Glutathione (GSH) Concentration and Developmental Competence of Korean Native Cow Oocytes Selected by Brilliant Cresyl Blue (BCB)

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ABSTRACT

This study was carried out to evaluate the nuclear, cytoplasmic maturation and developmental potential of bovine oocytes selected by brilliant cresyl blue (BCB) as indirect measurement of oocytes growth phase. Cumulus-oocyte complexes (COCs) were collected from 2 to 8 mm follicles from slaughterhouse Hanwoo ovaries. The COCs were divided into stained cytoplasm to blue (BCB+) and unstained (BCB−) according to their ooplasm BCB coloration stained by 26 μm of BCB after 90 min. Selected COCs were cultured in a TCM199 for 18 to 26 h. Nuclear maturation and total cell number was evaluated after *in vitro* maturation (IVM) or *in vitro* culture (IVC) using 10 μg/ml Hoechst 33342, and cytoplasmic maturation was evaluated by intracellular glutathione (GSH) assay before (0 h) and after (24 h) IVM. The oocyte diameters were not differed significantly between BCB+ (157.4±5.8 μm) and BCB− (149.0±31.0 μm) groups ($p>0.05$). However, the proportion of metaphase II oocytes in BCB+ group was significantly higher than BCB− group after IVM ($p<0.05$). GSH content of BCB+ group oocytes was significantly higher than that of BCB− group just after collection (7.3±0.6 vs. 4.8±0.6 pmol/oocyte, $p<0.05$), but not varied after IVM (13.1±0.9 and 12.6±2.5 pmol/oocytes for BCB+ and BCB− respectively; $p>0.05$). The proportion of blastocyst formation and total cell number in BCB+ group (23.5% and 105.5±28.6) was significantly higher than that in BCB− (9.8% and 72.4±26.1; $p<0.05$). The results indicate that BCB+ group oocytes may provide a cellular and functional basis for the greater developmental competence in Korean Native Cow (KNC) oocytes.

(Key words : BCB, GSH, bovine oocytes, nuclear maturation)

INTRODUCTION

For *in vitro* production (IVP) of bovine embryos, cumulus-oocyte complexes (COCs) are usually collected from heterogeneous follicles of slaughterhouse ovaries. Because of bovine estrous cycle is characterized by a series of two or three follicular waves (Pierson and Ginther, 1987; Sirois and Fortune, 1988) that the ovaries have mixture of several growing and atretic follicles. The ovaries are comprehensive of follicles of different growth stages and the collected COCs have been observed for an asynchronous nuclear configuration. Theses variation of nuclear configurations seems to cause an asynchronous progression of meiotic maturation and developmental competence in porcine oocytes (Funahashi *et al.*, 1997; Ye *et al.*, 2005). It has been speculated that follicular growth phase and oocyte diameters should be considered as selection parameters. There is general acceptance that the oocyte development competence are related to follicular and oocyte diameter (Crozet *et al.*, 2000; Ma *et al.*, 2003), estrus cycle stage (Machatkova *et al.*, 1996) and the level of atresia follicle (Hagemann, 1999). When COCs with uniform ooplasma and compact cumulus cells mass are selected for *in vitro* maturation (IVM) of bovine oocytes, the blastocyst development rates are limited to 20~30%. One of the main factors affecting the embryo yield is the intrinsic quality of the oocyte, while the embryo culture condition plays a crucial role in determining embryo quality (Rizos *et al.*, 2002). Therefore it has been necessary that the selection of COCs before IVM without destroy of oocytes for the selection of nonatretic follicles (Rodriguez-Gonzalez *et al.*, 2002).

