RESEARCH ARTICLE

Efficiency Evaluation of Two Media for *In Vitro* Maturation of Buffalo Oocytes

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Abstract

This study was conducted to evaluate morphometry of ovarian functional structures and effect of two different commercially available *in vitro* maturation (IVM) media on cytoplasmic and nuclear maturation of slaughter origin buffalo oocytes. The buffalo ovaries were collected from Ahmedabad Municipal slaughter-house, Ahmedabad and were transported at the laboratory within 2-3 h of slaughter at 33 to 35°C warm normal saline. The ovaries were washed and processed under laminar floor and evaluated for functional structures like mature corpus luteum (CL) and follicles i) <3 mm (small), ii) 3 to 6 mm (medium) and iii) >6 mm (large). The cumulus oocytes complexes (COCs) were aspirated with 18 G needle in 5 mL sterile disposable needle. The COCs were graded in to four categories (I to IV) and first three categories of COCs were used for IVM. For IVM 90 µL drops of BO IVM (IB; Bioscience) and IVM (VB; Vitrogen, Brazil) media were prepared and kept in benchtop incubator at 5 % O_2, 5% CO_2 and rest N_2 at 38.5 °C. The selected COCs were randomly allocated to two media treatments and incubated for 22 h. The cytoplasmic and nuclear maturation were observed using morphology and nuclear staining method (Hoechst 334452). The weight of ovaries with CL (6.63 ± 0.18 g) was significantly higher as compared to without CL (4.33 ± 0.14 g). Total 546 visible follicles with average 2.15 per ovary observed were aspirated from 254 buffalo ovaries and 440 COCs were collected. Overall oocyte recovery per ovary (OR/O), follicle recovery per ovary (FR/O) and oocyte retrieval per follicle (OR/F) were 1.73, 2.15 and 0.81, respectively. The small, medium and large follicles were 43.04, 41.94 and 15.01 %, respectively. The mean ± SE (n) count of oocytes with Grade I, Grade II, Grade III and Grade IV were 6.90 ± 0.61 (138), 6.50 ± 0.48 (130), 4.35 ± 0.40 (87) and 4.25 ± 0.54 (85) with recovery rate of 31.36, 29.54, 19.77 and 19.31 %, respectively. Total 133 and 137 COCs were randomly allocated to IB and VB media treatment. Total cytoplasmic maturation in IB and VB was 79.69 and 73.71 %. Similarly, the nuclear maturation was 78.19 and 71.53 % in IB and VB media. No significant differences in cytoplasmic or nuclear maturation between two media were observed. The study concludes that CL influences ovarian weight and both commercial medias can be used for successful *in vitro* maturation of COCs of buffaloes.

Key words: Buffalo, Cumulus oocytes complexes, *In vitro* maturation, Media

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Introduction

The higher feed conversion efficiency, resistance to diseases and better milk production with higher fat percentage made the buffaloes most favourable over cattle in Indian conditions. However, the factors such as silent or poor estrus expression, seasonality, late puberty, poor conception rate, prolonged inter-calving interval, and postpartum anoestrus reduce their reproductive efficiency (Suthar and Dhami, 2010; Barile *et al*., 2021). Therefore, intensive investigation on improving reproductive efficiency through use of an assisted reproductive technology is a need of time in India as well as at the global level (Barile *et al*., 2021). Manipulation of reproductive biology using *in vivo* hormonal treatment and *in vitro* embryo production using defined media is usual practice to improve reproductive efficiency and production. The widely and successfully applied first generation reproductive biotechnology is artificial insemination (Suthar and Dhami, 2010). The *in vitro* production of an embryo (IVEP) is rapidly growing in the animal reproduction field (Suthar and Shah, 2009). Compared to conventional superovulation and embryo transfer (ET), the production of embryos in the laboratory has many advantages, like, a) cost cutting for hormone, b) save man hours, c) more COCs harvest and greater chances of embryo conversion rate, d) more sire can be tested at a same time on harvest of COCs and e) slaughter origin COCs can be used for *in vitro* maturation (IVM), fertilization (IVF) and culture (IVC) (Suthar, 2008; Suthar and Shah, 2009).

