Global Veterinaria 4 (2): 176-184, 2010
ISSN 1992-6197

Developmental Competence of Buffalo Oocytes from Follicles of Different Diameters Selected by Brilliant Cresyl Blue Staining

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Abstract: The objective of this study was to increase efficiency of blastocysts production from buffalo by selection of more competent oocytes for *in vitro* embryo production. Oocytes were selected on the basis of brilliant cresyl blue (BCB) staining correlated with follicular origin. Buffalo Cumulus oocyte complexes (COCs) were recovered from ovaries by follicles isolation and classified into 3 categories according to their diameter (small, <3mm; medium 3-5 mm and large >5-8mm). In experiment I. oocytes were placed immediately into culture without exposure to BCB stain (control) or stained with BCB for 90 min before culture. One part of the control and treated oocytes were fixed and stained to determine chromatin configuration at the time of recovery. Other part was cultured in *in-vitro* maturation medium for 24 h and fixed to evaluate meiotic competence of oocyte. In experiment 2: oocytes were culture for 24 h fertilized *in vitro* for determined cleavage rate after 2 days post IVF and blastocyst obtained after 8 days from IVF. The results showed that there was no difference in meiotic configuration at time of recovery. In contrast, the maturation rate to metaphase II was significantly ($P < 0.05$) higher for BCB+ and control than BCB-oocytes in all follicles diameters. Furthermore the cleavage rate after IVF was significantly ($p<0.05$) low for BCB-oocyte originated from all follicles size. Moreover, the BCB^+ oocyte yielded a significantly ($p<0.05$) higher proportion of blastocyst rate from medium and large follicles. In contrast, the lowest proportion of blastocyst was obtained for BCB^-oocytes from small follicles. Both BCB^+ and control had significantly ($p<0.05$) higher blastocysts than BCB-oocytes in all follicle diameters. In conclusion, the staining of buffalo COCs from different follicle diameters before *in vitro* maturation may be used to select more developmentally competent oocytes for IVF (BCB^+ oocytes originated from 3-8 mm follicles).

Key words: Buffalo · Oocytes · Follicles Diameter · BCB · G6PDH

INTRODUCTION

Follicular oocytes recovered from ovaries of slaughtered buffalo are commonly used to study the processes of maturation and fertilization and the technique of *in vitro* production of embryos. Most buffalo oocytes failed to develop to blastocyst stage [1, 2]. The inefficiency of *In vitro* production (IVP) of embryos has been attributed to oocyte quality at the start of maturation. The immature oocytes are routinely selected based on compaction of the cumulus corona investment and the homogeneity of the ooplasm [3]; this may reduce the yield of transferable embryos. As some oocytes with apparently normal morphology are in the early stage of degeneration. The size of the follicles seems to be an important factor in the selection of potential oocytes [4]. Moreover, the developmental capacity of oocytes is closely related to follicular dimension [4-12]. Buffalo in developing countries are usually slaughtered due to subfertility, poor body condition and advanced age. Immature oocytes from buffaloes with reduced reproductive performance or at reproductive senescence were heterogeneous in quality with low developmental competence because they come from follicles at different stages of growth and atresia as well as animal varying age and stages of the estrous cycle [13]. For these reasons establishment of effective protocols for oocyte selection is vital for obtaining consistent results. Selection of more

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homogenous and developmentally competent oocytes for IVP, brilliant cresyl blue (BCB) staining has been used to identify the most competent oocytes in several species such as mice [14]; pigs [15-19]; prepubertal goats [20-22], buffaloes [23] and cattle [24-29].

During the course of their growth, immature oocytes are known to synthesize a variety of protein, among them, G6PDH [30, 31]. This enzyme is active in the growing oocyte, but has decreased activity in oocytes that have finished their growth phase [31, 32]. BCB has been used to measure G6PDH activity. BCB test is based on the capability of G6PDH to convert the BCB stain from blue to colorless [16]. This enzyme may synthesized within the oocytes during oogenesis and is a component of the pentose phosphate cycle which provides ribose phosphate for nucleotide synthesis and much of the NADPH utilized as hydrogen or electron donor in reductive biosynthetic reaction such as the formation of fatty acids., thus, oocytes that have finished their growth phase show a decreased G6PDH activity and exhibit a cytoplasm with a blue coloration (BCB+) while growing oocytes are expected to high level of active G6PDH which results colorless cytoplasm (BCB-). Therefore, the present study was conducted to assess the effect of oocytes selection, using BCB, in relation to the follicle diameter, on developmentally competent buffalo oocytes before IVM and thereby increase the efficiency of blastocyst development after IVM/ IVF.