During the course of ovum growth, immature oocytes are

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known to synthesis a variety of proteins (Wassarman, 1988) including glucose-6-phosphate dehydrogenase (G6PDH). The low expression of G6PDH emzyme is associated with oocytes those finished their growth, but the activity of G6PDH is higher among the growing oocytes (Tian *et al.*, 1998; Wassarman, 1988). The developmental competence of oocyte can be evaluated by intracellular activity of G6PDH determinate using BCB. Therefore, oocytes that have finished their growth show a cytoplasm with a blue coloration, but growing oocyte is expected to increase the G6PDH activity which results in unstained cytoplasm (Rodriguez-Gonzalez *et al.*, 2002). BCB compound were successfully used for the selection of competent oocytes in pig (Wongsrikeao *et al.*, 2006), mouse (Wu *et al.*, 2007), goats (Rodriguez-Gonzalez *et al.*, 2002) and cattle (Opiela *et al.*, 2008; Pujol *et al.*, 2004).

The GSH is the major non-protein sulphydryl compound in mammalian cells which plays a critical role in protecting the cell from oxidative damages, and is known to have many biological functions and has been shown to play an important role in oocyte maturation (El Mouatassim *et al.*, 1999). The oocyte maturation involves both nuclear and cytoplasmic maturation which is indispensible to normal fertilization and embryo development. In particular, cytoplasmic maturation processes numerous molecular events including synthesis of protein, protein phosphorylation and activation of many metabolic pathways (Eppig, 1996; Krisher and Bavister, 1998). The intracellular intracellular glutathione (GSH) synthesis is a critical part of oocyte cytoplasmic maturation, because the GSH function has mainly reactive oxygen species (ROS) scavenges resulting protection of ROS toxic effect in the oocytes (Eppig, 1996). Synthesis of GSH during oocyte maturation has been reported in pig (Yoshida *et al.*, 1993), hamster (Perreault *et al.*, 1988), cow (de Matos *et al.*, 1996) and buffalo (Gasparrini *et al.*, 2003), and postulated that may be a valuable indicator of cytoplasmic maturation of oocyte after IVM. In addition, the maintenance of adequate GSH levels is essential for oocyte maturation, fertilization and embryonic development (Guerin *et al.*, 2001). In many reports, the oocyte stained with BCB+ were more competent in maturation and development than those that with BCB-oocytes (Wongsrikeao *et al.*, 2006; Wu *et al.*, 2007; Rodriguez-Gonzalez *et al.*, 2002; Opiela *et al.*, 2008; Pujol *et al.*, 2004). Considering theses reports, we hypothesized that the BCB positive oocytes might have high accumulation of GSH than that of BCB negative oocytes during IVM.

Therefore, the objectives of the present work were carried out to improve the potential of *in vitro* embryo production from Korean Native Cow (KNC) oocytes selected by BCB before IVM and measurement of intracytoplasmic content of GSH before and after IVM both BCB positive and negative oocytes.

MATERIALS AND METHODS

All reagents used in this study were obtained from Sigma-Aldrich (St Louis, MO, USA) unless otherwise stated.

1. Oocytes Collection

The bovine ovaries were collected and transported from a local slaughter house to laboratory within 3~4 h at around 30℃ in physiological saline supplemented with 100 IU/ml penicillin/streptomycin. After washing 2~3 times with 0.9% physiological saline, COCs were recovered from 2~8 mm diameter follicles with an 18-gauge needle attached to a vacuum. Only oocytes with 2~3 layers of COCs and homogenously granulated cytoplasm were used in this study.

2. BCB Staining and IVM

To carry out the BCB test, immediately after COCs collection, the COCs were washed three times in Dulbecco's PBS (D-PBS) modified with 1,090 mg/l glucose, 35.2 mg/ml pyruvate, 0.4% BSA and 50 ug/ml gentamycin (mDPBS). Then the COCs were exposed to 26 μM of BCB diluted in mDPBS for 90 min at 38.5℃ in humidified air described as (Bhojwani *et al.*, 2007). After BCB exposure, the COCs were washed in mDPBS and were examined under a stereomicroscope and divided into two groups according to their cytoplasm coloration: oocytes with any degree of blue coloration to the cytoplasm (BCB+) and oocytes without blue cytoplasm (BCB−) (Fig. 1). The selected COCs were matured in TCM-199 supplemented with 10% FBS, 1 μg/ml β-estradiol, 10 μg/ml FSH, 0.6 mM cystein, 0.2 mM Na-pyruvate at 38.5℃ in a humidified atmosphere of 5% CO_2 in air for 18 to 26 h.