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In vitro maturation (IVM) of oocyte is one of the most critical steps in the successful *in vitro* production of bovine embryos. It is one of the most vital processes, as it includes the oocyte's ability to complete meiosis/nuclear maturation, as well as biochemical, cytoskeletal and structural changes that will be important for fertilization and subsequent embryonic development (Dadarwal *et al*., 2015). For any successful *in vitro* embryo culture program, aspiration of the cumulus-oocyte complexes (COCs) from the follicles, culturing, and maturing them is a primary requirement (Dadarwal *et al*., 2015). The selection of potential oocytes is important for both human and animal-assisted reproductive technology that needs IVM of oocytes. The success rate of IVM is reported from 70 to 80 % in buffalos and it affected with factors like supplements in the media, laboratory environment and experience of embryologist (Suthar and Shah, 2009: Ruhil *et al*., 2015). Media is a crucial environment that contributes most to the successful IVM of oocytes. To date, many in-house media were prepared and tested for the same (Ruhil *et al*., 2015). After introduction of defined media (commercial) the success rate of IVM improved and giving successful results in IVEP consistently. Limited literature is available on performance of commercial media for IVM confirmation using nuclear maturation. Further it is high time as many commercial media were introduced by different companies in India which has boosted the success of embryo production. Hence the objective of this experiment was to study the morphometry of ovarian structures and effect of two different commercially available IVM media on cytoplasmic and nuclear maturation of slaughter origin buffalo oocytes.

Materials and Methods

Ovarian Collection

Buffalo ovaries of unknown reproductive status were collected from the Municipal slaughterhouse, Ahmedabad during October 2021 to February 2022. The slaughter origin ovaries were transported within 3 to 5 h of slaughter to the laboratory at 33 to 35°C in a thermos flask containing 0.9 % normal saline solution, which was supplemented with 1 % (w/v) antibiotic/antimycotic (10000 IU/mL penicillin, 10000 µg/mL streptomycin and 25 µg/mL amphotericin B (Gibco, USA). The ovaries were trimmed for extra tissue and washed thrice in warm sterile normal saline. Then wash once with 70 % ethyl alcohol for 30 sec to eliminate surface organisms and again by three washes with warm sterile saline (37°C) to remove the remained part of ethyl alcohol.

Ovarian Structure and Morphometry

The weight of ovaries with or without CL was recorded during the study. Ovaries were weighed using a digital pre-calibrated mono pan weighing balance (Reptech EMFC technology, RA series) having a minimum 0.1 g and maximum 220 g capacity with an accuracy of 0.0001 g. Visible follicles on each ovary were counted and follicle size was measured with the help of a Vernier callipers and classified those follicles into three groups as i) <3 mm (small), ii) 3 to 6 mm (medium) and iii) >6 mm (large). Oocyte recovery/ovary (OR/O), Follicles recovery/ovary (FR/O) and Oocyte retrieval/aspirated follicles (OR/F) were calculated (Mehmood *et al*., 2011; Shahid *et al*., 2014; Chaudhari *et al*., 2014).

Oocyte Aspiration, Grading, Allocation to Two Treatment and *In Vitro* Maturation

Six h prior to ovarian collection, IVM media drops were prepared under the laminar airflow cabinet with all aseptic precautions. Four IVM drops of 90 µL droplets were prepared in a 35 mm petri dish each for IVM media (VB; Vitrogen, Brazil) and BO maturation media (IB; IVF-Bioscience, Mumbai, India). Drops were covered with about 4 mL sterile lightweight silicone oil (Bioscience), then the petri dish was equilibrated in a benchtop incubator (Planer BT37 Mark II, Cooper Surgical, Lautrupparken 42, 2750 Ballerup, Denmark) at 38.5°C under 5% CO_2, 5% O_2 and rest N_2.

Cumulus Oocyte Complex (COCs) were collected by aspiration of surface follicles using an 18 G disposable needle attached to a 5 mL syringe containing 1 mL of pre-warmed sterile OPU medium (IVF-Bioscience). COCs were searched under a stereo zoom microscope (SZX7, Olympus, Japan). The COCs were collected and transferred into 500 µL drops of OPU medium covered with silicon oil in a 60 mm pre-sterilized petri dish. The COCs were washed thrice in OPU media and transferred in to 90 µL droplets of pre-warmed IVM wash media covered with silicone oil. Subsequent grading of immature COCs were performed under a stereo-zoom microscope at 50 to 100 X using morphological criteria and number of layers of cumulus cells and ooplasm characters as described by Das *et al*. (1996); Grade I: Oocytes with ≥5 layers of compact cumulus cell mass with evenly granulated cytoplasm. Grade II: Oocytes with at least 3 to 4 complete layers of cumulus cell mass with evenly granulated cytoplasm. Grade III: Oocytes with 1 to 2 layer of cumulus cell mass with evenly granulated cytoplasm. Grade IV: Denuded oocytes with no cumulus cells or incomplete layer of cumulus cells with unevenly granulated cytoplasm.

