Cell Research (2007) 17:722-731.

www.nature.com/cr

ORIGINAL ARTICLE

Selection of oocytes for *in vitro* maturation by brilliant cresyl blue staining: a study using the mouse model

Yan-Guang Wu[1], Yong Liu[1], Ping Zhou[1], Guo-Cheng Lan[1], Dong Han[1], De-Qiang Miao[1], Jing-He Tan[1]

[1]*Laboratory for Animal Reproduction and Embryology, College of Animal Science and Vet Medicine, Shandong Agricultural University, Tai-an 271018, Shandong, China*

Selecting oocytes that are most likely to develop is crucial for *in vitro* fertilization and animal cloning. Brilliant cresyl blue (BCB) staining has been used for oocyte selection in large animals, but its wider utility needs further evaluation. Mouse oocytes were divided into those stained (BCB+) and those unstained (BCB−) according to their ooplasm BCB coloration. Chromatin configurations, cumulus cell apoptosis, cytoplasmic maturity and developmental competence were compared between the BCB+ and BCB− oocytes. The effects of oocyte diameter, sexual maturity and gonadotropin stimulation on the competence of BCB+ oocytes were also analyzed. In the large- and medium-size groups, BCB+ oocytes were larger and showed more surrounded nucleoli (SN) chromatin configurations and higher frequencies of early atresia, and they also gained better cytoplasmic maturity (determined as the intracellular GSH level and pattern of mitochondrial distribution) and higher developmental potential after *in vitro* maturation (IVM) than the BCB− oocytes. Adult mice produced more BCB+ oocytes with higher competence than the prepubertal mice when not primed with PMSG. PMSG priming increased both proportion and developmental potency of BCB+ oocytes. The BCB+ oocytes in the large-size group showed more SN chromatin configurations, better cytoplasmic maturity and higher developmental potential than their counterparts in the medium-size group. It is concluded that BCB staining can be used as an efficient method for oocyte selection, but that the competence of the BCB+ oocytes may vary with oocyte diameter, animal sexual maturity and gonadotropin stimulation. Taken together, the series of criteria described here would allow for better choices in selecting oocytes for better development.

Keywords: brilliant cresyl blue staining, glucose-6-phosphate dehydrogenase, *in vitro* maturation, oocyte quality

Cell Research (2007) **17**:722-731. doi: 10.1038/cr.2007.66; published online 7 August 2007

Introduction

In vitro matured (IVM) oocytes are increasingly demanded in both human *in vitro* fertilization (IVF) and animal *in vitro* embryo production and cloning. However, it is recognized that the developmental capacity of bovine IVM oocytes is inferior to that of the *in vivo* matured (IVO) oocytes [1]. The developmental competence of IVM human oocytes is also markedly lower than that of their IVO counterparts [2-7]. The low developmental competence of IVM oocytes is certainly related to their quality at the beginning of maturation. Therefore, selecting oocytes that are most likely to develop is crucial for both assisted human reproductive technology and animal embryo technologies involving IVM oocytes.

Follicular oocytes are routinely selected for IVM on the basis of the assessment of morphological features such as cumulus thickness and compactness and the homogeneity of the ooplasm [8, 9], and sizes of follicles [10, 11] or oocytes [12, 13]. However, it has been found that immature oocytes from cows with reduced reproductive performance or slaughtered at the end of their use are heterogeneous in quality [9]. The developmental capacity of oocytes from pre-pubertal animals was lower than that of oocytes from adult animals (cattle: [14-17]; sheep: [18, 19]; pig: [20]; goat: [21, 22]). Follicular waves and advanced atresia of the ovary also affected the developmental competence of oocytes [11, 23]. A limited number of studies have

Correspondence: Jing-He Tan
Tel : +538 8249 616; Fax: +538 8241419
E-mail: tanjh@sdau.edu.cn
Received 26 October 2006; revised 1 January 2007; accepted 12 March 2007; published online 7 August 2007

demonstrated that the developmental potential of human oocytes was also affected by the age of the woman [24], size of follicles [25, 26] or oocytes [27], follicle dominance [28] and ovarian stages [29]. Furthermore, it has been shown that fully grown mouse oocytes do not constitute a homogeneous population in terms of germinal vesicle (GV) chromatin configuration [30, 31]. Therefore, clearly characterizing ovarian oocytes biochemically to establish non-invasive and non-perturbing means for selection of oocytes prior to culture has become of prime importance.

