)	3(75)	0(0.00)
Total	89	63(70.79)	26(29.21)	18(69.23)	5(19.23)	10(38.46)	14(53.85)	3(11.54)
Mean $\pm$ SE		16.25 $\pm$ 1.03	8.50 $\pm$ 1.19	10.50 $\pm$ 4.50	1.25 $\pm$ 0.48	4.00 $\pm$ 0.71	4.25 $\pm$ 1.03	1.75 $\pm$ 0.25

Table 4: Replicate wise morphological changes of frozen-thawed COCs at 6 mins exposure time in V1 Vitrification media

Replicates	Devitrified COCs	Normal oocytes (%)	Damaged oocytes (%)	Zona crack (%)	Leaked zona (%)	Shrunk cytoplasm (%)	No. of vacuoles	
							<5 (%)	>5 (%)
1	25	15(60)	10(40)	6(60)	2(20)	4(40)	4(40)	2(20)
2	25	18(72)	7(28)	3(42.86)	1(14.29)	5(71.43)	4(57.14)	1(14.29)
3	25	14(56)	11(44)	6(54.55)	2(18.18)	5(45.45)	7(63.64)	2(18.18)
4	24	18(75)	6(25)	3(50)	0(0.00)	2(33.33)	2(33.33)	2(33.33)
Total	99	65(65.65)	34(34.34)	18(52.94)	5(14.71)	16(47.06)	17(50)	7(20.59)
Mean $\pm$ SE		15.75 $\pm$ 2.18	6.5 $\pm$ 1.32	4.50 $\pm$ 0.87	1.25 $\pm$ 0.63	2.50 $\pm$ 0.29	3.50 $\pm$ 0.65	0.75 $\pm$ 0.48

Table 5: Replicate wise morphological changes of fresh (Control group) COCs

Replicates	Devitrified COCs	Normal oocytes (%)	Damaged oocytes (%)	Zona crack (%)	Leaked zona (%)	Shrunk cytoplasm (%)	No. of vacuoles	
							<5 (%)	>5 (%)
1	24	21(87.5)	3(12.5)	0(0.00)	0(0.00)	1(33.33)	1(33.33)	1(33.33)
2	21	18(85.71)	3(14.29)	0(0.00)	0(0.00)	2(66.67)	1(33.33)	0(0.00)
3	27	23(85.19)	4(14.81)	0(0.00)	0(0.00)	3(75.00)	1(25.00)	0(0.00)
4	23	21(91.30)	2(8.70)	0(0.00)	0(0.00)	0(0.00)	1(50.00)	1(50.00)
Total	95	83(87.37)	12(12.63)	0(0.00)	0(0.00)	6(50.00)	4(33.33)	2(16.67)
Mean $\pm$ SE		20.75 $\pm$ 1.03	3.00 $\pm$ 0.41	0.00	0.00	1.50 $\pm$ 0.65	1.00 $\pm$ 0.00	0.5 $\pm$ 0.29