3. Fixation and Nuclear Configuration Observation

At the end of IVM (18 to 26 h), oocytes were denuded from cumulus cells in D-PBS supplemented with 1 mg/ml hyaluronidase. Denuded oocytes were washed three times in 0.1% PVA-PBS medium. Fixed in PVA-PBS supplement in 3.7% (w/v) formaldehyde and 10 μg/ml Hoechst 33342 at room temperature for 30 min. The fixed oocytes were rinsed in PVA-

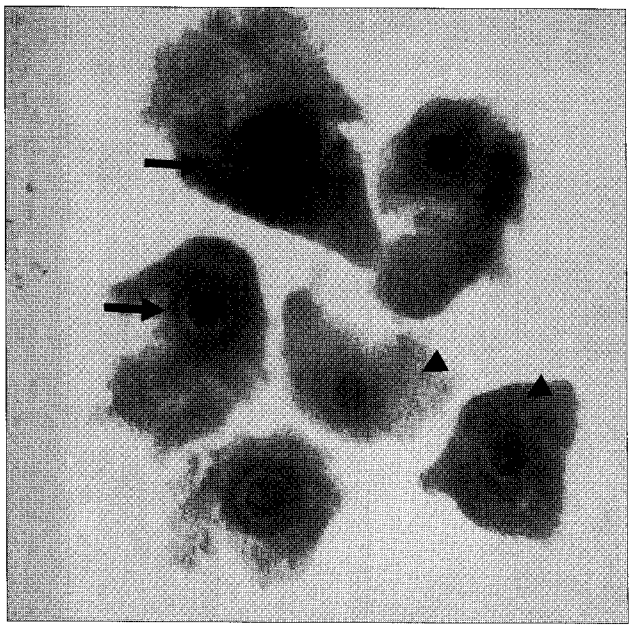

Fig. 1. COCs were stained by BCB before IVM. BCB+: blue- color (arrow) and BCB−: unstained (arrow head).

PBS to remove excess Hoechst 33342 and mounted on slides to evaluate the nuclear configuration. Oocytes were classified as being at MII stage.

4. Assay of GSH

Intracellular content of GSH was measured before (0 h) and after IVM (24 h) as described by Funahashi *et al.* (1994). Cumulus cells were removed completely using 0.1% hyaluronidase and denuded oocytes were washed three times in the assay buffer (0.2 M sodium phosphate buffer, 10 mM EDTA, pH 7.2). Groups of 30 oocytes in 5 μl of assay buffer were transferred to 1.5 ml microtubes, and 5 μl of 1.25 M phosphoric acid (Fluka, Buchs, Switzerland) were added. Samples were frozen at −80℃. Thawed oocytes were broken by pipetting and freezing again until assay. Concentrations of GSH in the oocyte were determined using the dithionitrobenzonic acid-glutathione disulphide (DTNB-GSSG) reductasa-recycling assay (Tatemoto *et al.*, 2000). Briefly, 700 ml of assay buffer containing 0.29 mg/ml NADPH and 100 ml of 0.75 mM 5,50-dithio-bis (2-nitrobenzonic acid) was mixed with 190 ml of water in the microfuge tube. After warming at room temperature for 15 min, 10 ml of 250 IU/ml GSH reductasa solution was added to the tube to start the reaction. The formation of 5-thio-2-nitrobenzonic acid, which has an absorption peak at 412 nm, was followed continuously with a spectrophotometer Beckman DU-40 a 412 UV for 2 min in 1 sec intervals. This amount was divided by the number of oocytes. Standards (0.02, 0.1, 0.2, and 1 mM) of GSH and a sample blank lacking GSH was also assayed.