The enzyme glucose-6-phosphate dehydrogenase (G6PDH) is active in the growing oocyte, but has decreased activity in fully grown oocytes [32]. Because G6PDH can convert the brilliant cresyl blue (BCB) stain from blue to colorless [33], BCB test has been used for selection of grown oocytes. Studies in large animals showed that oocytes stained with BCB (BCB+) were larger and more competent in maturation and development than those that were unstained (BCB−) [17, 34, 35]. However, only 70% of the BCB+ cow oocytes matured *in vitro*, with even less (34%) forming blastocysts [36]. The BCB+ oocytes from pre-pubertal animals performed even more poorly; only 12% and 4% of the BCB+ oocytes from heifers [17] and pre-pubertal goats [35] formed blastocysts, respectively, after IVF. This suggests that the BCB+ oocytes may not constitute a homogeneous population. Therefore, studies on additional factors affecting the developmental potential of BCB-selected oocytes will definitely contribute to the establishment of more specific criteria for oocyte selection and promote the utilization of the non-invasive and non-perturbing BCB test for oocyte selection. In addition, although the activity of G6PDH has been found to be high in follicular oocytes but to drop significantly in ovulated oocytes in mice [37, 38], BCB staining has not been performed in this species.

The objective of this study is to evaluate the utility of BCB staining for oocyte selection, using the most commonly used mouse oocyte model. The results showed that BCB+ oocytes gained better cytoplasmic maturity (determined as the intracellular GSH level and pattern of mitochondrial distribution) and higher developmental potential after IVM than the BCB− oocytes. The better cytoplasmic maturity and higher developmental potential of the BCB+ oocytes were correlated with their higher percentages of surrounded nucleoli (SN) chromatin configuration and higher frequencies of early atresia in comparison with the BCB− oocytes. The cytoplasmic maturity and developmental competence of the BCB+ oocytes were also affected by oocyte diameter, animal sexual maturity and gonadotropin stimulation. The series of criteria described here would allow for better choices in selecting oocytes for better development.

Materials and Methods

Chemicals were purchased from Sigma Chemical Co (St. Louis, MO, USA) unless otherwise specified.

Oocyte collection

Procedures for animal handling, superovulation and oocyte collection were those described previously [39, 40], with modifications. Briefly, mice of the Kun-ming breed, originally derived from ICR (CD-1), were kept in a room with 14 h/10 h light-dark cycles, the dark starting from 8 PM. To obtain follicular oocytes, female mice, 6-8 wk (or 20-25 days) after birth, were primed with pregnant mare serum gonadotropin (PMSG, 10 IU, ip) and were killed at 46 h after PMSG administration. Follicles on the ovary were ruptured in medium M2 and oocytes with complete, compact cumuli were collected. To recover ovulated oocytes, female mice, 6-8 wk after birth, were induced to superovulate with PMSG (10 IU, ip), followed 48 h later by human chorion gonadotropin (hCG, 10 IU, ip). They were killed at 12 h after hCG injection, and oocytes were collected by rupturing the oviduct ampula.

BCB staining

The cumulus-oocyte complexes (COCs) obtained were first washed three times in medium M2 and then incubated in M2 containing 26 μM BCB for 90 min at 37 ºC in humidified air atmosphere. Oocytes in the control group were cultured in M2 without BCB for the same period. This concentration and duration of BCB staining was used because our preliminary experiment had shown that 1 h was not enough for G6PDH to reduce BCB to a colorless compound and that mouse oocytes of any size could not be stained at the concentration of 13 μM. After staining, COCs were washed in M2 and examined under a stereomicroscope at magnification 50×. They were classified into different groups according to BCB coloration in the ooplasm and/or cumulus cells. Phenol red was omitted from medium M2 during BCB staining and observation because it interfered with BCB coloration of oocytes under the microscope.

Measurement of oocyte diameters

After BCB test, follicular oocytes were divided into large (>70 μm), medium (60-70 μm) and small (<60 μm) groups according to their diameters excluding the zona pellucida (DEZ). The DEZ of an oocyte was measured with cumulus cells under a microscope using a micrometer (Figure 1A). To validate the DEZ measurement, some of the measured oocytes of different sizes were denuded of cumulus cells and their diameters including the zona (DIZ) were measured again under a microscope. The results showed that the average DEZ and DIZ of the large (n=19), medium (n=16) and small (n=8) oocytes measured were 76.7 ± 0.7 and 87.9 ± 0.8 μm, 65.9 ± 1.1 and 76.1 ± 0.8 μm, and 55.9 ± 1.5 and 66.6 ± 1.2 μm, respectively.

In vitro maturation

The BCB+ and BCB− oocytes of different sizes were cultured separately in groups of around 10 in droplets of 50 μl, covered with mineral oil, at 37.5 ºC under 5% CO_2 in humidified air. The maturation medium was TCM-199 (Gibco, Grand Island, New York, USA) supplemented with 10% (v/v) FCS (Gibco), 1 μg/ml 17 β-estradiol, 24.2 mg/L sodium pyruvate, 0.05 IU/ml FSH, 0.05 IU/ml LH and 10 ng/ml EGF. At 24 h of culture, oocytes were stripped of their

