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Assessing the cleavage, blastocyst, and hatching rate of Buffalo origin cumulus oocyte complexes

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Abstract
Assisted reproductive techniques, such as *in vitro* embryo production (IVEP), have garnered significant attention due to the slow reproductive rates observed in buffaloes. The aim of this study was to assess cleavage, blastocyst formation, and hatching rates in ovarian cumulus oocyte complexes (COCs) obtained from slaughtered buffaloes. COCs were recovered from buffalo ovarian follicles using the aspiration method. A total of 655 ovaries were collected and utilized across 29 sessions (averaging 23±1.2 ovaries per session). Surface follicles were measured with a Vernier caliper and classified into three groups based on diameter: < 3 mm, 3-5 mm, and > 5 mm. These follicles were then subjected to *in vitro* maturation (IVM), *in vitro* fertilization (IVF), and *in vitro* culture (IVC) using frozen semen doses. The maturation of COCs of grades I to IV was evaluated based on morphological characteristics 22 hours after IVM. Cleavage, blastocyst formation, and hatching rates were assessed 72 hours, 6 days, and 6 to 9 days after IVC, respectively. Overall, 55.15% (1387) of follicles were small, 39.05% (982) were medium-sized, and 5.80% (146) were large. A total of 66.76% (1679/2515) COCs were recovered from these follicles. The distribution of COC grades was as follows: 36.09% (I), 36.21% (II), 24.66% (III), and 3.04% (IV). Out of 1640 presumptive zygotes, 1015 successfully cleaved, resulting in a cleavage rate of 63.1%. Of these, 295 (20%) reached the blastocyst stage by day 6. Among the blastocysts, 131 were observed for hatching, with 63 (48.1%) hatching between the 6th and 8th day post IVC. In conclusion, the study suggests that IVEP could be a valuable tool for salvaging the genetic potential of the buffalo population.

Keywords: *In vitro* embryo production, buffaloes, cleavage rate, blastocyst and hatching rate

Introduction
The buffalo population in India, numbering at 109.85 million, holds the distinction of being the largest worldwide. Buffaloes play a crucial role in Indian agriculture, contributing significantly to the milk industry with a 49% share, as well as providing meat and draught power. Despite their pivotal role, buffaloes exhibit sluggish reproductive efficiency characterized by delayed puberty, poor estrus expression, low embryonic survival rates, prolonged inter-calving intervals, extended postpartum ovarian quiescence, seasonal breeding patterns, and reduced conception rates, particularly when artificially bred (Gordon, 1996) [5].
To address these challenges, numerous research groups and breeding agencies in India, including NDDB, BIAF, JK Trust, veterinary institutions, cooperative dairies, and globally recognized organizations like EMBRAPA and WBC, have explored various Assisted Reproductive Technologies (ART) and their combinations. Superovulation and embryo transfer technologies (ETT) have shown limited success in buffaloes (Suthar and Shah, 2009) [25]. Consequently, there's a growing interest in utilizing third-generation ART, such as *in vitro* embryo production (IVEP) technology, to enhance offspring numbers from genetically superior buffaloes, thereby reducing generation intervals and promoting global germplasm dissemination (Suthar, 2008; Suthar and Shah, 2009; Doultani *et al*., 2019) [23, 25, 3].
According to the annual report of the International Embryo Technology Society (IETS) in 2021, 1,521,018 bovine *in vitro* produced embryos were generated and transferred worldwide, with IVEP replacing superovulation-based *in vivo* embryo production in bovines over the past two decades (Viana, 2022) [26]. Approximately 80% of transferred embryos were utilized fresh to maximize pregnancy rates.

However, obtaining suitable recipients with optimal nutrition, reproductive health, and synchronized statuses remains a challenge for IVEP practitioners.

Over the last decade, commercial media for IVEP has become available, leading to improved blastocyst production efficiency. Nevertheless, there remains a scarcity of data regarding buffalo species. Hence, the objective of this study is to evaluate cleavage, blastocyst formation, and hatching rates in ovarian cumulus oocyte complexes (COCs) obtained from slaughtered buffaloes.