MATERIALS AND METHODS

All chemicals, if not described separately, were obtained from sigma-Aldrich, Taufkirchen. Germany.

Oocyte Collection: Apparently normal ovaries (n=985) of buffalo were collected from slaughterhouse (Kafr El Sheikh, Tanta and Cairo, Egypt). Immediately after slaughtering the ovaries was transported in physiological saline (0.9 Nacl) held at 35-37 °C, to cell culture laboratory of the Faculty of Veterinary Medicine, Kafr El Sheikh within 3h. Upon reaching laboratory, the ovaries were washed in fresh Dulbecco's PBS (D 6650). The follicles were isolated from the ovaries and classified according their size into 3 categories small < 3 (2-3mm), medium (3-5mm) and large > 5 (5-8mm). Cumulus oocyte complexes (COCs) were recovered by rupturing the isolated follicles with fresh recovered media (TCM 199 with Earle's salts and 25mM Hepes (M 7528) supplemented with 10% (V/V) heat treated FCS (F7524), follicles were opened and pressed with tweezers and a needle under stereomicroscope in order to observe the release of COCs. Only oocytes having a compact cumulus investment were used in the experiment.

Brilliant Cresyl Blue Staining: Immediately after collection of the buffalo compact COCs from different follicle diameters, they were washed 3 times in Dulbecco's PBS modified by the addition of 0.4% BSA (mDPBS). Then COCs were exposed to 26μM of BCB (B-5388) [23] diluted in mDPBS for 90 min. at 38.5°C in humidified air atmosphere. Following BCB exposure, the COCs from different follicle diameters were transferred to mDPBS and washed twice. After washing, the COCs were examined under a stereomicroscope at X50 and divided into two groups' according their cytoplasm coloration: oocytes with any degree of blue coloration to the cytoplasm (BCB+) and oocyte without blue cytoplasm (BCB-).

***In vitro* Maturation (IVM):** The classified COCs were washed three times in maturation medium [TCM 199 supplemented with 20% (V/V) heat-treated FCS and 10 μg/ ml FSH (ovagen, icp, New Zealand]. Oocytes were transferred in groups (5-10 in each group) into 50μL droplets of IVM culture medium. The droplets containing oocytes were covered with pre-warmed (38.5°C) mineral oil and incubated for 24h at 38.5°C in CO_2 incubator (5% CO_2 in air,-100% relative humidity).

***In vitro* Fertilization (IVF):** After IVM, oocytes were fertilized *in vitro* using frozen-thawed buffalo semen. The frozen semen of buffalo bull, known for high rates of IVF and cleavage was used. A motile sample of sperm was obtained by swim-up separation based on the method of Lonergan *et al.* [9]. Two straws of frozen semen (0.25ml/ straw; 20 X 10^6 sperm/ straw) were thawed in a water bath (73°C) for 1 min and emptied into 15 ml conical tube. The frozen-thawed semen was layered under 2 ml capacitation base medium (modified Ca++-free Tyrode's). Following incubation for 1h., the uppermost 0.5-1. 0 ml of the medium containing motile spermatozoa was removed and washed twice with 2-3 ml of capacitation base medium followed by centrifugation at 500xg for 7min. The resulting pellet was measured using adjustable micropipette. A 50-60 aliquot of the swim-up separated spermatozoa were then diluted with an equal volume of capacitation medium containing 200μg/ ml heparin (H 3393). After incubation for 15 min, the suspension was further diluted with capacitation base medium to reduce the concentration of capacitation inductors and to obtain the desired final concentration of spermatozoa for IVF. After maturation, the oocytes were transferred to modified

TALP medium and most of cumulus was removed mechanically by gentle pipetting. Five oocytes were placed in a 50 ml droplet of fertilization medium (TALP; 21) and 5-8 µl of final sperm suspension were added to each droplet to give final concentration of approximately 1.0 X 10^6 motile sperm/ ml in fertilization droplet. Fertilization was carried out for 24 h at 38.5°C under 5% CO_2 in 100% humidified air.

