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Assessing factors influencing *in vitro* maturation and success of vitrification in buffalo oocytes

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Abstract
This study was conducted on buffalo oocytes collected from slaughtered ovarian specimen to evaluate the effect of season and presence of corpus luteum (CL) on *In vitro* maturation rate along with examining the survivability of oocytes after processing for vitrification and thawing (devitrification). COCs were aspirated from surface follicles after noting the presence of CL on ovaries. Maturation rate was compared between winter (November to February) and summer (March to June) seasons. Out of total 944 COCs, 830 (87.79±0.9%) COCs have shown the *In vitro* maturation. Maturation rate in winter and summer season was 89.76±1.08 (437/486) and 85.35±0.56 (391/458) per cent, respectively. Maturation rate in COCs collected from the ovaries with CL and without CL was 82.98±1.22 (292/348) and 90.34±1.25 (536/596), respectively. Oocytes were vitrified using vitrogen vitrification kit according to different time exposure and stored in liquid nitrogen (LN_2). Thereafter, oocytes were devitrified, stained with Hoechst 33342 staining and evaluated for viability under inverted microscope in fluorescent light. A total of 112 devitrified and 63 fresh oocytes were examined and 50.89% (57/112) and 96.83% (61/63) live oocytes among them, respectively. This study concludes that no effects of factors like season or morphological structure like CL was observed on COCs recovery or maturation and revival rate of vitrified COCs was not up to the mark and needs to be reinvestigate with modification of vitrification media.

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

Introduction
India holds the highest number of domestic buffaloes (*Bubalus bubalis*). According to the 20th Indian Livestock Census (2019), the total population of buffaloes in the country is 109.85 million, which is 1.1% higher than the previous census conducted in 2012, where the population was 108.7 million. Buffaloes are valued for their multipurpose utility, including milk, meat, and draft purposes. They exhibit excellent adaptability to harsh environmental conditions and demonstrate notable feed conversion efficiency, which helps to lower the management and treatment costs for farmers. However, there are several drawbacks associated with domestic buffaloes. These include late puberty, silent or poor estrus expressions, seasonally breeding behavior, poor conception rates, poor embryonic survival, prolonged inter-calving intervals, and postpartum anestrus (Barile, 2005; Suthar and Dhami, 2010; Doultani *et al*., 2022) [1, 27, 5]. Despite these challenges, the overall contribution of buffaloes to India's livestock sector remains significant.

In vitro Embryo Production (IVEP) is a rapidly advancing field in animal reproduction (Suthar, 2008; Suthar and Shah, 2009) [26, 28]. Several factors contribute to the development of IVEP. These include improved media composition, faster turnover compared to conventional Multiple Ovulation Embryo Transfer (MOET), the introduction of sexed semen, and the ability to use semen from multiple bulls on oocytes from a single donor simultaneously. Through *In vitro* maturation (IVM) of oocytes and *In vitro* fertilization (IVF), it is possible to generate a large number of embryos, even from gametes of dead or slaughtered animals (Wani and Wani, 2003) [30]. The initial and crucial stage of the IVEP process is IVM, which grants oocytes the ability to support ongoing embryonic development (Hatırnaz *et al*., 2018) [13]. Successful IVM of bovine oocytes requires modifications to the maturation medium, supplements such as