5. IVF and IVC

The frozen semen was thawed in a water bath at 35℃ for 1 min and then was centrifuged with D-PBS at 1,800 rpm for 5 min. To induced sperm capacitation, the pellet was diluted carefully in 500 μl heparin (200 μg/ml, sodium salt) for 15 min before insemination. The spermatozoa were diluted in Tyrode's lactate solution supplemented with 6 mg/ml BSA, 0.2 mM/ml sodium pyruvate and 100 IU/ml penicillin/streptomycin. After IVM, the oocytes were fertilized with capacitated sperm at a concentration of 2×10^6 sperm/ml of *in vitro* fertilization (IVF) medium and incubated at 38.5 °C in 5% CO_2 in air for 20 h. After IVF, the cumulus cells were denuded, washing and presumptive zygotes were placed in 4-well dish into 700 μl of modified CR1-aa medium supplemented with, 3 mg/ml BSA and incubated at 38.5℃ in 5% CO_2 in air for 3 days and then cultured additional 4 days in replaced 3 mg/ml BSA with 10% FBS. At the end of the culture period (day 8), the total cell number of each blastocyst was assessed by fluorescence microscopy after 10 μg/ml Hoechst 33342 staining for 15 min.

6. Statistical Analysis

This experiment was replicated at least three times. The GSH concentrations were analyzed by Student *t*-test, the nuclear maturation and blastocyst formation rates were analyzed by chi-square analysis using the SAS 8.01 program (SAS Institute, Inc., Cary, NC, USA). Differences with a probability value of 0.05 or less were considered significant.

RESULTS

The diameter of BCB+ and BCB− oocytes was shown in Table 1. There were no significant differences in oocyte diameter between BCB+ and BCB− groups (157.4±5.8 vs. 149.0± 31.0 μm; $p>0.05$). As shown in Fig. 2, the percentage of oocytes reached to MII (nuclear maturation) were significantly higher ($p<0.05$) in BCB+ oocytes compared to BCB− oocytes after 18 to 26 h of IVM culture. Most of the BCB+ oocytes were reached to MII stage after 18 h of IVM (83%) whereas 71% of BCB− oocytes were reached to MII after 26 h of IVM. The GSH concentrations of BCB + and BCB− bovine oocytes were

Table 1. The diameter of bovine oocytes selected by BCB staining

BCB	Oocyte	Diameter (μm)	Cytoplasm without ZP (μm)
+	31	157.4± 5.8	116.0± 6.2
−	21	149.0±31.0	112.3±22.9

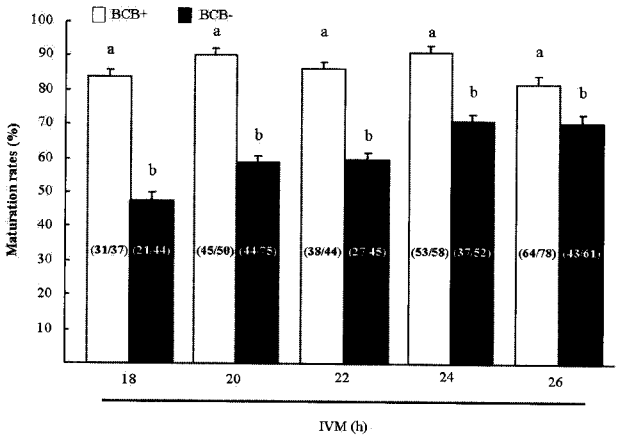

Fig. 2. Nuclear maturation rates (MII) of bovine oocytes during IVM.
[ab] Values with same column with different letters differ significantly (p<0.05).