Materials and Methods

In the present study, buffalo ovaries were obtained from the slaughterhouse facility of Ahmedabad Municipal Corporation, Ahmedabad, Gujarat, between November 2022 and May 2023. The ovaries were meticulously washed with normal saline containing 1% (w/v) antibiotic solution (10000 IU/ml penicillin, 10000 µg/ml streptomycin, and 25 µg/ml amphotericin B; Gibco, USA) and stored in an isothermal container with normal saline at 34-36°C during transit. Within 3 hours of collection, the ovaries were processed at the *in vitro* fertilization (IVF) laboratory facility of the Gujarat Biotechnology Research Centre, Gandhinagar, Gujarat.

Cumulus-oocyte complexes were aspirated from follicles with diameters ranging from 3 to 12 mm. These COCs were then classified based on their morphology following the classification described by Das *et al*., (1996) [2]. Afterward, the COCs were washed with wash media (Vitrogen, Brazil) and transferred to pre-equilibrated *in vitro* maturation (IVM) media (Vitrogen, Brazil). Subsequently, the COCs were placed into pre-equilibrated 90 µl IVM drops. The petri dish containing IVM drops with COCs was then placed in a benchtop incubator set at 38.5°C with 5% CO_2, 5% O_2, and balanced N_2 for 22 hours. The maturation of COCs was assessed based on cumulus expansion, and matured COCs were then transferred to 90 µl IVF drops containing fertilization media (Vitrogen, Brazil). Frozen semen doses (FSDs) from buffalo bull were procured from Sabarmati Ashram Gaushala Bidaj, Gujarat. The semen straws were carefully thawed by shaking in the air for 10 seconds and then submerged into a pre-warmed 37°C water bath for 30 seconds. The semen was evaluated for post-thawed motility, and only samples with motility above 50% were used. After centrifugation and washing, the sperm cells were diluted to a concentration of 2 x 106 sperm/ml and introduced into the IVF media drops containing COCs. The gametes were co-incubated at 38.5°C in a benchtop incubator with 5% O_2, 5% CO_2, and 90% N_2 for 18-22 hours. After co-incubation, the presumptive zygotes were gently washed and denuded to remove all cumulus cells using IVM wash media. The denuded zygotes were then transferred to pre-equilibrated 90 µl drops of *in vitro* culture (IVC) media (Vitrogen, Brazil) and placed in a benchtop incubator for 6 days. Cleavage and blastocyst rates were assessed after 72 hours and on day 6 of IVC, respectively. Hatching rate was observed every 12 hours from day 6 to day 8 after IVC. All the session data were recorded in excel sheet which includes sessions, number of ovaries procured, grading of oocytes, maturation grade, cleavage and blastocyst rate. The 72-h cleavage, blastocyst conversion rate on day 6 and hatching rate during day 6 to 8 after IVC was calculated using descriptive tools in SPSS software (SPSS 25 IBM, Bangaluru, India). The difference between COC grades was evaluated using one-way ANOVA and LSD post-hoc test.

Results and Discussion

Total 655 ovaries were procured with average of 23 ± 1.21 ovaries/session from slaughterhouse for COCs aspiration (Table 1). Over the 29 sessions 1679 COCs were recovered from 2515 observed follicles of 655 buffalo ovaries. During 29 sessions a total of 1679 COCs were recovered with an average recovery rate of 2.56 COCs per ovary. This result is in accordance with the earlier findings of 2.04 (274/134; Hussain *et al*., 2005) [7], 2.94 (655/206; Hammad *et al*., 2014) [6] and 2.6 (409/155; Pitroda *et al*., 2021) [17]. Furthermore, some studies reported a higher recovery rate of COCs than the present study such as 3.31(635/192; Khan *et al*., 1997) [9], 3.22 (574/172; Kandil *et al*., 2023) [8]. Similarly studies also reported lower recovery rate of COCs then the present study such as 0.93 (425/457; Nandi *et al*., 2002) [12], 1.69 (490/290; Manjunatha, 2008) [10], 1.79 (335/187; Elbaz *et al*., 2019) [4], and 1.73 (440/254; Patel *et al*., 2023) [15].