proteins and hormones, and improvements in the quality of cumulus-oocyte complexes (COCs), which enhance *In vitro* culture and eventual fertilization (Bavister and Rose-Hellekant, 1992) [2]. Additionally, high ambient temperatures adversely affect the quality of extracted oocytes and further reduce embryo formation (Nain *et al*., 2004) [20]. Nowadays, vitrification is a very crucial process in the field of reproduction for long-term preservation of genetic material, in humans as well as animals including farm animals. Gautam *et al*. (2008) [11] have demonstrated that slow freezing is not suitable for cryopreservation of immature buffalo oocytes and vitrification is more effective than slow freezing of IVM buffalo oocytes. Vitrification is a relatively new and successful approach, which is defined as "a physical process by which a highly concentrated solution of cryoprotectants solidifies during cooling, without formation of ice crystals". Embryo vitrification was first reported by Rall and Fahy (1985) [22]. Advantages of vitrification over slow freezing are faster and simplified freezing and thawing procedures and no requirement for an expensive freezing machine. High variability in culture conditions, biological material, concentration of cryoprotectants and physiological properties of oocytes across species are responsible factors for oocyte survival and further development (Dujíčková *et al*., 2021) [6]. However, getting higher success rate in embryo cryopreservation, oocytes vitrification is more difficult, mainly because of chromosome abnormalities, microtubule depolymerization, changes in spindle structure and disruptions in the actin cytoskeleton structure (Saunders and Parks, 1999) [24]. Considering above justification and dearth of information this experiment was planned with objective to evaluate the effect of season, quality of the COCs and presence of corpus luteum (CL) on IVM rate along with examining the survivability of oocytes after processing for vitrification and thawing (devitrification). Considering above justification and dearth in the area this study was planned on buffalo oocytes collected from slaughtered ovarian specimen to evaluate the effect of season, quality of the COCs and presence of CL on IVM rate along with examining the survivability of oocytes after processing for vitrification and thawing (devitrification).

Materials and Methods

Season

In tropical countries like India especially towards western part i.e., Gujarat, during winter season (November to February) the weather remains relatively cooler compared the summer months March to July. During this period of the year 2022-23 the experiment was carried out.

Ovary collection and recovery of cumulus-oocytes-complexes

Ovaries were obtained from slaughtered buffaloes at a slaughterhouse managed by the Ahmedabad Municipal Corporation (AMC) Ahmedabad, Gujarat, India. The ovaries were carefully washed with pre-warmed normal saline solution (NSS) containing 1% (w/v) antibiotic solution (10000 IU/ml penicillin, 10000 µg/ml streptomycin, and 25 µg/ml amphotericin B) and transported to the laboratory in a thermos at a temperature of 34 to 36 °C. Upon arrival at the IVF laboratory, Gujarat Biotechnology Research Centre, Gandhinagar the ovaries were again washed with NSS and placed in a beaker at 36 °C until the aspiration of cumulus-oocyte complexes (COCs). COCs were aspirated using a sterile 18 G needle and a 5 ml syringe filled with pre-warmed OPU fluid (IMV France), and then collected in a 90 mm petri dish. Immediately after collection, the COCs were examined under a stereo-zoom microscope and transferred into a 35 mm petri dish containing 3 ml of pre-warmed OPU/wash media.

Morphology of Ovaries

The ovaries were classified as with and without corpus luteum (Figure 1) and follicles on ovaries were observed. The side of ovaries with or without CL (left or right) was also noted.

Grading of Cumulus Oocyte Complexes

COCs were categorized and separated into grades I to IV according to the method described by Das *et al*. (1996) [4], with slight modifications as illustrated in Figure 2. In brief, COCs with four or more layers of cumulus cells were classified as grade I, those with 3 to 2 layers of cumulus cells were classified as grade II, COCs with 1 layer of cumulus cells were classified as grade III, and denuded, damaged, or degenerated oocytes were classified as grade IV. *In vitro* maturation of COCs was conducted including grade I to III. The graded COCs were washed three times in drops of pre-equilibrated IVM media (90 µl each drop) in separate 35 mm petri dishes and then transferred into previously prepared equilibrated IVM media drops covered with oil in petri dishes (with a maximum of 20 oocytes in each 90 µl drop). Grade I to III COCs aspirated from ovaries with and without corpus luteum (CL), were kept separately in different drops in the petri dish. The IVM petri dishes were then incubated at 38.5 °C with 5% O2, 5% CO2, and 90% N2 in a benchtop incubator (Cooper Surgical, USA) for 22 hours. The degree of cumulus expansion was used to assess the maturation rate of COCs. Maturation of COCs was evaluated based on Cumulus Cell Expansion (CCE) following the criteria outlined by Kobayashi *et al*. (1994) [16]. In summary, cumulus expansion was assessed based on the following degrees: Degree 0 indicated minor or no expansion, where the cumulus mass remained adhered to the zona pellucida; Degree I represented partial cumulus expansion, characterized by non-homogenous spreading of the cumulus mass with the presence of cell clusters; Degree II indicated complete cumulus expansion, with homogenous spreading of all cumulus cells and the absence of cell clusters. Only COCs achieving a maturation degree of I or II were considered mature. The influence of various factors such as COCs grade, seasonal variations (winter: November to February, and summer: March to June), and the presence or absence of CL on the ovary were evaluated.