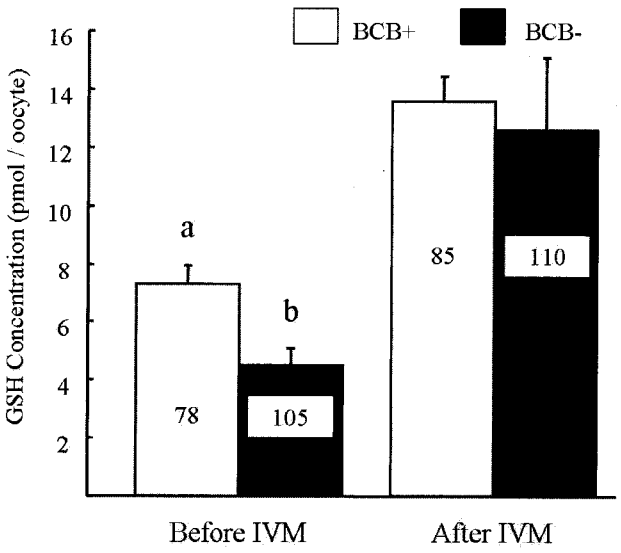

Fig. 3. GSH concentration of bovine oocytes selected by BCB before (0 h) and after IVM (24 h).
[ab] Values with same column with different letters differ significantly (p<0.05).

Table 2. *In vitro* development of bovine oocytes selected by BCB after IVF

BCB	No. of oocyte used	No. of embryos developed to		Total cell	Total cell range
		Cleavage (%)	Blastocyst (%)		
+	226	157 (69.5)	53 (23.5)[a]	105.5±28.6[a]	83~188
−	225	156 (69.3)	22 (9.8)[b]	72.4±26.1[b]	53~115

[a,b] Values with same column with different letters differ significantly (p<0.05).

presented in Fig. 3. The GSH concentration of BCB+ (7.3±0.6 pmol/oocyte) oocytes was higher than BCB− (4.8±0.6 pmol/oocyte) oocytes before IVM (p<0.05). However, there were no significant differences in GSH concentration after IVM (MII stage) (BCB+: 13.1±0.9 pmol/oocyte, BCB−: 12.6±2.5 pmol/oocytes; p>0.05).

The developmental competence of BCB+ and BCB− oocytes following IVM/F and embryo culture was shown in Table 2. The cleavage rate was not significantly differed between BCB+ and BCB− groups (69.5 and 69.3%), but the blastocyst development rate and total cell number of the BCB+ group (23.5% and 105.5±28.6) was significantly higher than that of the BCB− group (9.8% and 72.4±26.1; p<0.05).

DISCUSSION

Bovine estrous cycle is characterized by two or three follicular waves and within several days of initiation of a follicle wave one follicle is selected as the dominant follicle which continues grows and differentiates before ovulation. However, subordinate follicles are undergone to atresia because of direct inhibitory effect exerted by the dominant follicle on their development (Pierson and Ginther, 1987; Sirois and Fortune, 1988). Therefore, the ovaries consist of a mixture of different stage follicles (growing and atretic follicles) for *in vitro* study; the COCs collected from heterogeneous follicles of slaughterhouse ovaries. Various studies revealed that the developmental competence of oocytes is related to the status of the follicle and oocytes diameter (Blondin and Sirard, 1995; Seneda *et al.*, 2001). Bovine oocytes acquire the capacity of resuming meiosis when they reach in a size of at least 110 μm. The mRNA synthesis is ceased at that size, which is an indication of completion of oocyte growth phase and there is a decline in G6PDH activity (Fair *et al.*, 1995; Alm *et al.*, 2005). The BCB test was based on the activity of G6PDH to convert BCB stain from blue to colorless whereas the cytoplasm of mature