According to COCs grade I, II, III, and IV total 608, 606, 414 and 51 COCs were recovered, respectively. One-way ANOVA analysis demonstrated significant difference between four grades of recovered COCs ($P = 0.0001$). The post-hoc test revealed COCs of grade I was higher than grade III ($P = 0.0001$) and IV ($P = 0.0001$), however, no difference was observed between grade I and II COCs ($P = 0.876$). These results are in accordance with past studies (Elbaz *et al*., 2019; Patel *et al*., 2021) [4,] In the present study, total 1640 presumptive zygotes were kept in IVC medium, after 72 h the cleavage was assessed which is depicted in Table 2. The different cleavage stages are elucidated in Figure 1. Total 1015 presumptive zygotes were cleaved, with observed cleavage rate of 63.1%; 1015/1640) and 295 (20%; 295/1640) reached either early blastocysts or blastocyst stages (BLs) on day 6. The reported cleavage rate in different studies ranging from 14 to 84.4% (Suthar and Shah, 2009; Ahmed *et al*., 2023) [25, 1]. Ahmed *et al*. (2023) [1] reported cleavage rate from 79 to 84.4% in blastocyst of buffaloes which is higher than the present study while others have reported 46 to 55% (Mostager *et al*. (2017) [11], 39.2% (Patil *et al*., 2022) [16] and 40.84 ± 2.51% (Pitroda *et al*., 2021) [17] which is lower than the present study. Many factors can influence cleavage rate such as media quality, drop size, incubator environment, semen quality, capacitation, season, etc. These factors, among others, are likely considered in the meta-analysis conducted by Suresh *et al*. (2009) [22] and discussed in classic reviews by Palta and Chauhan (1998) [14] and Nandi *et al*. (2002) [12]. These studies provide valuable insights into the multifaceted nature of cleavage rate regulation in buffalo embryos, synthesizing findings from various research endeavours.

Understanding the intricacies of cleavage rate regulation is essential for optimizing assisted reproductive technologies in buffalo breeding programs, ultimately enhancing reproductive outcomes and genetic progress. The blastocyst rate in the present study is in accordance with 19.9 ± 4.2% (Neglia *et al*. 2003) [13] and 20.4±2.5% (Sadeesh *et al*., 2016) [20]. Further few studies also reported blastocyst rate 22.9 to 26.98% (Nandi *et al*., 2002; Ravindranatha *et al*., 2003) [12, 18]. Many factors can affect blastocyst rate such as source of oocytes (i.e., slaughter origin or OPU), media, culture conditions, sire, capacitation, oocyte and zygote handling and incubation environment. These factors, along with others, have been scrutinized in studies such as those by Suthar and Shah (2009) [25] and Suresh *et al*. (2009) [22] which delve into the multifaceted determinants of blastocyst formation in buffalo embryos. By comprehensively understanding the intricate interplay of these factors, researchers and practitioners can

refine assisted reproductive techniques tailored to buffalo breeding programs. This holistic approach facilitates the optimization of blastocyst formation rates, thereby advancing genetic progress and bolstering reproductive success in buffalo populations.

To observe hatching rate 131 blastocysts were examined for their hatching rate at 2h, 12h, 24h and 48 h. An effect of hours was observed on the hatching rate of BLs. At 12h, 24h, 48 h 2,32 and 63 blastocysts hatched, respectively. Overall, 48.1% hatching rate achieved in this study. The observed hatching rate is in accordance with past study. The results are lower than the studies (Saha *et al*., 1996; Rodero *et al*., 2021) [21, 19]. from cows and higher hatching rate (Manjunatha *et al*., 2008) [10] in buffaloes.