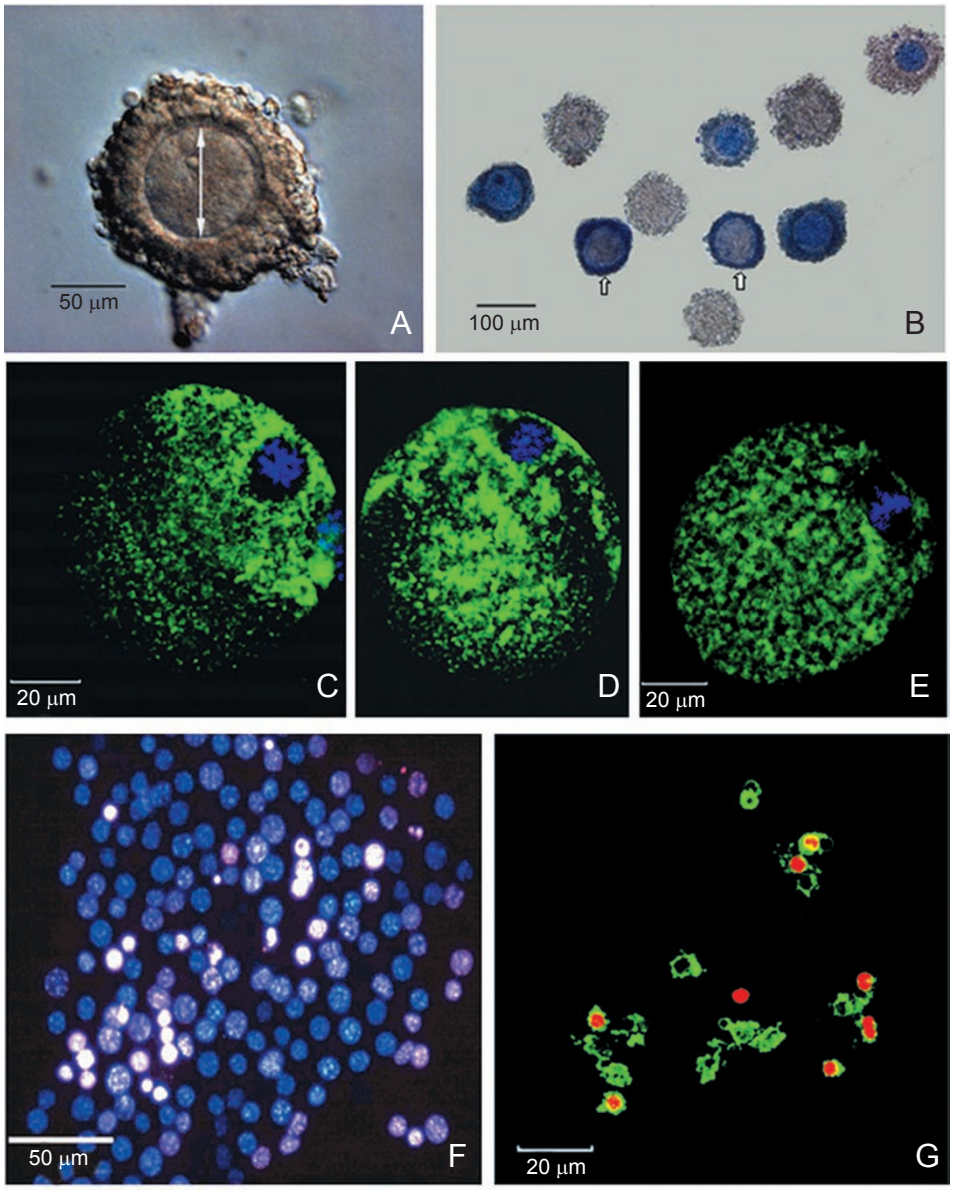

Figure 1 (A) The diameter excluding the zona pellucida (DEZ) of an oocyte was measured with cumulus cells; oocyte plasma membrane was brought into the clearest focus under a phase contrast microscope and the DEZ was measured with a micrometer. **(B)** Four groups of COCs were observed under a microscope after BCB staining: (a) those with a blue color in both the ooplasm and cumulus cells, (b) those showing a blue color in neither ooplasm nor cumulus cells, (c) those with blue color in ooplasm but not in cumulus cells and (d) those with blue color in cumulus cells but not in ooplasm (arrows). **(C-E)** Oocytes with fully polarized, partially polarized and non-polarized patterns of mitochondrial distribution, respectively (refer to text for detailed description). **(F)** After double staining, all the nuclei of cumulus cells were stained blue by Hoechst 33342, but only the nuclei of apoptotic cells were stained red by PI and hence looked pink under the fluorescence microscope. **(G)** A merged picture after Annexin V and PI staining, in which early apoptotic cumulus cells were stained exclusively by Annexin V (green), late apoptotic cells were stained by both Annexin V and PI (red), while necrotic cells were labeled by PI, but were negative for Annexin V.

cumulus cells by pipetting in M2 containing 0.1% hyaluronidase. Denuded oocytes with a polar body were considered matured and selected for different treatments.