***In vitro* Culture of Embryos:** Twenty four hours after IVF, the presumptive zygotes were denuded and transferred to Menezo B_2 medium (Laboratories C.C.D., Paris and France). Twenty-four hours later after placement in the culture, the cleaved embryos were transferred to previously prepared buffalo oviductal epithelial cell (BOEC) monolayer formation were done as described by Nandi *et al.* [2]. After 3 days of culture, 40 µl of the culture medium was replaced with fresh embryo culture medium. On day 8 (day 1= day of fertilization), the percentage of blastocyst were recorded.

Evaluation of Oocytes Before and after IVM and IVF: To evaluate the stage of nuclear development, oocytes were fixed in buffered formol saline (BFS) and stained with Hoechst 33258 (B-1155) [24]. Oocytes in the control and treated groups of different follicles diameter were stained before 24h IVM. Based on their nuclear status described by Torner *et al.* [33] oocytes were classified as immature (germinal vesicle), in resumption of meiosis (germinal vesicle break down, metaphase I and anaphase I), mature (Telophase I and Metaphase II) or degenerating (pyknotic chromation at various meiotic stages or degenerated chromatin spread through oocyte). After IVF cleavage rate (number of eggs which had cleaved to the two-cell stage or beyond at 48h after IVF) and the proportion of the blastocysts developing at the end of the 8 day of culture period were compared among groups of different follicles diameters. The number of blastomeric (nuclei) in embryos was determined using Hoechst staining technique [34].

Experimental Design: Two experiments were carried out to investigate the importance of G6PDH activity in buffalo oocytes from different follicles diameter after classification of their G6PDH status by BCB staining.

Experiment I: The objective was to determine chromatin configuration and maturation capacity of buffalo oocytes in relation to BCB staining and follicle diameter. Immediately after follicles isolation and oocytes recovery, COCs were incubated in mPBS supplemented with (treated groups) or without (control) 26 µM of BCB for 90 min. After incubation, oocytes were washed 3 times in PBS and classified according to G6DH status. After exposure to BCB, some COCs were fixed to assess the chromatin configuration at time of recovery and the rest of the COCs were matured *in vitro* for 24h and after IVM, the oocytes were denuded from cumulus cells and fixed to determine maturation capacity in relation to BCB and follicle diameter. The experiment was replicated 8 times.

Experiment 2: To evaluate the developmental competence of the selected buffalo oocytes by BCB test from different follicle diameters. Oocytes from all groups were pooled to IVM and IVF as described above. The cleavage and blastocyst rates were recorded. The experiment was replicated 11 times.

Statistical analysis: The difference between the treatment groups (control, BCB+ and BCB-) and within each group was calculated by means of chi-square analysis or Fisher's exact test where appropriate. The overall chi square was calculated and found to be significant before performing the Fisher's exact test to detect the difference among treatment groups. One way analysis of variance (ANOVA) was performed by procedure of SAS/STAT to calculate simple liner rank statistics for the Wilcoxon, medium, Van der Warden and Savage scores to test was performed to analysis the differences between groups in number of cells per blastocyst. The difference having P values of the test equal to less than 0.05 ($p<0.05$) were considered statistically significant.

RESULTS

A total of 1846 buffalo compact COCs was recovered from different follicle diameters and used for the investigation, 393 COCs for chromatin configuration at the time of recovery based on BCB staining and follicles diameter. Also, 428 COCs to evaluate meiotic competence after 24 *in vitro* maturation and 1025 COCs for *in vitro* maturation, *in vitro* fertilization and further development.

Overall all experiments, a total of 1225 compact COCs exposed to BCB solution and was classified as BCB+ (blue stained oocytes) or BCB-(colorless oocytes) in different follicle groups. Through the experiments the mean proportion of COCs classified as BCB+ in small, medium and large follicles were 45.2, 58.9 and 53.9%, respectively (no significant difference between follicles group). As well as no difference in proportion of BCB^+ and BCB-for all oocytes exposed to BCB test regardless follicles diameter was 53.9 and 46.1%, respectively.

