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Effect of Prolactin on Developmental Competence of Bovine Oocytes Selected by Brilliant Cresyl Blue Staining

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Abstract: The aim of the present study was to evaluate the influence of prolactin (PRL) supplemented maturation medium on the development of brilliant cresyl blue (BCB) selected bovine oocytes. Before IVM compact cumulus oocyte complexes (COCs) were incubated in BCB solution for 90 minutes. Treated oocytes were then divided into BCB- (colorless cytoplasm, increased G6PDH) and BCB^+ (colored cytoplasm, low G6PDH) on their ability to metabolize the stain. The selected COCs were matured in TCM 199 + 10% (v/v) heat-treated FCS and 10^6/ml granulosa cells (GC) either without (control) or with the addition of 50 ng/ml PRL (experimental group). After IVM oocytes were fertilized *in vitro* and embryos were cultured by standard protocols. The cleavage rate including status of chromatin and the blastocyst rate was evaluated. Both in the control and the experimental group the cleavages rates were significantly increased in the BCB+ oocytes (75.9 and 63.1%) in comparison to the BCB- oocytes. The addition of PRL increased the number of embryos, in BCB+ and BCB- oocytes. The BCB+ oocytes yielded a significantly higher proportion of blastocysts (37.6%) by the addition of PRL in comparison to the control (21.3%) and both BCB+ oocytes had significantly higher blastocyst development than did BCB- oocytes (19.5 and 4.5%). The number of nuclei in the blastocysts was significantly increased in BCB+ oocytes of the PRL group (112.8 ± 2.3 vs. 100 ± 21.0) in comparison with BCB+ control groups. The addition of PRL improved the development and nuclei number in BCB+ and BCB- oocytes. In conclusion, oocytes selection using BCB staining was a useful tool to classify competent bovine occytes. Furthermore, the addition of PRL to maturation medium highly improve the developmental capacity of bovine oocytes selected by BCB staining.

Key words: Bovine · Oocytes · Prolactin · BCB · G6PDH

INTRODUCTION

Follicular oocytes recovered from the ovaries of slaughtered cattle are commonly used to study the in vitro technique of embryos production. The relatively low level of efficiency achieved using *in vitro* embryo production, manifested by the frequent failure of up to 60-70% of recovered oocytes to reach the blastocyst stage after fertilization, is almost certainly related to the quality of the oocyte at the beginning of maturation [1].

Effective *in vitro* maturation (IVM) is essential for successful *in vitro* embryo production. During IVM, oocytes undergo two maturation changes, nuclear and ooplasmic maturation. In bovine oocytes, nuclear maturation as a high frequency, although insufficient cytoplasmic maturation can affect the completetion of IVM. Incomplete cytoplasmic maturation in bovine oocytes after IVM causes fertilization to fail [1,2]. For *in vitro* embryo production, prospective detection of competent oocytes is critical. However, oocytes are commonly recovered from ovaries of slaughtered animals with reduced reproductive performance or slaughtered at the end of their use, are heterogenous in quality and developmental competence [3]. Furthermore, immature oocytes in cattle, as in other mammals, are routinely selected for IVM on the basis of the visual of morphological features such as thickness of compactness of cumulus investment and homogensity of the cytoplasm

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[3] and sizes of follicles [4-6], oocytes growth [7]. Although morphological criteria provide a reasonable means of identify oocyte quality and suitability for IVM, there is a considerable variability among oocytes and morphological criteria are not sufficient for the identification of competent oocytes appropriate for *in vitro* development to the blastocyst stage [1,8]. With urgent need for establishing non-invasive and non-pertubing means for selecting more homogenous and competent oocytes, the brilliant cresyl blue (BCB) stain test has been evaluated previously in cattle [1, 9-11], buffalo [12], Goat [13], Pig [14, 15]. BCB is a vital blue dye which determines the intracellular activity of glucose-6-phosphate dehydrogenase (G6PDH), an enzyme synthesized during the oocyte growth phase but decreased activity in oocytes that finished their growth phase [16]. The cytoplasm of these oocytes turns blue (BCB^+) because they do not reduced BCB to colorless compound. Hormonal supplements to IVM medium or known to be required to promote cytoplasmic maturation of bovine oocytes [17]. Compounds like prolactin have shown to enhance developmental competence of *in vitro* matured rabbit [18], human [19] and bovine oocytes [6].