Assay of intracellular glutathione

Intracellular content of glutathione (GSH) was measured as described by Funahashi *et al*. [41]. IVM oocyes were denuded of cumulus cells and washed three times in M2. A volume of 5 μl of distilled water containing 35-40 oocytes was transferred to a 1.5 ml microfuge tube, and then 5 μl of 1.25 M phosphoric acid was added to the tube. Samples were frozen at −80 °C and thawed at room temperature. This procedure was repeated three times. Then, the samples were stored at −20°C until analyzed. Concentrations of GSH in the oocyte were determined by the DTNB-GSSG reductase recycling assay. Briefly, 700 μl of 0.33 mg/ml NADPH in 0.2 M sodium phosphate buffer containing 10 mM EDTA (stock buffer, pH 7.2), 100 μl of 6 mM 5,5′ dithio-*bis* (2-nitrobenzoic acid) (DTNB) in the stock buffer and 190 μl of distilled water were added and mixed in a microfuge tube. A volume of 10 μl of 250 IU/ml GSH reductase (Sigma, G-3664) was added with mixing to initiate the reaction. The absorbance was monitored continuously at 412 nm with a spectrophotometer for 3 min, with reading recorded every 0.5 min. Standards (0.01, 0.02, 0.1, 0.2 and 1.0 mM) of GSH and a sample blank lacking GSH were also assayed. The amount of GSH in each sample was divided by the number of oocytes to get the intracellular GSH concentration per oocyte.

Observation of mitochondria distribution

Oocytes denuded of cumulus cells were washed in M2 and incubated in 10 μg/ml Rhodamine 123 in M2 at 37 ºC for 15 min. Then, they were washed three times in M2 and stained for 5 min at room temperature with 10 μg/ml Hoechst 33342 in M2. After being washed three times in M2, oocytes were mounted on a slide and observed with a Leica laser scanning confocal microscope (TCS SP2). The Rhodamine 123 fluorescence was excited with the 488 nm line of an Ar/ArHr laser, and the emitted light was passed through a 488 nm filter.

Parthenogenetic activation and embryo culture

Oocytes were first treated with 10 % (v/v) ethanol in M2 for 5 min at room temperature and then incubated in CZB medium [42] containing 2 mM 6-DMAP and 5 μg/ml cytochalasin B (CB) in a CO_2 incubator at 37.5 ºC for 4 h. At the end of incubation, oocytes were examined under a microscope for activation. Activated oocytes with pronuclei were cultured for 4 d in the regular CZB medium without CB at 37.5 ºC in a humidified atmosphere with 5% CO_2 in air. Glucose (5.5 mM) was added to CZB when embryos were cultured beyond the 3- or 4-cell stages. Embryonic development was observed and numbers of embryos at different stages were recorded at the end of embryo culture.

Observation of GV-chromatin configuration

The BCB+ and BCB− oocytes of different sizes were denuded of cumulus cells separately. The denuded oocytes were labeled by incubation for 5 min in M2 containing 10 μg/ml Hoechst 33342 at 37.5 ºC under 5% CO_2 in humidified air. Oocytes of the same group were then placed on a glass slide and squashed with coverslips to visualize GVs. Observation was performed with a Leica DMLB microscope equipped with an MPS 30 camera. The GVs were examined first by phase-contrast microscopy to visualize the morphology of nucleoli and nuclear envelopes, and then by fluorescence optics. Hoechst 33342 fluorescence was obtained by excitation at 220-360 nm using a mercury lamp (50 W) with neutral filters. The GV chromatin configuration of oocytes was classified into the non-surrounded nucleoli (NSN) pattern with diffuse chromatin throughout the nucleus and the SN pattern with chromatin condensed into a perinucleolar rim [43].

Detection of cell apoptosis

After BCB test, the BCB+ and BCB− oocytes of different diameters were subjected to a double or a triple staining for assessment of cumulus cell apoptosis. For double staining, COCs were incubated individually for 2 min in drops of medium M2 containing 2 μg/ml Hoechst 33342 and 5 μg/ml propidium iodide (PI) at room temperature. At the end of incubation, COCs were washed individually in drops of fresh M2 and placed on a glass slide, squashed with a coverslip and observed under a Leica DMLB microscope. For triple staining, COCs were washed three times in the binding buffer (150 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1.8 mM $CaCl_2$, 10 mM Hepes, pH 7.4) before being incubated in the dark for 10 min in 10 μg/ml Annexin V-FITC contained in the buffer. After being washed three times in the buffer, the COCs were incubated in M2 containing 10 μg/ml Hoechst 33342 and 10 μg/ml PI for 10 min at room temperature. At the end of incubation, the COCs were pipetted individually in M2 to release cumulus cells. The released cumulus cells were smeared on a slide and observed under a Leica laser-scanning confocal microscope. Hoechst 33342-labeled nuclear chromatin was excited with the 405 nm line of a diode laser. The FITC and PI fluorescence was obtained by excitation with the 488 nm line of an Ar/ArHr laser and the emitted light was passed through a 488 nm filter. The individual optical sections were pseudo-colored and digitally recombined into a single composite image using the Leica Confocal Software. Percentages of the apoptotic cells were calculated from 200 cells in each oocyte following double or triple staining.

