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Thickness of cumulus cell layer is a significant factor in meiotic competence of buffalo oocytes

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This study evaluated the meiotic competence of buffalo oocytes with different layers of cumulus cells. A total of 588 oocytes were collected from 775 ovaries averaging 0.78 oocytes per ovary. Oocytes with homogenous cytoplasm (n = 441) were selected for *in vitro* maturation (IVM) and divided into four groups based on their cumulus morphology: a) oocytes with ≥ = 3 layers of cumulus cells, b) 1-2 layers of cumulus cells and oocytes with partial remnants or no cumulus cells to be co-cultured c) with or d) without cumulus cells. Oocytes in all four groups were matured in 100 μL drop of TCM-199 supplemented with 10 μg/mL follicle stimulating hormone (FSH), 10 μg/mL luteinizing hormone (LH), 1.5 μg/mL estradiol, 75 μg/mL streptomycin, 100 IU/mL penicillin, 10 mM Hepes and 10% FBS at 39°C and 5% CO_2 for 24 hours. After IVM, cumulus cells were removed from oocytes using 3 mg/mL hyaluronidase, fixed in 3% glutaraldehyde, stained with DAPI and evaluated for meiotic competence. The oocytes with ≥ = 3 layers of cumulus cells showed higher maturation rates ($p < 0.05$: 64.5%) than oocytes with partial or no cumulus cells (8.6%) and oocytes co-cultured with cumulus cells (34.5%) but did not differ from oocytes having 1-2 layers of cumulus cells (51.4%). The degeneration rates were higher ($p < 0.05$) for oocytes with partial or no cumulus cells (51%) than rest of the groups (range: 13.8% to 17.4%). These results suggest that buffalo oocytes with intact layers of cumulus cells show better IVM rates than oocytes without cumulus cells and the co-culture of poor quality oocytes with cumulus cells improves their meiotic competence.

Key words: buffalo, oocyte, cumulus, IVM

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Introduction

The successful *in vitro* maturation, fertilization, and culture (IVM/IVF/IVC) of bovine oocytes has brought interest to implement this technique in water buffalo (Bubalis bubalis) for *in vitro* production of embryos. The inherent problem of low oocyte yields from buffalo ovary [8,11,27,32] makes the use of IVF procedures questionable given the cost of IVF and low oocyte yield. This poor oocyte yield has been attributed to low number of primordial follicles (10,000 to 19,000) in the buffalo ovary [10,26] compared to 150,000 in cattle [14]. Despite this major factor, high rates of 70-90% for IVM [4,8,17,22], 60-70% for IVF [8,17,21,33] and 40-50% for cleavage rate [5,17,20, 21] have been observed. However, blastocyst development is still very poor and ranges between 10-30% [3,4,6,21,23]. The number and quality of oocytes further decreases during summer months [22,29] allowing fewer oocytes available for IVF studies. Considering the low yield of oocytes from the buffalo ovary, this investigation was carried out to utilize all the available oocytes with homogenous cytoplasm with varying layers of cumulus cells for IVM.

Materials and Methods

This study was conducted during the months of July and August when minimum temperature varied between 27°C (80.6°F) to 32°C (89.6°F) and maximum temperature varied between 31°C (87.8°F) to 37°C (98.6°F). The relative humidity varied between 66 to 89% during trial period. Unless specified, the reagents were from Sigma Chemicals (St. Louis, MO, USA).

Collection of oocytes

Ovaries from adult buffalos of Nili-Ravi breed were collected immediately after slaughter and transported to the laboratory within two hours of collection in an insulated container at 35-37°C. Upon arrival, ovaries were washed twice with normal saline containing 100 IU/mL of penicillin

and 100 µg/mL of streptomycin. Oocytes were aspirated from 2-8 mm follicles with an 18-gauge needle fitted to a 12 mL disposable syringe and transferred to 15 mL polystyrene centrifuge tubes in a 37°C water bath. Oocytes were recovered from the settled sediment after 15-20 minutes using a low power (20X) stereomicroscope. The data regarding number of ovaries used, number of oocytes collected, quality of the oocytes and the number of oocytes used in each replicate was recorded.

In vitro maturation of oocytes

Oocytes with homogenous cytoplasm were divided into four groups based on their cumulus morphology: a) oocytes with ≥ = 3 layers of cumulus cells, b) 1-2 layers of cumulus cells and oocytes with partial remnants or no cumulus cells to be co-cultured c) with or d) without cumulus cells. Oocytes in all four groups were washed thrice in medium 199 and transferred to 100 µL droplets (5-8 oocytes/drop) of maturation medium (TCM-199: Gibco Life Technologies, NY, USA) under sterile mineral oil in plastic dishes and incubated at 39°C and 5% CO_2 in air for 24 hours. The maturation medium was supplemented with 10 µg/mL FSH, 10 µg/mL LH, 1.5 µg/mL estradiol, 75 µg/mL streptomycin, 100 IU/mL penicillin, 10 mM Hepes, and 10% fetal bovine serum (FBS: Hyclone Laboratories Inc. Logan, UT, USA). Freshly detached cumulus cells were washed twice by centrifugation at 300 g in maturation medium and 1-1.2×10^6/mL cells were added to each microdrop of maturation medium in the co-culture group.