Table 1: Chromatin configuration of buffalo oocytes from different follicles diameter at the time of recovery (control) and after 90 min. of incubation in BCB (n=393)

Follicular diameter (mm)			Chromatin Configuration		
Groups	Parameter	Number of oocytes	Immature n (%)	In progression of meiosis n	Degenerated Oocytes
Small <3	Control	65	45 (69.2)	9	10
	BCB+	42	31 (73.8)	3	8
	BCB-	56	33 (58.9)	9	14
Medium 3-5	Control	48	36 (75.0)	5	7
	BCB+	59	48 (81.4)	3	8
	BCB-	38	25 (65.7)	5	8
Large >5-8	Control	29	19(65.6)	7	3
	BCB+	38	29 (76.3)	4	5
	BCB-	18	10 (55.6)	1	7

Table 2: Meiotic configuration of buffalo oocytes of different follicles diameter selected by BCB after 24 h. *in vitro* maturation (n=428)

Follicular diameter (mm)					
Groups	Parameter	Number of oocytes	Metaphase II n (%)	In progression of meiosis	Degenerated
Small <3	Control	43	29 (67.4) a	9	5
	BCB+	47	34 (72.3) a	9	4
	BCB-	52	28 (53.8) b	17	7
Medium 3-5	Control	63	51 (80.9) a	5	7
	BCB+	76	65 (85.5) a	0	11
	BCB-	51	34 (66.7) b	5	12
Large > 5-8	Control	32	26 (81.1) a	3	3
	BCB+	43	35 (83.3) a	1	7
	BCB-	21	12 (57.1) b	4	5

Within column, values with different letters (a:b) different significantly (P <0.05)

Table 3: Developmental competence of *in vitro* maturated and *in vitro* fertilized buffalo oocytes selected by BCB from different follicles diameter (n=1025)

Follicular diameter (mm)				
Groups	Parameter	Number of oocytes	Cleavage days-2 n (%)	Blastocyst day-8 n (%)
Small <3	Control	121	89 (73.5) a	10 (11.2) abB
	BCB+	109	87 (79.8) a	16 (18.3) aD
	BCB-	132	70 (53.0) b	4 (5.7) bE
Medium 3-5	Control	148	118 (79.7) a	25 (21.2) dAB
	BCB+	182	159 (87.3) a	56 (35.2) cC
	BCB-	132	83 (62.8) b	9 (10.8) eE
Large > 5-8	Control	72	58 (80.5) a	15 (25.8) fgA
	BCB+	81	69 (85.0) a	27 (39.1) fC
	BCB-	48	31 (64.5) b	4 (12.9) gE

Within columns, values with different letters (a: b, c: d, c: e, f: g, A: B, C:D) different significantly (P <0.05)

Table 4: Number of nuclei *in vitro* produced buffalo blastocyst on day 8 after IVF (n=168)

Follicular diameter (mm)			
Groups	Parameter	No of blastocyst	No of nuclei in blastocyst (x ± S.D)
Small <3	Control	10	90.8 ±8.48 a
	BCB+	16	94.2 ±8.88 a
	BCB-	4	68.2 ±4.20 b
Medium <3-5	Control	25	101.8 ±8.18 a
	BCB+	56	96.9 ±8.65 a
	BCB-	9	67.0 ±7.53 b
Large >5-8	Control	15	101.8± 7.68 a
	BCB+	27	99.3± 6.46 a
	BCB-	4	74.7± 7.55 b

Within column, values with different letters (a:b) different significantly (P <0.05)

The initial nuclear stages of oocytes from different follicles diameter selected after BCB exposure to the control before or after *in vitro* maturation (experiment I) as shown in Tables 1 and 2. Exposure of COCs from all follicle groups to BCB for 90 min was not associated with differences in proportion of immature oocyte at germinal vesicle stage of evaluated oocytes in all follicle diameters. As well as, no differences in chromatin configuration between BCB+ and BCB-oocytes in the same follicle diameters and between different follicle groups (Table1).