Data analysis

For each treatment, at least three replicates were run. Statistical analyses were carried out by ANOVA. Differences between treatment groups were evaluated with the Duncan multiple comparison test. Data are expressed as mean±SE and $P<0.05$ is considered significant.

Results

Classification and distribution of BCB+ and BCB− mouse oocytes of different sizes

Four groups of COCs were observed after BCB staining of the mouse follicular oocytes: (A) those with a blue color in both the ooplasm and cumulus cells, (B) those showing a blue color in neither ooplasm nor cumulus cells, (C) those with blue color in ooplasm but not in cumulus cells, and (D) those with blue color in cumulus cells but not in ooplasm. In this study, COCs with blue color in ooplasm (groups A and C) were classified as BCB+, whereas those with no blue color in ooplasm (groups B and D) were classified as

BCB−. The BCB+ proportion in the large oocytes (83.3%, n=288) was significantly (P<0.05) higher than that in the medium oocytes (55.6%, n=247). However, 95% (n=78) of the small oocytes were of BCB+ coloration. To test whether cumulus cells would interfere with or facilitate BCB staining of the ooplasm, some oocytes were stained with BCB after denudation of cumulus cells. The result showed that none of the follicular oocytes in group B displayed a blue color when stained for the second time after denudation, and that over 98% of the ovulated oocytes were BCB+ when stained after removal of cumulus cells (data not shown). This indicates that cumulus cells neither interfere with nor facilitate BCB staining of the ooplasm.

IVM and embryo development of oocytes with different BCB coloration and sizes

In both the large- and medium-size groups of follicular oocytes, rates of blastulation were significantly ($P < 0.05$) higher in BCB+ oocytes than in BCB− oocytes and than in control oocytes that had not been exposed to BCB (Table 1). Blastulation rates of BCB+ oocytes were significantly ($P < 0.05$) higher in the large-size group than in the medium one. The BCB+ oocytes in the small-size group failed to mature *in vitro*.

Effects of BCB coloration and size of oocytes on their concentration of intracellular GSH after IVM

The intracellular concentration of GSH was measured after IVM of BCB+ and BCB− oocytes of large and medium sizes. Three samples (35-40 oocytes/sample) from three replications were assayed for each treatment. The intracellular concentrations of GSH in BCB+ oocytes were significantly ($P < 0.05$) higher than those of BCB− oocytes in both the large- (7.44 ± 0.15 vs. 3.57 ± 0.26 pmol/oocyte) and medium-size groups (5.27 ± 0.15 vs. 3.69 ± 0.13 pmol/oocyte). The GSH concentration was higher ($P < 0.05$) in BCB+ oocytes of large sizes than in those of medium sizes.

Effects of BCB coloration and size of oocytes on their distribution of mitochondria after IVM

Three patterns of mitochondrial distribution were observed in mouse oocytes after Rhodamine 123 staining. In the fully polarized (FP) pattern, most of the mitochondria aggregated in the animal hemisphere around the M II spindle of the oocyte (Figure 1C). In the partially polar-

Table 1 Maturation and embryo development of BCB+ and BCB− mouse follicular oocytes of different diameters

Oocyte diameter (μm)	BCB staining	Oocytes observed	% MII oocytes	Embryos cultured	% Embryos	
					4-cell	Blastocyst
> 70	Control	101	85.2±1.6[a]	84	68.1±1.3[a]	15.7±1.3[a]
	+	240	90.3±1.3[a]	98	79.5±2.8[b]	20.3±1.0[b]
	−	48	46.5±2.1[b]	55	47.9±3.7[c]	1.9±1.9[c]
60-70	Control	99	69.5±1.3[c]	59	49.8±1.4[c]	6.9±0.6[d]
	+	138	81.5±1.8[a]	72	63.1±2.6[a]	12.5±0.2[a]
	−	109	47.0±1.2[b]	59	35.7±4.2[d]	0.0±0.0[c]
< 60	Control	20	5.0±5.0[d]			
	+	74	5.5±3.6[d]			
	−	4	0.0±0.0[c]			

[a-d]Values with common letters in their superscripts in the same column did not differ significantly ($P > 0.05$).

Table 2 Patterns of mitochondrial distribution in the BCB+ and BCB− oocytes of different diameters following IVM

Oocyte diameter (μm)	BCB	Oocytes observed	Patterns of mitochondrial distribution	
			Fully polarized	Non-polarized
IVO control		33	88.1±2.4[a]	0.0±0.0[a]
> 70	+	39	64.8±2.3[b]	4.2±2.4[a]
	−	36	39.2±3.4[c]	2.9±2.9[a]
60-70	+	38	53.1±3.1[d]	4.6±2.5[a]
	−	33	33.5±3.7[c]	0.0±0.0[a]

[a-e]Values with common letters in their superscripts in the same column did not differ significantly ($P > 0.05$).