The first three grades (GI, GII and GIII) of COCs were selected and randomly allocated to two IVM media treatments (VB and IB) and were placed in a benchtop incubator (Cooper Surgical, Planner BT37) at 38.5°C under 5% CO_2, 5% O_2 and rest N_2 with 90% relative humidity for 20-22 h. The degree of cumulus expansion (degree 0, 1 and 2) was observed and the cytoplasmic maturation rate (CMR) was calculated.

CMR (%) = No. of oocytes expanded (degree 1 + degree 2) X 100 / Total oocytes cultured

The evaluation of nuclear maturation was confirmed by placing COCs in 200 µL of 0.25 % trypsin solution and incubated for 5 min at 38.5°C in benchtop incubator. After

5 min of incubation, COCs were washed twice with PBS. The cumulus cells were detached from the oocytes by repeated pipetting through a fine bore glass capillary and the denuded oocytes were placed separately in 20 µL of working solution of Hoechst 33342 stain (Sigma-Aldrich, SAFC, St. Louis, Missouri, United States) and incubated for 15 min in a benchtop incubator at 38.5°C temperature. The oocytes were examined under a 20X epifluorescence microscope (Olympus, BX53 Upright Microscope, Shinjuku, Tokyo, Japan) equipped with excitation filter around 350 nm, emission filter had a maximum 461 nm wave length for the nuclear maturation (MII) stages (Doultani *et al*., 2022). The denuded oocytes which demonstrated germinal vesicle (GV) stage or germinal vesicle breakdown (GVBD) were considered as an immature, while oocytes with anaphase, telophase or polar body (Metaphase II) in perivitelline space were considered as nuclear maturation for IVM.

Nuclear maturation (%) = Number of matured stained oocytes X 100 / Total oocytes stained

Statistical Analysis

Observations for ovarian weight, follicles and COCs retrieved were recorded in an excel sheet and analysed using IBM SPSS 26.0 software. The descriptive statistics function of SPSS was used to fetch descriptive values. The effect of presence of functional structure on ovarian weight, different size of follicles and effect of two different media on COCs maturation (cytoplasmic and nuclear) was evaluated using χ^2 test.

Results and Discussion

Ovarian Collection, Follicle Size and CL Weight

A total of 254 ovaries in 20 replicates were processed during collection period at IVF lab of Gujarat Biotechnology Research Centre, Gandhinagar (Gujarat), India. A careful examination of the ovaries revealed that 116 ovaries were with CL and 138 without CL. The overall mean ± SE ovarian weight of total ovaries (n=254) was 5.38 ± 0.13 g. There was significant effect of presence of CL on ovarian weight ($p < 0.001$). The average weight of ovary with CL (6.63 ± 0.18 g) was greater than ovary without CL (4.33 ± 0.14 g). This finding is in accordance of Zaber *et al*. (2020). However, the lower mean ovarian weight (3.5 ± 0.2 g) was also reported in buffaloes by Hasanzadeh and Sadeghinejad (2012).

The morphometry of ovarian follicles is presented in Table 1. The study revealed that the mean numbers of small and medium size follicles were significantly ($p<0.01$) higher as compared to large size follicles (0.93, 0.90 vs. 0.32), with the 43.04, 41.94 and 15.01 % distribution, respectively. Out of total 546 follicles, the overall number of follicles per ovary was 2.15. The percentage of small, medium and large size of follicles in present study was almost similar to the observations reported earlier (Mistry and Dhami, 2009; Pitroda *et al*., 2021).

Table 1: Three different sizes of ovarian follicles and no. of follicles/ ovary with different size

Size of follicles	Total No. of follicles	Follicle/ ovary	Percentage
<3 mm (small)	235^b	0.93	43.04
3 to 6 mm (medium)	229^b	0.90	41.94
>6 mm (large)	82^a	0.32	15.02
Overall	546	2.15	-

Data are the cumulative value of 20 replicates; Values bearing different superscripts within the column (a,b) differ significantly ($P<0.01$).