Therefore, the aim of the present study was to evaluate (i) the influence of PRL supplemented to maturation medium on the developmental competence of bovine oocytes selected by BCB, (ii) determine the influence of PRL on the ongoing embryonic quality of bovine oocytes by estimating the number of blastomeric (nuclei) embryos.

MATERIALS AND METHODS

Recovery of Oocytes and Preparation of Granulosa Cells: Ovaries from dairy cows were obtained at a local slaughterhouse (St. Petersburg-Pushkin Russia) and transported within 2 h to the laboratory in sterile PBS maintained at 35 to 37°C. The cumulus-oocyte complexes (COCs) were recovered by slicing the surfaces of ovaries. The oocytes were collected in TCM 199 with 10% fetal calf serum (FCS). Only oocytes having complete compact, multilayered cumulus and homogenous ooplasm were used for culture. Before cultivation, the oocytes were washed twice in TCM 199 with 10% FCS.

Granulosa cells (GC) were collected from the medium from which COCs were dissected from follicles (3 to 5 mm in diameter) and centrifuged twice for 5 min at 500 g. The final pellet of granulosa cells was resuspended in maturation medium. Co-culture of oocytes and granulosa cells was carried out in Petri dishes in 2 ml of medium at 38.5°C in an atmosphere of 5% CO_2 for 24 h.

Brilliant Cresyl Blue Staining: Immediately after collection of the bovine compact COCs, 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) [1] diluted in mDPBS for 90 min. at 38.5°C in humidified air atmosphere 5% CO_2. Following BCB exposure, the COCs were transferred to mDPBS and washed twice. After washing the COCs were examined under a stereomicroscope at X50 and divided into two groups according to 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) in CO_2 incubator (5% CO_2 in air 100% relative humidity). In different treatment groups based on the BCB and culture system as following:

Group 1: Oocytes (n=141 BCB+) matured in TCM199 (25 mM HEPES with Earle's salt and L-glutamine, Sigma USA) supplemented with 10% heat treated fetal calf serum (FCS; Sigma) and 50 μg/ml gentamycin (Sigma) plus 1 x 10^6 ml granulosa cells (GC) [20].

Group 2: Oocytes (n = 137 BCB-) matured in TCM199 + 10% FCS plus 1 x 10^6/ml GC.

Group 3: Oocytes (n = 133 BCB+) matured in TCM199 + 10% FCS + 1 x 10^6 GC plus 50 ng/ml PRL x (20 IU/mg, institute of Endocrinology, Moscow, Russia, [6]).

Group 4: Oocytes (n = 139 BCB-) matured in TCM199 + 10% FCS + 1 x 10^6 GC plus 50 ng/mL PRL.

***In vitro* Fertilization (IVF):** After IVM, matured oocytes from different culture systems were fertilized *In vitro* using frozen-thawed bovine semen. The frozen semen of bull, known for high rates of IVF and cleavage was used. A motile samples of sperm was obtained by swim-up separation based on the method of Lonergan *et al.* [21]. 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 fertilization droplet to give final concentration of approximately 1 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, France). Twenty-four hours later after placement in the culture, the cleaved embryos were transferred to previously prepared bovine oviductal epithelial cell (BOEC) monolayer formation. After 3 days of culture, 40µl of the culture medium was placed with fresh embryo culture medium. On day 8 (day 1 = day of fertilization), the percentage of blastocyst were recorded.

Blastocyst Evaluation: At the end of the culture period, the blastocyst were fixed in buffered formal saline (containing 5% formaldehyde) and subsequently stained with Hoechhst 33342 (Sigma B-2261). The number of nuclei in the blastocysts were then recorded under fluorescence microscope.