Vitrification

Partial denudation of COCs

Matured COCs displaying degree I and II cumulus expansion were chosen for vitrification and underwent three washes with pre-warmed washing media. The expanded cumulus mass was partially removed from the COCs using a denuding pipette fixed with the denuding handle.

Vitrification and devitrification of matured COCs

The vitrification process for oocytes was conducted using commercially available vitrification media (Vitrification Kit, Vitrogen, Brazil) at room temperature (approximately 25 °C), following the manufacturer's instructions. The COCs were first transferred to V1 medium for either 4 minutes (Group I) or 6 minutes (Group II), and then both groups were moved to the V2 medium drop and kept there for 40 seconds. Subsequently, the COCs were immediately loaded into the

vitrification device (VitriFit, Cooper Surgical, USA; Figure 3), and any excess media was removed using a denuding pipette, leaving approximately 0.3 µl of media on the tip of the device. The devices were promptly submerged in liquid nitrogen and sealed with a pre-cooled lid. They were then transferred into pencil goblets containing identification details and moved into a liquid nitrogen tank for storage until devitrification.

The devitrification process for the vitrified oocytes from both timing groups was conducted using devitrification media (D1, D2, and D3; Vitrogen, Brazil) according to the manufacturer's guidelines. First, a D1 media drop was warmed to 37 °C, and the vitrified device containing COCs was opened in liquid nitrogen (LN_2). The COCs were immediately transferred into the D1 media drop and collected by visual inspection under a stereo zoom microscope, where they were kept for 1 minute. Subsequently, the COCs were transferred to D2 media for 3 minutes and then to D3 media for 5 minutes at room temperature (25 °C) using a denuding pipette (size 270 µm). Following this, the COCs were washed in three drops of 100 µl of pre-warmed wash media. The devitrified COCs were then incubated for 2 hours in pre-equilibrated IVF media drops.

Effect of vitrification on viability of Cumulus Oocyte Complexes

Evaluation of viability of Cumulus Oocyte Complexes

COCs from two groups, vitrified-thawed and non-vitrified (Fresh matured oocytes), were assessed for viability. Initially, they were washed in pre-warmed filtered PBS and treated with pre-warmed trypsin solution for 5 minutes at 38.5 °C to remove cumulus cells. After denudation, the oocytes underwent triple washing with PBS before being incubated in Hoechst 33342 stain solution (10 µg/ml) in the dark at 38.5 °C for 15 minutes. Following incubation, the oocytes were examined under an inverted fluorescent microscope (Olympus, U-CMAD3, Japan) in a dark room. Live oocytes absorbed the stain, emitting fluorescence, while dead COCs showed no fluorescence. This method allowed for the evaluation of oocyte viability based on their fluorescence response, as described by Chazotte (2011) [3].

Experiment design

Effect of season

During November 2022 to July 2023 ovaries were collected from slaughterhouse. The November to March is relatively cool period while April to June is relatively hot period in Gujarat. During the two period 7 each ovarian collection session (Replicates) was performed.

Effect of Presence or absence of Corpus Luteum

Ovaries with CL or without CL were segregated and recovery of COCs was noted. The effect of presence or absence of CL on COCs recovery and maturation rate was investigated as mentioned above.