Cumulus Oocyte Complexes Recovery Rate, Grading and Maturation *In Vitro*

A total of 546 follicles were aspirated from 254 ovaries and total of 440 COCs were recovered. The overall oocytes recovered per ovary (OR/O), follicles recovered per ovary (FR/O) and oocytes recovered per follicle (OR/F) were 1.73, 2.15 and 0.81, respectively. Similar OR/O was recorded earlier in buffaloes by Das *et al*. (1996), and Jamil *et al*. (2008). The higher OR/O was also reported by Pitroda *et al*. (2021). The present results of FR/O matched with the reports of Jamil *et al*. (2008) in buffaloes. However, the higher FR/O in buffaloes has been also reported (Rahman *et al*., 2015). Further, OR/F was lower compared to the report of Shahid *et al*. (2014). The FR/O was dependent on the presence or absence of CL on ovary and the changes in the local microenvironment of reproductive tract like hormones concentrations and pH which may affect the follicular dynamics and ovarian quality (Zaber *et al*., 2020).

The overall recovery rate of COCs was 5.50 ± 0.29, while the Grade I, Grade II, Grade III and Grade IV COCs (Fig. 1A) were 6.90 ± 0.61 (138), 6.50 ± 0.48 (130), 4.35 ± 0.40 (87) and 4.25 ± 0.54 (85), respectively, with corresponding percentages as 31.36, 29.54, 19.77 and 19.31, respectively. In our study we achieved greater number of Grade I and Grade II than that of Grade III and Grade IV COCs ($P < 0.001$). Overall mean no. of COCs in our study were lower than the observation of Das *et al*. (1996), Mistry and Dhami (2009), and Rahman *et al*. (2015). This result might be due to the factors like environment, unknown nutritional status of slaughtered buffaloes and method itself.

The total cytoplasmic (Fig. 1 B and D) and nuclear maturation (Fig. 1 F and G) of COCs in IB and VB media are demonstrated in Table 2. No significant difference was observed between cytoplasmic or nuclear maturation of two media groups. The cytoplasmic maturation rate of present study agreed with Alsalim *et al*. (2021) in buffaloes. Interestingly lower per cent of maturation was reported in buffaloes by Chaudhari *et al*. (2014) and Magdy *et al*. (2018). Compared to our study, higher rate of cytoplasmic maturation was also reported in buffalo COCs (Mehmood *et al*., 2011; Pitroda *et al*., 2021) and in cows (Fukuda *et al*., 1990). The nuclear maturation rate of buffalo in the present study was in accordance to the report of Ruhil *et al*. (2015),