Statistical Analysis: The difference between groups (BCB+ and BCB-) 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 groups. One way analysis of variance (ANOVA) was performed by procedure of SAS/STAT to calculate simple linear 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

In order to evaluate the effect of PRL on the developmental competence of bovine oocytes selected by BCB (Fig. 1) were assessed until day 8 following IVM, *In vitro* fertilization (IVF) and *in vitro* culture (IVC) as shown in Table 1. After 2 days from IVF cleavage rate in absence (control) and presence of PRL (experimental) was significantly higher ($p<0.05$) in BCB+ oocytes (75.9 and 63.1%) in comparison to BCB- oocytes (48.2 and 62.6%) in control and PRL groups.

The addition of PRL increased the number of embryos in BCB+ and BCB- oocytes. The BCB+ oocytes yielded a significantly higher proportion of blastocyst (37.6%) by addition of PRL in comparison to control (21.3%) and BCB- oocytes had significantly higher blastocyst development in experimental group than BCB- oocytes in control (19.5 and 4.5%) (Table 1).

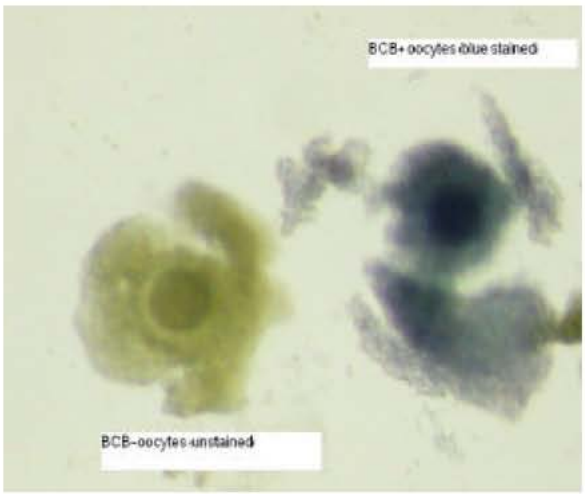

Fig. 1: Differentially stained bovine COCs after exposure to BCB stain (BCB + blue coloured; BCB-, unstained)

Table 1: Effect of prolactin on developmental competence of bovine oocytes selected by brilliant cresyl blue staining

Treatment during IVM	BCB	N. oocytes	8-16 cells embryo n (%)	Blastrocyst n (%)
TCM 199 + 10% FCS + 10^6 GC	+	141	89(63.1)[a]	19 (21.3)[a]
TCM 199 + 10% FCS + 10^6 GC	-	137	66(48.2)[b]	3 (4.5)[b]
TCM 199 + 10% FCS + 10^6 GC + 50 ng/ml PRL	+	133	101 (75.9)[c]	38 (37.6)[c]
TCM 199 + 10% FCS + 10^6 GC + 50 ng/ml PRL	-	139	87 (62.6)[d]	17 (19.5)[d]

a:b, a:c, b:d, c:d $P<0.05$

Table 2: Number of nuclei in bovine blastocysts obtained after IVF of BCB selected oocytes

Treatment during IVM	BCB	No. blastocyst	No. of nuclei in blastocyst (mean + SD)
TCM 199 + 10% FCS + 106 GC	+	18	100+21a
TCM 199 + 10% FCS + 106 GC	-	3	51.3+2.5b
TCM 199 + 10% FCS + 106 GC + 50 ng/ml PRL	+	32	112.8+2.3c
TCM 199 + 10% FCS + 106 GC + 50 ng/ml PRL	-	15	105.9+2.2d

a:b, a:c, b:d, c:d $P<0.001$

Embryos in the blastocyst stage from all groups had normal morphology through they appeared to comparatively small in the BCB- group. when the cell numbers in the blastocysts were recorded (Table 2), it was found that all the four groups (control, PRL, BCB+ and BCB- oocytes) were significantly different from each other. The highest number of blastocyst nuclei were recorded in the BCB+ group with PRL (112.8±2.3) and the lowest number in the BCB-group without PRL (51.3 ± 2.5).