oocytes retain BCB dye (BCB+) due to their inability to convert BCB to a colorless compound while growing oocytes metabolize and clear BCB (BCB−) (Ericsson *et al.*, 1993). Many research groups successfully used the BCB compound for the selection of competent oocytes of pig (Wongsrikeao *et al.*, 2006), mouse (Wu *et al.*, 2007), goats (Rodriguez-Gonzalez *et al.*, 2002) and cattle (Opiela *et al.*, 2008; Pujol *et al.*, 2004). In the present study, the oocytes diameter was similar between BCB+ and BCB− groups. In contrast to the present findings, the BCB+ oocytes for heifer (Pujol *et al.*, 2004), goat (Rodriguez-Gonzalez *et al.*, 2002) and pig (Roca *et al.*, 1998) had a greater diameter than that for BCB− oocytes. The different results on oocyte diameter and its relationship to BCB staining of this study compared to previous studies may be due to origin of the oocytes (Korean Native Cow).

The developmental competence of mammalian oocytes depends on high levels of RNA involved both in maturation and in subsequent cellular events and protein synthesis from mRNAs including reassembly of cytoskeletone and their molecular maturation corresponds to the phases of oocyte growth (Eichenlaub-Ritter and Peschke, 2002). These proteins storage in the oocyte cytoplasm is of great importance until the appropriate time for their utilization (Uzbekova *et al.*, 2008). In addition, the acquisition of meiotic competence coincides with a decrease of general transcriptional activity at the end of oocyte growth phase, particularly ribosomal RNA (rRNA) synthesis in the nucleolus (Bjerregaard *et al.*, 2004) in porcine oocytes. In this study most BCB+ oocytes reached to MII stage within 18 h of IVM (83.0 %), but the BCB− oocytes were reached to MII stage (18 h; 46.3%) and steadily increased up to 26 h (71.2%) of IVM (Fig. 2). Therefore, our results suggested that the BCB− oocytes probably collected from unfinished growth phase follicles following delayed nuclear maturation than that of BCB+ oocytes. Evidence has shown that oocytes are enriched with RNA transcripts and protein biosynthesis compared to their dominance phase counterparts (Ghanem *et al.*, 2007).

We found that the GSH levels of BCB− oocytes were lower than that of BCB+ oocytes before IVM, but not differed after IVM (Fig. 3). The percentage of cleaved oocytes were not varied between two groups, but the percentage of blastocyst development and total cell number were significantly higher in BCB+ group than that in BCB− group (Table 2). Intracytoplasmic GSH level in oocytes could be considered as an important marker for cytoplasmic maturation in several species (Luberda, 2005). The GSH function in oocytes is mainly related to antioxidative properties and protection against ROS toxic activity. GSH also protect meiotic spindle morphology of oocyte from oxidative damage consequently, ensures normal zygote formation and develop to the blastocyst (Luberda, 2005; Yoshida *et al.*, 1993). In pigs, the oocytes from small follicles are unable to synthesize intracellular GSH and insufficient RNA syntheses are related to their low developmental competence (Yoon *et al.*, 2000).

In this study we found that the GSH levels of BCB− oocytes were lower than that of BCB+ oocytes before IVM, but after IVM there were no differences between two groups (Fig. 3). The percentage of cleaved oocytes were not differed between BCB+ and BCB− groups, but the percentage of blastocyst development and total cell number in BCB+ group was significantly higher than that in BCB− group (Table 2). This results are in agreement with previous studies that BCB+ oocytes showed higher fertilization and embryo development rates than that of BCB− oocytes in goats (Rodriguez-Gonzalez *et al.*, 2002), cattle (Pujol *et al.*, 2004; Alm *et al.*, 2005), pig (Roca *et al.*, 1998) and mice (Wu *et al.*, 2007). The relationship between BCB staining and the developmental competence of oocytes provide the opportunity to study the cellular features of BCB+ and BCB− oocytes.

In conclusion, the present study showed that BCB+ oocytes are better nuclear and cytoplasmic maturation rates than that of BCB− oocytes, and greater developmental competence to blastocyst.

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