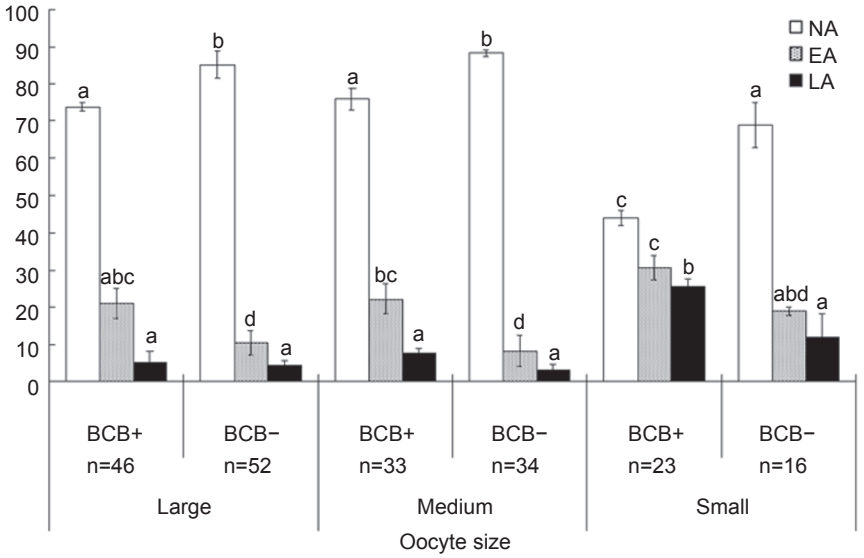

Figure 2 Distribution of non-atretic (NA), early atretic (EA) and late atretic (LA) mouse oocytes of different diameters in the BCB+ and BCB− coloration groups. The number underneath bars represents the number of oocytes observed for each treatment. Values with common letters above the bars within the same atretic (NA, EA or LA) group did not differ significantly ($P > 0.05$).

ized (PP) pattern, while many mitochondria aggregated in the animal hemisphere, still many were located in the vegetal hemisphere (Figure 1D). The non-polarized (NP) pattern was characterized by a uniform mitochondrial distribution throughout the ooplasm (Figure 1E). Around 90% of the ovulated (IVO) oocytes showed a FP pattern (Table 2). Percentages of FP patterns after IVM were significantly ($P < 0.05$) higher in BCB+ than in BCB− oocytes. The proportion of BCB+ oocytes with FP patterns was significantly higher in the large-size group than in the medium-size one.

Correlation of oocytes' BCB coloration and size with their GV chromatin configurations

While most of the BCB+ follicular oocytes were of SN pattern, most of the BCB− oocytes were of NSN configuration in both large- and medium-size groups (Table 3). All oocytes smaller than 60 μm in diameter were of NSN configuration, regardless of BCB coloration. Significantly more BCB+ oocytes were of SN configuration in the large-size group than in the medium-size one ($P < 0.05$).

Effects of cumulus cell apoptosis on BCB coloration of oocytes of different sizes

Following double staining, all nuclei of cumulus cells were stained blue by Hoechst 33342, but only nuclei of late apoptotic and necrotic cells were stained red by PI and hence looked pink (Figure 1F) under the wave length adopted (220-360 nm). To confirm the accuracy of double staining, a triple staining with Annexin V, PI and Hoechst 33342 was conducted. After triple staining, all cumulus cells were stained blue by Hoechst; viable cells were negative for both Annexin V and PI; early apoptotic cells were labeled by Annexin V (green), while being negative for PI; late apoptotic cells were positive for both Annexin V and PI; necrotic cells were labeled by PI (red), while being negative for Annexin V (Figure 1G, [44]). Among the follicular COCs analyzed (n = 15), percentages of the total and late apoptotic and necrotic cells were 51.8 ± 5.1%,

Table 3 GV-chromatin configurations of BCB+ and BCB− mouse oocytes of different diameters

Oocyte diameter (μm)	BCB	Oocytes observed	% SN
> 70	+	90	84.6±4.9[a]
	−	45	38.0±4.6[b]
60-70	+	56	69.6±1.9[c]
	−	40	36.1±4.0[b]
< 60	+	30	0.0±0.0[d]
	−	8	0.0±0.0[d]

[a-d]Values with common letters in their superscripts in the same column did not differ significantly ($P > 0.05$).