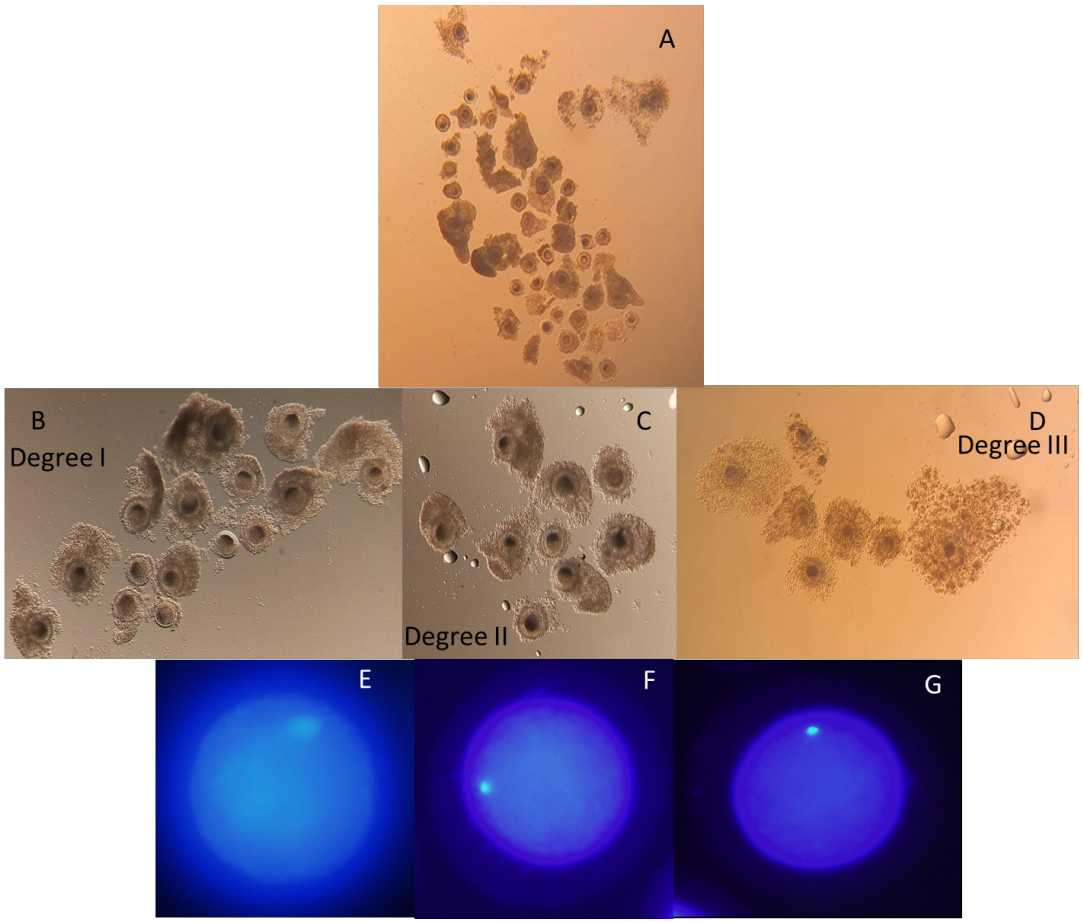

Fig. 1: Recovered buffalo immature Cumulus Oocytes Complexes-COCs (A), degree of *in vitro* maturation of COCs (B, C and D) and immature GVBD stage of oocyte (E) and nuclear maturation stage Metaphase II (F and G).

Table 2: Percentage (n) cytoplasmic and nuclear maturation of buffalo COCs in two different commercially available maturation media

Name of media	Total No. of COCs selected for maturation (n)	Percentage (n) of cumulus expansion and Cytoplasmic maturation				Percentage (n) of nuclear maturation
		Degree 0 (D0)	Degree 1 (D1)	Degree 2 (D2)	Pooled (D1 + D2)	
IB / BO IVM	133	20.30^{a} (27)	40.60^{b} (54)	39.09^{b} (52)	79.69^{c} (106)	78.19 (104)
VB/Vitrogen IVM	137	26.27^{a} (36)	34.30^{ab} (47)	39.41^{b} (54)	73.71^{c} (101)	71.53 (98)

Data are the cumulative value of 20 replicates; Figures in parentheses indicate numbers; Percentage values bearing different superscripts within the row (a,b,c) differ significantly (P<0.05) for cytoplasmic maturation.

who observed 80 % nuclear maturation in Way mouth MB media. However, lower nuclear maturation rates (36.68 to 74.06 %) using TCM-199 + 10 µg/mL FSH + 10 µg/mL LH with antibiotics media in buffalo oocytes with different stages have been reported by Ruhil *et al.* (2015), while higher rates of nuclear maturation using Ham's F 10 or Way mouth MB or TCM – 199 media in buffalo oocytes (77 to 80.13 %) have been also reported by others (Suthar and Shah, 2009; Mehmood *et al.*, 2011). The difference in the maturation rate may be due to the factors like transportation time of ovaries, temperature fluctuation, oocytes health at the time of collection, nutritional status of animal during slaughter, time interval in ovarian sample transportation to the laboratory, maturation media (Suthar and Shah, 2009), oocytes recovery method, presence or absence of cumulus cells, season, age of animal etc. Cumulus cells have more importance in cytoplasmic maturation as they support the maturation by providing energy, supply nutrients and prevent hardening of zona pellucida (Rahman *et al.*, 2015).

The time for oocyte maturation was identically 22 h in both the media under study. However, Rosario *et al.* (2022) stated that buffalo oocytes are more sensitive to the maturation time and they found that optimal time for maturation of oocytes was 18 h. Oocytes left in maturation media for more than 21 h leads to degenerative changes. In our study, the maturation studied around 22 h, may be a reason for the lower maturation rate in IB (79.69%) and VB (73.71%). However, with similar time the maturation rate was

reported 95 to 100 % in buffalo (Chauhan *et al*., 1998) for COCs collected from abattoir ovaries.

Conclusions

The buffalo ovaries with CL have the significantly higher weight than the ovaries without CL. Both commercial media (BO and Vitrogen) demonstrated almost similar cytoplasmic as well as nuclear IVM rate in accordance with past reports. Hence either of the commercial media can be used for *in vitro* maturation of buffalo oocytes.

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