After 24 h *in vitro* maturation, there no significant difference in the proportion of oocytes reaching metaphase II between control and BCB+ in all follicles groups. The rate metaphase II was significantly lower ($P < 0.05$) in BCB-than BCB+ oocytes in all follicles diameter. Results after IVF and further development (Experiment 2) are shown in Table 3. There were no significant differences between control and BCB+ in cleavage rate two days after IVF in all follicles diameter. Conversely, significant differences ($p<0.05$) were recorded between BCB+ and BCB-in cleavage rate in all follicular group (Table 3). Significant differences ($p <0.05$) in the percentage of blastocyst were observed in day 8 after IVF between BCB+ and BCB-among all follicular diameter. The proportion of blastocyst from BCB+ selected was oocytes significantly higher ($p <0.05$) than control in medium and large follicles (35.2-39. 1 and 21.2-25.8 %, respectively) Table 3. Embryos in all blastocyst stage had normal morphology in all follicles diameter, the cell number in blastocysts were comparable in the control group and BCB+ selected group, but significantly decreased($p <0.05$) in BCB-group when compared to control and BCB+ in embryos from different follicles group (Table 4).

DISCUSSION

In vitro production of buffalo embryos provides an excellent opportunity for cheap and abundant embryos carrying out basic research and for application of emerging biotechnologies like cloning and transgenesis. The origin of the oocyte plays an important role in their *in vitro* fertilization (IVF) and subsequent developmental competence. Indeed, the first problem facing researcher's *in vitro* production of buffalo embryo is the small number of oocytes recovered per ovary and the most of these oocytes of poor quality [35]. Moreover, the most buffalo oocytes failed to develop to blastocyst stage after *in vitro* fertilization [1, 13]. The low developmental competence of *in vitro* matured buffalo oocyte is attributed to their quality at the beginning of maturation [3]. From distinguishable ovarian population, only fully grown oocytes are able to resume meiosis and progress into maturation. Therefore, identification and selection of developmental competent oocyte is crucial for success of embryo technologies. To our knowledge, there is the first parallel evaluation of influence follicles diameter and BCB staining in buffalo oocytes. Our investigation, as previous studies [8, 13, 23, 36] validated that follicles diameter and BCB selection after collection has beneficial effects on the percentage of matured and fertilized oocyte after IVM and IVF in buffalo. The results of the present study showed that about 45.2, 58.9 and 53.9. % from small, medium and large follicles diameter, respectively of BCB+ oocytes indicating that they finished growing and could be used for IVM /IVF. Our results revealed that, the staining with BCB are independent on follicle size whereas the methods of oocytes recovery may influence degree of heterogeneity of recovered COCs and oocyte from abattoir derived ovaries were heterogeneous coming from follicles in various stages of growth and atresia [37]. Moreover, regardless of follicle diameters only 53.4% of buffalo oocytes stained positively with BCB. This finding is in agreement with the result of Manjunatha *et al.* [23] in buffalo oocytes using 26μM of BCB, who observed that 57.2% of morphologically selected oocyte had blue coloration with BCB. Nearly similar results reported (58%) in cow [24] and in 66.5% heifer [29]. Higher values (75.6%) [37] 91.0% [16] of BCB^+ classified oocytes were observed in pigs. In prepubertal goat oocytes the proportions of fully grown to growing oocytes were 30.1% BCB^+ and 64.4% BCB-[20]. The discrepancy in the results may be due to the fact that BCB staining was influenced by oocyte morphological grade; this difference in morphological selection may associate with differences in observed BCB staining oocytes among laboratories.

In the present study, good quality immature oocytes at germinal vesicle stage were higher in BCB+ oocytes than BCB-oocytes in all follicles diameter, but this different statistically not significant. Similar results obtained in bovine [24] and in buffalo [23]. On the other hand, Heleil *et al.* [8] proved that competent good quality buffalo oocytes at time of recovery was obtained from ? 3 and 3-5mm follicles.

In this study, nuclear maturation was affected by exposure to BCB. The proportion of oocytes reaching metaphase II after IVM in the BCB-COCs was significantly lower ($p<0.05$) than control and BCB+ COCs from all follicle diameters. There are no reports concerning influence of BCB and follicle diameter together.