Statistical analysis

All data were recorded in excel spreadsheets of Microsoft windows. The effect of season, presence of CL on varies on COCs recovery and maturation rate was analyzed using independent test. All the analysis was performed in SPSS 25 (IBM pvt ltd., Bangaluru, India).

Results and Discussion

In the present study, a total of 399 ovaries were collected over 14 sessions and aspirated 1255 follicles during November to June 2023. It includes 217 ovaries during Winter and 182 ovaries during Summar season. A total of 970 COCs were aspirated including 26 of Grade IV, 944 COCs consisting of Grade I, II, and III over the period of two seasons. The overall cytoplasmic maturation rate of COCs was 87.79±0.9% (830/944). Various researchers have reported IVM rate of COCs using different maturation media preparations. Here in this study commercial media was used to maintain consistency. In the past a pioneer study by Totey *et al*. (1992) [29] utilized Hams F-10 supplemented with 20% BES and gonadotropins, Gupta *et al*. (2001) [12] used follicular fluid with TCM-199, and Gad *et al*. (2018) [10] examined the effect of 9-cis retinoic acid. The reported maturation range 81 to 92% is in accordance with the present study. However, the maturation rate in this study was lower than that reported by Dutta *et al*. (2013) [7]. Conversely, it was higher than the maturation rate found by Mehmood *et al*. (2011) [19], who also used Estrus Buffalo Serum (EBS) and fetal calf serum (FCS), and Patel *et al*. (2023) [21], who compared two commercially available media (BO-IVM and Vitrogen IVM).

Effect of season on maturation rate

In the present study, conducted during winter (November 2022 to February 2023) and summer (March 2023 to June 2023), a total of 486 and 458 COCs were processed for IVM, respectively. Out of these, 437 COCs from the winter batch and 391 COCs from the summer batch reached maturation (Table 1). The calculated maturation rates (Mean±S.E.) during winter and summer were 89.76±1.08% (437/486) and 85.35±0.56% (391/458), respectively (Table 1). No significant effect of season was observed on either COCs recovery or maturation rate during the study. Although Sadhan *et al*. (2010) [23] documented a greater maturation rate of buffalo COCs during winter, our findings are consistent with those of Mansor, (2019) [18], who reported no impact of season on the maturation rate of buffalo COCs. Conversely, Elbaz *et al*. (2019) [8] and Kandil *et al*. (2023) [15] observed a significantly higher maturation rate during cool weather. It is worth noting that various factors such as media quality, nutrition, and incubator conditions can influence the maturation rate (Suthar and Shah, 2009) [28].

Effect of presence of CL on maturation rate

Total 399 ovaries were collected during the present study, among them 157 ovaries having CL on surface and 242 ovaries did not have CL. As no effect of season was observed on COCs recovery or maturation rate effect of CL was assessed independently on pooled data of two season. Out of total 348 and 596 numbers of COCs aspirated from ovaries with and without CL, 292 and 536 COCs were matured, respectively. The maturation rate (%; Mean±S.E.) in COCs recovered from ovaries with CL or without CL were 82.98±1.22 and 90.34±1.25 (Table 2), respectively. Interestingly no effect of presence of CL was observed on COC recovery or maturation rate. This study results are in accordance of (Singh *et al*. 2001; Patel *et al*., 2023) [25, 21]. However, there was a significantly higher maturation rate was found by Mansor, (2019) [18] in group of ovaries without CL than with CL but the maturation rate was lower compared to present study. Finally, it could be concluded that the season (hot or cold), quality of COCs in respect to numbers of cumulus and homogeneity of oocytes of oocytes cytoplasm and presence/absence of CL on the ovary from which the COCs were collected affect the maturation rate in immature oocytes of buffalo.