Table 1: Number of follicles aspirated, and cumulus oocyte complexes recovered from slaughtered buffalo ovaries during 29 sessions

Sr. No	No of ovaries	Follicles	Cumulus Oocyte Complexes				
			G I (%)	G II (%)	G III (%)	G IV (%)	Total
1	38	96	29 (61.70)	13(27.66)	5 (10.64)	0 (0)	47
2	20	87	10 (50.00)	8 (40.00)	2 (10.00)	0 (0)	20
3	26	79	21 (58.33)	12(33.33)	3 (8.33)	0 (0)	36
4	30	96	26 (41.27)	22(34.92)	15 (23.81)	0 (0)	63
5	30	107	31(39.74)	25(32.05)	19 (24.36)	3 (3.85)	78
6	26	132	42 (39.62)	28(26.42)	32 (30.19)	4 (3.77)	106
7	15	98	17 (42.5)	7(17.5)	12 (30)	4 (10)	40
8	26	103	23 (53.49)	12(27.91)	6 (13.95)	2 (4.65)	43
9	26	97	32 (42.67)	30 (40)	13 (17.33)	0 (0)	75
10	24	93	27 (35.53)	31(40.79)	15 (19.74)	3 (3.95)	76
11	24	86	18 (31.03)	29(50)	10 (17.24)	1 (1.72)	58
12	22	92	24 (38.71)	28(45.16)	9 (14.52)	1 (1.61)	62
13	20	95	29 (37.18)	30(38.46)	16 (20.51)	3 (3.85)	78
14	30	96	32 (32.65)	36(36.73)	27 (27.55)	3 (3.06)	98
15	20	89	18 (33.96)	21(39.62)	12 (22.64)	2 (3.77)	53
16	26	85	22 (31.88)	23(33.33)	21 (30.43)	3 (4.35)	69
17	36	96	25 (32.05)	28(35.9)	23 (29.49)	2 (2.56)	78
18	8	43	11 (32.35)	12(35.29)	9 (26.47)	2 (5.88)	34
19	26	84	24 (40.68)	18(30.51)	16 (27.12)	1 (1.69)	59
20	12	60	20 (46.51)	11(25.58)	11 (25.58)	1 (2.33)	43
21	14	79	14 (29.79)	22(46.81)	9 (19.15)	2 (4.26)	47
22	20	73	16 (30.19)	23 (43.4)	12 (22.64)	2 (3.77)	53
23	20	81	11 (23.4)	22(46.81)	11 (23.4)	3 (6.38)	47
24	20	86	10 (20.41)	20(40.82)	17 (34.69)	2 (4.08)	49
25	20	91	13 (21.31)	24(39.34)	23 (37.7)	1 (1.64)	61
26	20	82	12 (20.69)	29 (50)	17 (29.31)	0 (0)	58
27	20	76	11 (20.75)	22(41.51)	19 (35.85)	1 (1.89)	53
28	20	69	20 (40.82)	10(20.41)	16 (32.65)	3 (6.12)	49
29	16	64	18 (39.13)	12(26.09)	14 (30.43)	2(4.35)	46
Total (Mean ± SE)	655 (23 ± 1.21)	2515 (87 ± 2.97)	606(36.09) (21 ± 1.47)	608 (36.21) (21± 1.46)	414(24.66) (14 ± 1.25)	51(3.04) (02 ± 0.23)	1679 (58 ± 8.2)

Table 2: Number of presumptive zygotes in IVC and their cleavage and blastocyst rate achieved during 29 sessions

Sr. No	Zygote in IVC	No. of zygote cleaved	Cleavage rate%	No. of Blastocyst	Blastocyst%
1	47	37	78.7	14	29.79
2	20	15	75	8	40
3	36	29	80.6	13	36.11
4	63	40	63.5	8	12.7
5	75	60	80	10	13.33
6	102	77	75.5	14	13.73
7	36	24	66.7	12	33.33
8	41	30	73.2	9	21.95
9	75	31	41.3	11	14.67
10	50	28	56	10	20
11	58	37	63.8	12	20.69
12	62	32	51.6	10	16.13
13	78	48	61.5	7	8.97
14	98	61	62.2	14	14.29
15	53	26	49.1	16	30.19
16	69	36	52.2	6	8.7
17	78	31	39.7	8	10.26
18	34	26	76.5	12	35.29
19	59	29	49.2	11	18.64
20	43	27	62.8	9	20.93
21	47	36	76.6	11	23.4
22	53	29	54.7	12	22.64