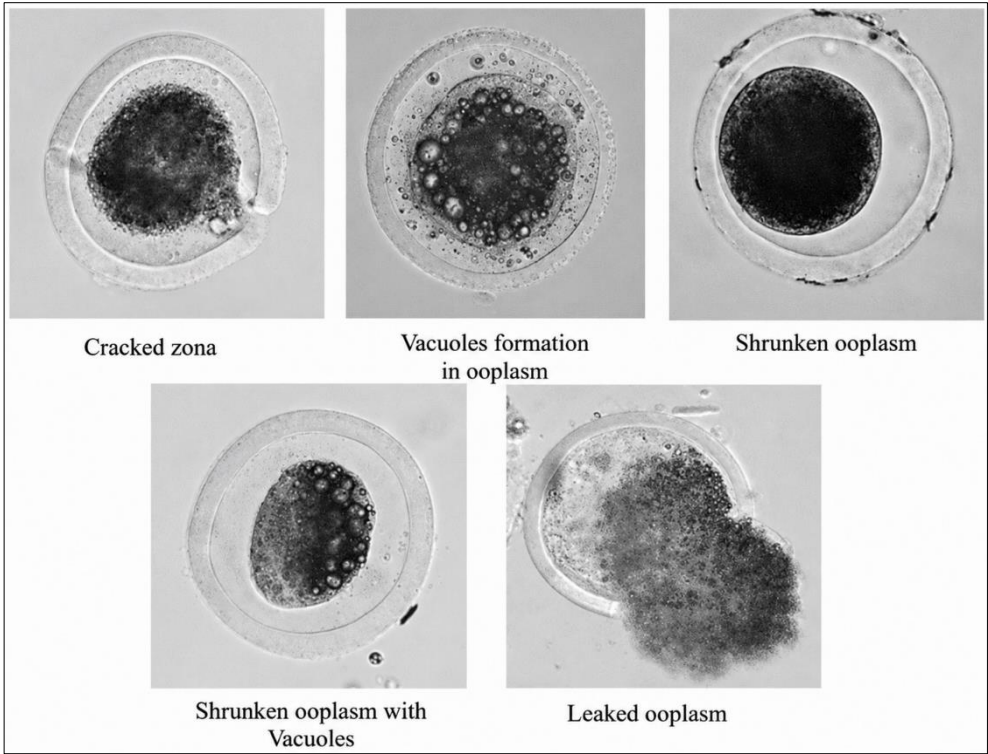


Fig 4: Post-devitrification damaged oocytes

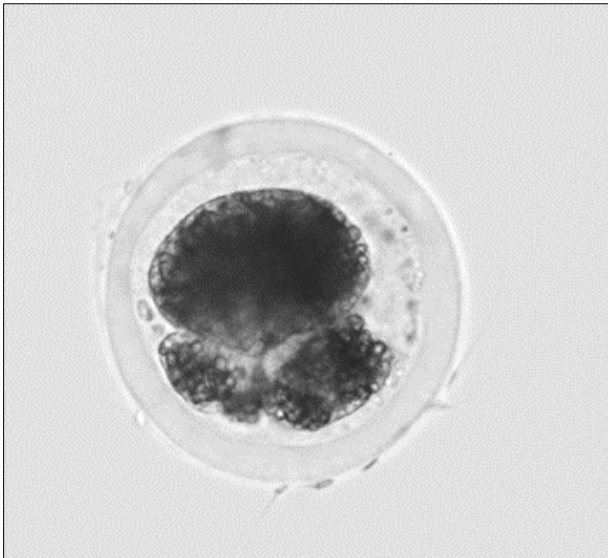


Fig 5a: Cleavage stage embryo at 72 hours incubation



Fig 5b: Blastocyst stage embryo at 6 days incubation

Table 6: Developmental competence in fresh and vitrified oocyte

	Presumptive Zygotes in IVC	Number of zygotes cleaved (72 hrs.)	Cleavage rate%	Number of blastocyst	Blastocyst%
Fresh oocytes	465	292	62.80 ^b	76	16.34 ^b
Vitrified oocytes (4 mins)	89	24	27.40 ^a	3	3.37 ^a
Vitrified oocytes (6 mins)	99	23	22.92	2.00	2.02 ^a

a, b superscripts within the columns differed significantly ($p < 0.05$)

Table 7: Oocyte viability of fresh and vitrified COCs

	Matured COCs	Live (%)	Dead (%)
Fresh COCs	63	61* (96.83)	2(3.17)
Vitrified COCs	112	57* (50.89)	55(49.12)

*. The mean difference is highly significant at the 0.01 level.

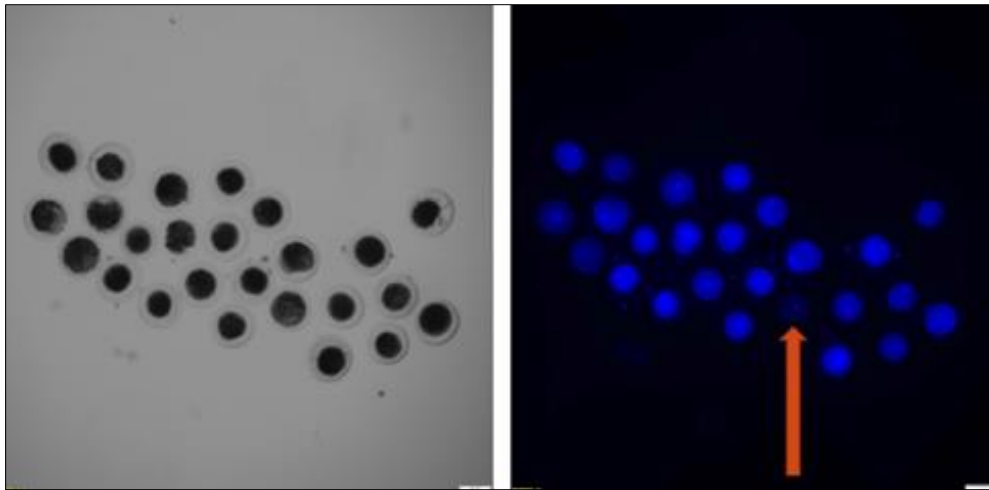


Fig 6a: Hoechst staining in group of fresh oocytes Arrow indicates oocytes not showing fluorescence

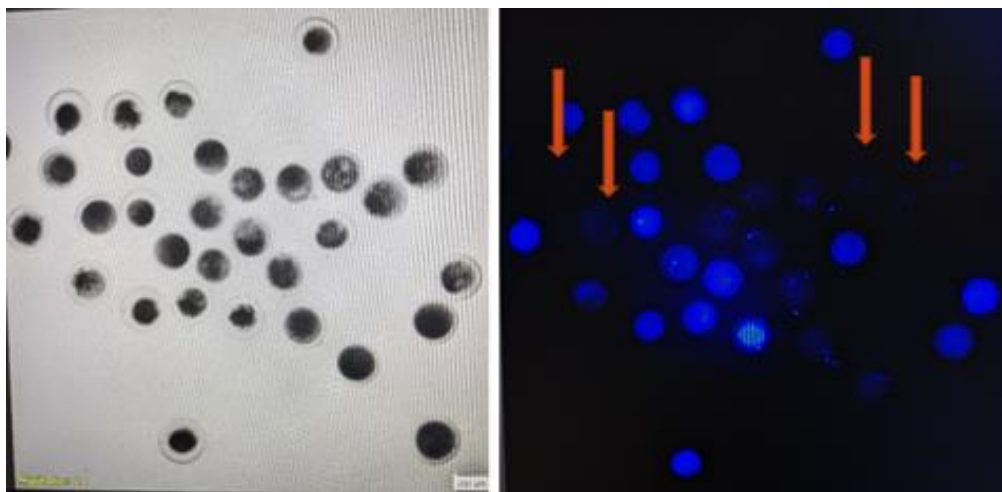


Fig 6b: Hoechst staining in group of vitrified oocytes after devitrification Arrows indicates oocytes not showing fluorescence

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