The results on buffalo (in the present study) were in agreement with that on porcine oocytes [16, 19] but different from those obtained from other species (cattle, buffalo and goat). On the other hand, Heleil *et al.* [7] and Torner *et al.* [33], in cattle, reported that maturation competence were independent of follicle size. Meiotic competence is closely correlated with oocytes sizes, which in turn correlated with follicles size [38]. Moreover, Manjunatha *et al.* [23] reported that buffalo BCB^+ oocytes had a significantly greater diameter (or volume) than BCB-. Oocyte diameter is determinant factor in acquiring meiotic competence in various species, cattle [39], buffalo [13] and pig [15]. These results proved that metaphase II rate was significantly higher in BCB+ oocytes than BCB^-oocytes within the same follicles diameter. The lower nuclear maturation rate of BCB-could be due to incomplete protein synthesis and abnormal cytoplasmic maturation.

Previous studies on bovine COCs found no difference in cleavage rate based on BCB status [23, 24]. This disagrees with our results, whereas difference in cleavage rate in BCB^+ range 79-85% in all oocytes from different diameters and BCB^-53-64% whereas this differences were significant ($p<0.05$). In studies investigating blastocyst development from oocytes sorted by BCB status, more mature BCB+ oocytes consistently yielded higher rate blastocyst development compare to BCB-oocytes [21, 24-27, 29]. In the present study, the percentage of BCB^+ oocytes that develop to blastocyst was significantly higher than in BCB-oocytes in all follicle groups. The low rate (5.7%) of blastocyst development for BCB^-oocytes in compare to (18.2%) blastocyst rate in BCB^+ oocytes was recorded in small follicles. Average values of 18.2, 35.2 and 39. 1% were recorded for blastocyst development of BCB+ oocytes in small, medium and large follicles, respectively, whereas the corresponding values were 5.7, 10.3 and 12.9% for BCB-oocytes. The BCB-oocytes exhibit a delay in mitochondria DNA (mt DNA).This replication due to delayed onset of expression of their nuclear encoded replication factors [18, 26]. Although BCB-oocytes make up their losses in expression of replication factors during IVM period, it is not enough to sustain embryonic development to blastocyst stage [40]. This reflected in the current result by lower cleavage and blastocysts rates as compared to BCB^+ and BCB-oocytes from each follicles diameter (Table-3) which agreed with result previous works [23] in buffalo, [24-26, 29] cows, [21, 41], goats and [15,19, 37, 42] in pig. Furthermore, there was direct correlation between cytoplasmic volume (diameter) and increase in DNA content in bovine [43] and porcine oocytes [15] in the studies of mouse and human indicated an important role for mtDNA copy number in fertilization and embryonic development [14]. A developmental competence of mammalian oocytes depend on a high rates of RNA synthesis, imprinting processes and biogenesis of organelles [44] transcript abundance for ribosomal RNA genes below the optimal threshold would jeopardies the oocytes competence for development [45, 46].

The quality of blastocyst assessed in this investigation as a number of cells was significantly better for blastocyst derived from control and BCB^+ than blastocyst from BCB-oocytes, similar results of other studies [24, 25] who reported that blastocyst in BCB-group had significantly lower cell number than controls. Pujol *et al.* [29] similarly found that BCB staining was accurate for selecting against incompetent heifer oocytes. The cell number in the embryos is an important indicator of embryonic development and health. It has been suggested that embryos with a large number of cells and more likely to important and give rise to live off spring [47]. Therefore, these results confirmed that BCB test enhanced the reliability and efficiency of selecting for high quality homologous oocytes for buffalo IVF.

In conclusion, the classification of buffalo cumulus-oocyte complexes on the basis of BCB staining (or G6POH) and follicle diameters before *in vitro* maturation could be effectively used to select buffalo oocytes in terms of further developmental competence and could thereby positively influence the blastocyst development from different follicle diameters. Further researches are needed to explain the developmental competence of buffalo oocytes from small follicles and BCB^-group.

ACKNOWLEDGEMENT

The authors would like to thank Dr. Helmut Torner, Research institute for biology of farm animals, 18196 Dummerstorf, Germany for providing the necessary facilities to conduct this research.

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