23	47	30	63.8	6	12.77
24	49	36	73.5	6	12.24
25	61	34	55.7	6	9.84
26	58	29	50	9	15.52
27	53	34	64.2	10	18.87
28	49	30	61.2	11	22.45
29	46	33	71.7	10	21.74
Total	1640	1015	63.1%	295	20%
(Mean ± SE)	(56.55 ± 3.33)	(35 ± 2.27)	(63.10±2.09)	(10.17 ±0.49)	(19.97 ± 1.58)

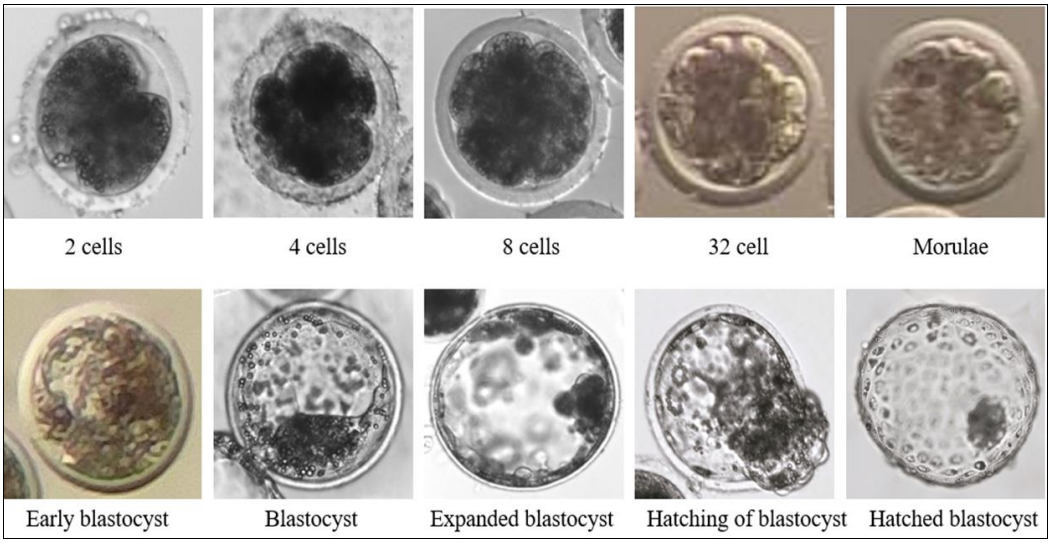

Fig 1: Different cleavage stages of buffalo origin zygotes from 2 cell to hatched blastocysts

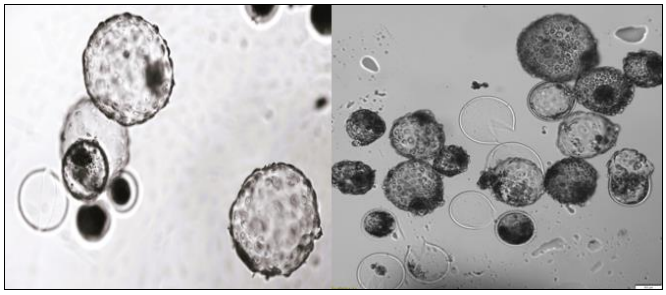

Fig 2: The figure illustrates the hatching and hatched blastocysts after *in vitro* culture between day 6 to 8.

Conclusion

The findings of this study are quite promising, indicating that technologies such as IVEP in buffaloes could prove to be a valuable asset in the field. This innovative approach holds the potential to salvage and amplify the genetic prowess of elite buffalo populations. By harnessing advanced reproductive techniques, we may unlock greater genetic diversity and enhance desirable traits within these populations. This advancement could have far-reaching implications for both conservation efforts and animal productivity, opening up new avenues for optimizing buffalo breeding programs.

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Conflict of Interest: There is no any conflict among the authors.

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