Evaluation for meiotic development

After IVM, oocytes in all groups were exposed to 3 mg/mL hyaluronidase in saline and cumulus cells were removed by repeated pipetting. Denuded oocytes were fixed in 3% glutaraldehyde in saline at room temperature for 15 minutes, rinsed and incubated in 0.001% 4, 6 diamidoino-2-phenylindole (DAPI), a fluorescent stain specific for nuclear material for 20 minutes at room temperature. Oocytes were rinsed in saline to remove DAPI particles, mounted on glass slides, and evaluated for meiotic development (oocytes reaching metaphase-II) at 400x using a Nikon microscope equipped with fluorescent illumination and filters giving maximum transmittance at 405 nm. An oocyte was classified as degenerated if the nuclear material was scattered in the ooplasm indicating spindle damage.

Statistical analysis

Data for meiotic development of oocytes among all groups was analyzed by chi square procedure using Statistix Analytical Software, Tallahassee, FL, USA.

Results

A total of 588 oocytes were aspirated from 755 ovaries in nine replicates of which 441 oocytes with homogenous cytoplasm and varying layers of cumulus cells were used for IVM while the remaining were used for cumulus cells or discarded due to poor quality. The number of oocytes collected averaged 0.78 per ovary (Table 1). The IVM rates for oocytes with ≥ = 3 layers (64.5%) and 1-2 layers of cumulus cells (51.4%) were significantly ($p < 0.05$) higher than oocytes with partial remnants or without cumulus cells (8.6%) and oocytes co-cultured with cumulus cells (34.5%). No difference was observed for IVM rates between oocytes having ≥ = 3 and 1-2 layers of cumulus cells. The degeneration rates were higher ($p < 0.05$) for oocytes without cumulus cells (51%) than all the other groups (Table 2).

Discussion

Cumulus cells have been considered to play an important role in oocyte maturation by keeping the oocyte under meiotic arrest, inducing meiotic resumption and by supporting cytoplasmic maturation. These functions have been attributed to their gap junctions and their specific metabolizing capabilities [31]. Physical contact between

Table 1. Oocyte yield and quality of oocytes collected from buffalo ovaries

Replicate Number	Total Ovaries	Total Oocytes	Oocytes with =3 layers cumulus cells	Oocytes with 1-2 layers cumulus cells	Oocytes with partial or no cumulus cells
1	70	56 (0.80)	14 (0.20)	12 (0.17)	30 (0.42)
2	102	82 (0.80)	22 (0.21)	20 (0.19)	40 (0.39)
3	88	72 (0.81)	22 (0.25)	20 (0.22)	30 (0.34)
4	82	66 (0.80)	18 (0.21)	16 (0.19)	32 (0.39)
5	68	53 (0.77)	18 (0.26)	15 (0.22)	20 (0.29)
6	83	51 (0.61)	14 (0.17)	12 (0.14)	25 (0.30)
7	89	66 (0.74)	17 (0.19)	16 (0.18)	33 (0.37)
8	102	82 (0.80)	19 (0.18)	20 (0.19)	43 (0.42)
9	71	60 (0.85)	22 (0.31)	17 (0.24)	21 (0.30)
Total	755	588 (0.78)	166 (0.22)	148 (0.20)	274 (0.36)

Numbers in parenthesis are average number of oocytes collected per ovary in respective group.

Table 2. *In vitro* maturation of buffalo oocytes with varying layers of cumulus cells

Groups	Oocytes	GV	GVBD	Metaphase-I	Metaphase-II	Degenerated
		Percentage				
Oocytes with ≥ = 3 layers of cumulus cells	118	4.2^{a} (5)	6.8^{ab} (8)	10.2^{a} (12)	64.5^{a} (76)	14.4^{a} (17)
Oocytes with 1-2 layers of cumulus cells	103	10.6^{ab} (11)	4.9^{a} (5)	15.6^{ac} (16)	51.4^{a} (53)	17.4^{a} (18)
Oocytes with partial or no cumulus cells	104	10.6^{ab} (11)	14.4^{b} (15)	15.4^{ac} (16)	8.6^{b} (9)	51.0^{b} (53)
Oocytes with partial or no cumulus cells + cumulus cells	116	15.5^{b} (18)	14.6^{b} (17)	21.6^{bc} (25)	34.5^{c} (40)	13.8^{a} (16)

abcd = Denote differences with in columns ($p < 0.05$) by Chi square.
Numbers in parenthesis show the number of oocytes.
GV = Germinal vesicle, GVBD = Germinal vesicle break down.

oocyte and cumulus cells has been considered necessary for the transfer of nutrients and factors essential for oocyte development [2]. However, dissociated cumulus cells have been reported to produce paracrine factors, which resume meiosis in denuded oocytes [13]. The results of this study showed that buffalo oocytes with homogenous cytoplasm surrounded by compact layers of cumulus cells had a significantly higher maturation rate than oocytes with partial remnants or no cumulus cells matured with or without additional cumulus cells. The modest increase in IVM rates of co-cultured oocytes in this study can be justified by the fact that paracrine factors produced by the added cumulus cells might have been only transferred to partially denuded oocytes via available gap junctions whereas such communication seems absent in similar oocytes matured without cumulus cells. This is also evident from the results that addition of cumulus cells not only improved IVM but also rescued oocytes from degeneration whereas more oocytes without somatic cell support underwent degeneration.