Evaluation of viability of Cumulus Oocyte Complexes

In the present study, denuded COCs were stained with Hoechst 33342 staining for assessment of post-devitrification viability of *In vitro* matured vitrified oocytes compared with fresh oocytes. The oocytes which gained stain, considered as live (Figure 4). A total of 112 devitrified oocytes and 63 fresh oocytes were examined for the viability. Out of total 112 devitrified COCs, 57 COCs (50.89%, 57/112) were considered live as they indicated blue fluorescents, while 55 COCs (49.12%, 55/112) were considered dead as indicated no fluorescents. In consideration of viability of fresh COCs, 61 (96.83%, 61/63) were live and 2 (3.17%, 2/63) were dead out of total 63 COCs (Table 7; Fig. 4.11). The statistical analysis revealed highly significant (P<0.01) difference between viability of fresh and vitrified COCs. Hoechst 33342 stain is a membrane permeant stain that gives fluorescence increases by binding with adenine thymine rich regions of DNA in the minor groove which can only stain live cells (Chazotte, 2011) [3]. After a short-term exposure to the ultraviolet (UV) wavelengths essential for Hoechst 33342 stain excitation, it is a vital DNA stain (Hinkley *et al*., 1986) [14]. Many authors used different stains for evaluating the viability of oocytes. El-Sokary *et al*. (2013) [9] stained oocytes with 0.4% (w/v) trypan blue and López *et al*. (2021) [17] incubated oocytes in methyl-thiazolyl-tetrazolium (MTT) for staining and reported that there was decrease in viable numbers of oocytes following to vitrification.

From this study we concluded that seasons like winter or summer, presence of functional structure like CL could not alter the COC recovery or maturation rate. Further vitrification of oocytes significantly altered the viability of the post vitrified oocytes.

Table 1: Session wise *In vitro* maturation rate of aspirated COCs during two winter and summer season.

Replicate	Winter (November-2022 to February-2023)				Summer (March-2023 to June- 2023)			
	Number of ovaries	Immature oocytes	Matured oocytes	Maturation rate (%)	Number of ovaries	Immature oocytes	Matured oocytes	Maturation rate (%)
1	34	44	39	88.64	32	69	59	85.51
2	34	69	63	91.30	28	75	63	84.00
3	32	76	70	92.11	26	55	48	87.27
4	26	68	61	89.71	22	63	54	85.71
5	30	78	66	84.62	24	59	49	83.05
6	33	89	83	93.26	20	54	46	85.19
7	28	62	55	88.71	30	83	72	86.75
Total	217	486	437	89.92	182	458	391	85.37
%Mean±SE				89.76±1.08				85.35±0.56

Table 2: Session wise *In vitro* maturation rate in COCs collected from ovaries with/without CL

Session	Ovaries with CL			Ovaries without CL		
	Immature oocytes	Matured oocytes	Maturation rate (%)	Immature oocytes	Matured oocytes	Maturation rate (%)
1	15	12	80.00	29	27	93.10
2	27	22	81.48	42	41	97.62
3	41	36	87.80	35	34	97.14
4	24	21	87.50	44	40	90.91
5	33	26	78.79	45	40	88.89
6	43	39	90.70	46	44	95.65
7	29	24	82.76	33	31	93.94
8	17	14	82.35	52	45	86.54
9	29	25	86.21	46	38	82.61
10	17	13	76.47	38	35	92.11
11	11	9	81.82	52	45	86.54
12	20	15	75.00	39	34	87.18
13	18	15	83.33	36	31	86.11
14	24	21	87.50	59	51	86.44
Total number (%)	348	292	83.91	596	536	89.93
Mean±SE			82.98±1.22			90.34±1.25
The difference is non-significant.						