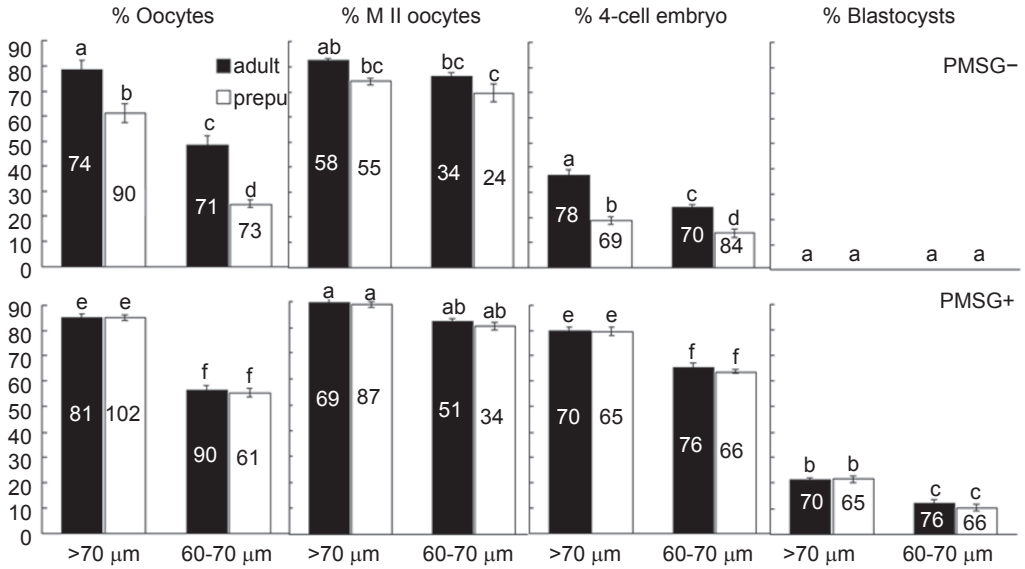

Figure 3 Effects of sexual maturity and PMSG treatment on proportion and development of mouse BCB+ oocytes. This figure consists of 4 units showing % oocytes, % MII oocytes, % 4-cell embryos and % blastocysts, respectively. Data in the upper row of each unit figure were collected from animals which had not been primed with PMSG (PMSG−), while those in the lower row were from the PMSG-primed mice (PMSG+). The number within each bar represents the number of oocytes observed for each treatment. Values with common letters above their bars within the same row or column of each unit figure did not differ significantly ($P > 0.05$).

9.13 ± 1.7% and 2.3 ± 0.3%, respectively. A correlation analysis showed that the percentage of total apoptotic cells was highly correlated with the percentage of late apoptotic/necrotic cells (r = 0.95, $P < 0.01$), indicating that the percentage of late apoptotic/necrotic cells can reflect the atretic status of an oocyte.

Follicular oocytes of different diameters were classified into three categories according to their percentages of late apoptotic/necrotic cumulus cells. Oocytes with less than 5% of late apoptotic/necrotic cells were classified as non-atretic (NA), those with more than 20% were classified as late atretic (LA), and those with 5-20% were early atretic (EA). The LA percentages did not differ significantly ($P > 0.05$) among different groups of oocytes, except for those of the BCB+ oocytes in the small-size group, which were significantly ($P < 0.05$) higher (Figure 2). The EA percentages of BCB+ oocytes were always significantly higher ($P < 0.05$) than those of BCB− oocytes in the same-size group, indicating that the greater developmental competence of the BCB+ oocytes might be related to their higher EA proportion.

Effects of sexual maturity and PMSG treatment on BCB coloration and developmental potential of mouse oocytes

Adult (6-8 wk after birth) and prepubertal (20-25 days old) mice were killed for follicular oocytes with or without prior PMSG priming. When not primed with PMSG (PMSG−), the adult mice produced significantly ($P < 0.05$) more BCB+ oocytes and a higher rate of 4-cell embryos of the BCB+ oocytes than prepubertal mice, although blastulation did not occur in either age group (Figure 3). When primed with PMSG (PMSG+), however, prepubertal mice did not differ significantly from adult mice in both proportion and development of BCB+ oocytes. In general, PMSG priming increased both proportion and development of BCB+ oocytes in both age groups. This indicates that adult mice produced more BCB+ oocytes with higher developmental competence than the prepubertal mice when not primed with PMSG, and that PMSG priming increased the proportion as well as the developmental potency of BCB+ oocytes in adult and, particularly in, prepubertal mice.

Discussion

The present results showed that BCB+ mouse oocytes gained better cytoplasmic maturity (determined as the intracellular GSH level and pattern of mitochondrial distribution) and higher developmental potential after IVM than the BCB− oocytes. This confirmed previous reports that BCB+ oocytes presented higher normal fertilization and embryo development rates than BCB− oocytes in goats [35, 45], cattle [17, 36] and pigs [34]. This study also showed that the better cytoplasmic maturity and higher developmental potential of the BCB+ oocytes were correlated with their

larger sizes, higher percentages of SN chromatin configuration and higher frequencies of early atresia in comparison with the BCB− oocytes. Besides, we found in this study that animal sexual maturity and gonadotropin stimulation had an impact on the proportion and cytoplasmic maturity and developmental competence of BCB+ oocytes. In summary, the present results indicate that BCB staining can be used as an efficient method for oocyte selection but the competence of the BCB selected oocytes may vary with oocyte diameter, animal sexual maturity and gonadotropin stimulation.