Present study yielded 0.78 oocytes per ovary, which is low compared to previous findings of 1.49 [8] and 1.76 [27] for the same breed of buffalo. This difference in oocyte yield is due to seasonal variation because the other studies were conducted during the cooler months of winter. Fewer follicles were found on buffalo ovaries at slaughter during summer than winter months [25] and buffaloes under heat stress produced fewer good quality oocytes than unstressed buffaloes [29]. The number of oocytes decreased from 1.7 to 0.9 [11] and from 0.7 to 0.4 [32] per ovary when selected for IVM on the basis of cumulus morphology. These findings are in agreement to cumulative number of 0.42 oocytes per ovary recovered with ≥ = 3 and 1-2 layers of cumulus cells in present study. Datta and Goswami [9] observed a significant drop from 1.02 to 0.84 in oocyte yield and a decrease from 0.21 to 0.14, 0.45 to 0.33 and 0.46 to 0.35 for good, average and poor quality oocytes per ovary in buffalo when temperatures increased from <25°C to >25°. Nandi *et al*. [22] also found a decline from 1.22 to 0.85 oocytes per ovary when oocytes were collected during cool (1-10°C) and hot (> 30°C) months, respectively. Their IVM rates also differed between cool (89%) and hot (72%) seasons but no difference was observed for fertilization, cleavage and blastocyst development because only matured oocytes were used for IVF/IVC. A marked decrease in oocyte yield, quality and developmental ability has been also reported in Bos taurus cows [24].

Our IVM rates for oocytes with ≥ = 3 and 1-2 layers of cumulus cells are lower than IVM rates of 84-91% previously reported for the same breed of buffalo [8]. This difference is due to season as well as the use of quality oocytes. In a previous study, Chauhan *et al*. [4] found significantly different IVM rates of 85, 54, and 26% for grade 1 (≥ = 5 layers of cumulus cells and homogenous cytoplasm), grade 2 (≤ = 4 layers of cumulus cells and homogenous cytoplasm) and grade 3 (without cumulus cells and irregular shrunken cytoplasm) buffalo oocytes. The subsequent fertilization, cleavage and blastocyst rates were also different in relation to the quality of the oocytes and no blastocyst was formed from grade 3 oocytes. They suggested that the embryo yield can be predicted after IVMFC by gross morphological appearance of the aspirated oocytes and grade 2 oocytes can be effectively used in buffalo IVF system however their paper lacks the information about the season of study which is a critical factor in buffalo reproduction. Similar observations were recorded for good, fair, and poor quality oocytes in Egyptian buffaloes [1]. Though buffaloes cycle throughout the year, they show a very significant seasonality in breeding that only 4% come into estrus from April through July [18]. Considering the low availability of quality oocytes from buffalo ovary, attempts have been made to utilize the oocytes recovered in denuded form during aspiration in an IVF system. In a previous study [12], addition of cumulus cells in maturation medium restored the nuclear maturation of

artificially denuded oocytes (64%) close to the compact cumulus enclosed oocytes (66%) but oocytes recovered in denuded form (46%) never reached the same levels of IVM. In another study [30], buffalo oocytes with compact and dense cumulus cells showed higher IVM rate ($p < 0.01$: 67.3%) than oocytes with thin cumulus layer (27.5%) or with small remnants of cumulus cells and poor naked oocytes (3%). These findings are in agreement to present results but again lack the information about season of study. Lower IVM and IVF rates have been also reported for cumulus free oocytes compared to cumulus enclosed oocytes in cattle [7,15,19,28,34].

In summary, the results of present study suggest that nuclear maturation can be restored in a substantial number of buffalo oocytes recovered in denuded form or with partial remnants of cumulus cells by addition of cumulus cells in maturation medium. However, further studies are required to understand the cytoplasmic maturation of such oocytes, which is essential for male pronucleus formation and subsequent embryonic development. We also suggest that IVM/IVF/IVC procedures in water buffalo should be preferably performed during the cool months of winter and season of experiment be reported in publications. Our understanding is that the studies on abattoir ovaries may not be truly representative of the potential of the buffalo ovary as mostly aged, underfed and post lactation animals are slaughtered. Therefore, further studies should be focused on IVMFC of oocytes recovered by ultrasound guided transvaginal aspiration from young live animals in good body condition.

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