Table 3: Replicate wise *In vitro* maturation rate in different grades of COCs

Replicate	Grade I COCs			Grade II COCs			Grade III COCs			Total		
	Immature oocytes	Matured oocytes	Maturation rate (%)	Immature oocytes	Matured oocytes	Maturation rate (%)	Immature oocytes	Matured oocytes	Maturation rate (%)	Immature oocytes	Matured oocytes	Maturation rate (%)
1	15	15	100.00	18	17	94.44	11	7	63.64	44	39	88.64
2	28	27	96.43	25	24	96.00	16	12	75.00	69	63	91.30
3	32	32	100.00	30	29	96.67	14	9	64.29	76	70	92.11
4	25	25	100.00	24	21	87.50	19	15	78.95	68	61	89.71
5	33	30	90.91	29	26	89.66	16	10	62.50	78	66	84.62
6	35	35	100.00	35	32	91.43	19	16	84.21	89	83	93.26
7	22	21	95.45	26	25	96.15	14	9	64.29	62	57	91.94
8	29	28	96.55	23	20	86.96	17	11	64.71	69	59	85.51
9	33	30	90.91	24	20	83.33	18	13	72.22	75	63	84.00
10	18	18	100.00	22	20	90.91	15	10	66.67	55	48	87.27
11	23	21	91.30	29	25	86.21	11	8	72.73	63	54	85.71
12	25	24	96.00	19	16	84.21	15	9	60.00	59	49	83.05
13	14	13	92.86	19	17	89.47	21	16	76.19	54	46	85.19
14	21	21	100.00	38	35	92.11	24	16	66.67	83	72	86.75
Total number (%)	353	340	96.32	361	327	90.58	230	161	70.00	944	830	87.92
Mean±SE			96.46 ^{a}±0.99			90.36 ^{a}±1.18			69.43 ^{b}±1.91			87.79±0.90
a, b superscripts within the row differed highly significantly (P< 0.01)												

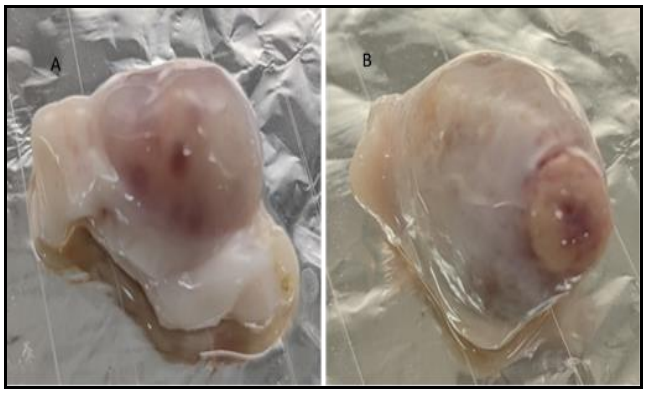

Fig 1: Buffaloe ovaries without (A) and with (B) Corpus luteum

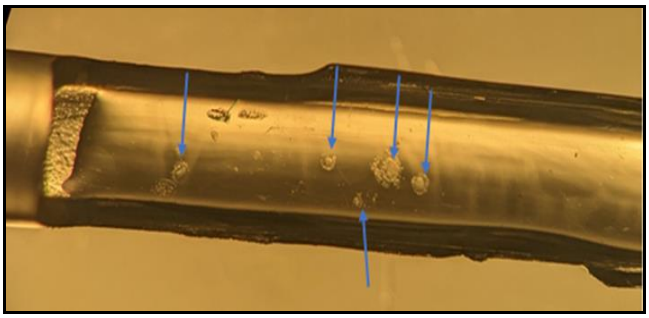

Fig 3: Cumulus Oocyte Complexes loaded on Vitrification device (VitriFit, ref. 42802001A, Cooper Surgical, USA) for Vitrification of *In vitro* matured buffalo oocytes