In this study, we found that the post-IVM concentration of intracellular GSH and the proportion of oocytes with FP pattern of mitochondrial distribution were significantly higher in the BCB+ than in the BCB− mouse oocytes, and in the BCB+ oocytes of large sizes than in those of medium sizes. Embryo development was correlated positively with intracellular GSH levels of oocytes [46-48]. Ovulated oocytes showed much higher GSH contents than IVM oocytes in hamsters [49], pigs [50] and goats [51]. Besides, several studies have shown that the intracellular GSH level could be a good marker for cytoplasmic maturity in porcine [41], bovine [47], ovine [52] and caprine [51]. Nishi *et al*. [53] found that the rates of mitochondrial polarization in MI and MII oocytes were significantly higher in IVO than in IVM mouse oocytes. Furthermore, Miki *et al*. [54] reported that mouse oocytes matured in optimized medium essentially resembled IVO oocytes in mitochondrial distribution. El Shourbagy *et al*. [55] demonstrated that BCB− porcine oocytes had significantly lower numbers of mtDNA copies than the BCB+ oocytes. In summary, for the first time, we have correlated the BCB coloration with intracellular GSH level, mitochondrial distribution and embryo development after IVM of oocytes. This further substantiates the use of BCB test for selection of oocytes prior to IVM.

In this study, the BCB+ proportion in the large-size group of mouse oocytes was significantly higher than that in the medium-size group. Previous studies in swine [34], caprine [35] and bovine [17] also found that BCB+ oocytes were larger than BCB− oocytes. Many authors studying the relationship between oocyte diameter and developmental potency have concluded that the developmental potential of oocytes increases with increasing oocyte diameters [12, 56-60]. Furthermore, it has been demonstrated that mouse oocytes with SN configurations were larger, more advanced toward ovulation and more competent in development than oocytes with NSN pattern [30, 31, 43, 61]. This would also help to explain why the BCB+ oocytes of large sizes have higher developmental potential than those of medium sizes.

We found that adult mice produced significantly more BCB+ oocytes with higher competence than prepubertal mice when not primed with PMSG. Percentages of BCB+ oocytes in gilts [33, 34] and heifers [17] were higher than those observed in goat oocytes [35]. Pigs and heifers slaughtered at commercial weight (approximately 6 and 10 months old, respectively) were older and closer to puberty than goats (2-month-old). In addition, oocytes from heifer and gilt ovaries were found to be smaller than oocytes from cows and sows [59, 60, 62, 63]. However, the proportion of BCB+ oocytes did not differ significantly between the prepubertal and adult mice when both were primed with PMSG in this study. Consistent with our results, oocytes recovered by laparoscopic ovum pick-up from gonadotrophin-primed prepubertal goats developed to blastocysts in a similar percentage as oocytes from adult goats [22].

The present results demonstrated that the EA percentages of BCB+ oocytes were significantly higher than those of BCB− oocytes in all three size groups. Many studies have suggested that a low level of atresia tends to improve the *in vitro* competence of oocytes [23, 64-66]. Our recent work showed that early atresia was beneficial to both competent and incompetent goat oocytes [67].

In this study, we differentiated BCB coloration between the ooplasm and the attached cumulus cells. We found that BCB coloration of the ooplasm is consistent with that of cumulus cells in most of the oocytes, and that the ooplasm and cumulus cells are independent for BCB staining. Therefore, the BCB coloration synchronization between ooplasm and cumulus cells would suggest that the pentose phosphate pathway (PPP) metabolism of glucose is completely coupled between these cells, while the inconsistency in BCB coloration might suggest a disruption of metabolic couplings between some oocytes and their cumulus cells. The importance of metabolic couplings between oocytes and granulosa cells in mammals is well known [68].

In conclusion, we have evaluated the utility of BCB staining for oocyte selection in the most commonly used mouse oocyte model, and have confirmed the BCB test as an efficient method for oocyte selection. For the first time, we have correlated the developmental potential of BCB-selected oocytes with their cytoplasmic maturity (GSH level and mitochondrial distribution) and with chromatin configurations and frequencies of early follicular atresia. In addition, we have found that the competence and cytoplasmic maturity of the BCB-selected oocytes were affected by oocyte diameter, animal sexual maturity and gonadotropin stimulation. Taken together, the series of criteria described here would allow for better choices in selecting oocytes for better development. However, the mechanisms for the BCB+ coloration of small incompetent oocytes and the significance of BCB coloration synchronization between ooplasm and surrounding cumulus cells need further investigation.

Acknowledgments

This study was supported by grants from the China National Natural Science Foundation (Nos. 30430530 and 30571337) and from the Momentous Research Project of the China Ministry of Science and Technology (No. 2006CB944003).

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