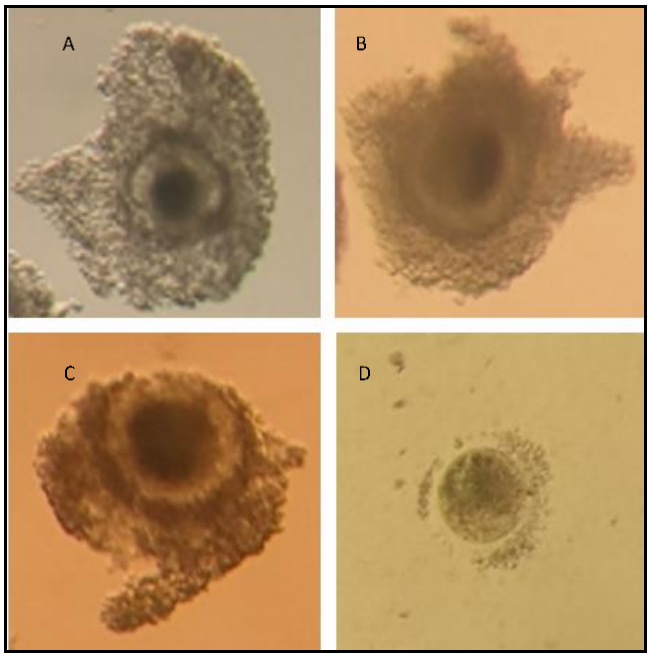

Fig 2: Different grades of Cumulus oocytes complex include grad I (A); grade II (B); Grade III (C); and Grade IV (D) aspirated from Buffalo ovaries

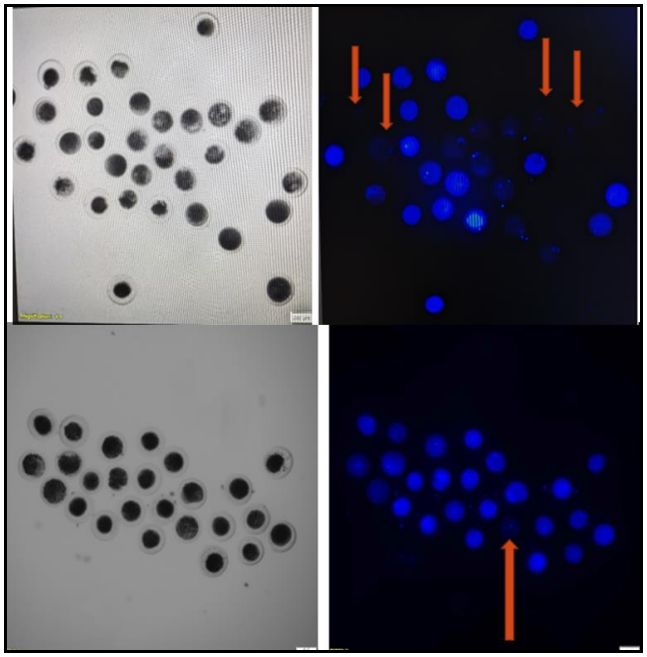

Fig 4: Viability of *in vitro* matured and denuded oocytes with and without vitrification and devitrification using Hoechst 33442 stain. Vitrified group (A), Non-Vitrified group (B)

Conclusion

In conclusion, our study spanning November 2022 to June 2023, involving 399 ovaries and 1255 follicles, yielded valuable insights into the *in vitro* maturation (IVM) of buffalo cumulus-oocyte complexes (COCs). Utilizing commercial media for consistency, we achieved an overall cytoplasmic maturation rate of 87.79%, aligning with previous reports ranging from 81% to 92%. Seasonal variation did not significantly affect COC recovery or maturation rates, consistent with some studies but contrary to others. Additionally, the presence of corpus luteum (CL) on ovaries didn't influence COC outcomes. However, our assessment of post-vitrification viability revealed a significant difference compared to fresh COCs. Hoechst 33342 staining proved effective in discerning live from dead cells, emphasizing the impact of vitrification on oocyte viability. Ultimately, this study underscores the complex interplay of factors such as season, CL presence, and vitrification in the maturation process of buffalo COCs, offering valuable insights for further research and reproductive technologies.

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We express our sincere gratitude to Municipal Slaughterhouse, Ahmedabad for providing reproductive sample (buffalo ovaries) for evaluation. We are also grateful to the Director of Research, Kamdhenu University, Gandhinagar, Gujarat for providing support and free hand to carry out the experiment.

Conflict of Interest

There is no any conflict among the authors.

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