DISCUSSION

The success of *In vitro* production of bovine transferable blastocysts using oocytes aspirated from slaughterhouse ovaries does not exceed 40%. Various studies have shown the quality of the oocyte to be the main determinant of the blastocyst rate, while culture environment affect their quality [2,22]. Oocytes recovered from ovaries of slaughtered animals are heterogenous [3], coming from follicles in different stages of growth and atresia. Therefore, the selection and further use of good quality or developmentally competent oocytes is vital for the success of various embryo technologies.

In the current study, classifying COCs by BCB improve developmental competence. In agreement with previous reports [1, 9, 12, 14]. Comparing oocytes exposed to BCB test, BCB+ oocytes showed higher percentage cleared embryos and number of blastocysts than BCB- in control and PRL group. In both control and PRL groups indicated that BCB+ oocytes more competent for development than BCB- oocytes. the bovine oocyte, zygote and embryo have a profound need for protein synthesis . However, the mRNA transcript for these proteins are not synthesized through development [23]. In mammals, synthesis of RNA, up to 60-65% during oocyte growth and reach a peak at the beginning of follicular antrum formation [24]. This is in accordance with our investigation concerning cleavage and blastocyst rate in BCB- oocyte (Table 1). The oocyte was insufficient cytoplasmic maturation, under control of high G6PDH activity. In contrast, in BCB+ oocytes finished RNA synthsis for establishment sufficient ribosomes for following protein synthesis and sufficient cytoplasmic maturation for greater developmental potential [25]. It was reported that large number of BCB- oocyts failed to fertilize because of their low capacity for sperm penetration [14] and because they were fertilized with numerous spermatozoa [12].

The reduced developmental capacity of early embryonic development has been associated with mitochondrial dysfunction and low ATP in mammalian oocytes and embryos [26,27]. Recently the amount of mitochondrial DNA and transcripts has been quantified in bovine oocytes and embryos [28] showing that bovine oocytes failed to cleave containing significantly lower transcripts implicated in mitochondrial biogenesis [25]. In the pig, competent BCB+ oocytes contain more copies of mitochondria DNA (mtDNA) and are more likely to be fertilized than incompetent BCB- oocytes [29]. Moreover, most recent research of Susor *et al.* [30]. They concluded that BCB- oocytes are not fully developed due to insufficiency of their cytoplasmic maturation. Additionally Spiking *et al.* [31] proves that BCB- oocytes are delayed in the onset of expression of proteins in comparison with BCB+ oocytes. As a result of significant differences in expression of nuclear-encoded replication proteins between BCB+ and BCB- protein oocytes, a delayed mitochondrial DNA replication was observed [31]. The delayed mitochondrial DNA replication was correlated with reduced fertilization and embryonic development in BCB- oocytes due to delayed onset of expression of their replication factor [31]. This reflected in current results by lower cleavage and blastocyst rates in BCB oocytes in comparison to BCB+ oocytes in control and PRL group (Table 1), which in agreement with previous work in cows [1, 9, 10, 32] goat [13, 33] and pig [14, 15, 29, 31, 34, 35].

Prolactin was shown to play important role in female reproductive function. In human beings PRL can support oocyte development competence during maturation prolactin is essential for physiological function of corpus luteum. Leroy Martin *et al.* [36] proved that PRL recognized by PRL receptors which are localized on granulosa cells . The results of Yoshimura *et al.* [18] indicated that addition of PRL to IVM enhances developmental competence of *in vitro* matured rabbit oocytes to morulae and blastocysts stage in dose dependent manner. This in accordance with our results, the addition of PRL to maturation media significant increase of cleavage and blastocyst rate in both BCB+ and BCB- oocytes. PRL prolong the time of maturation and make synchrony between nuclear and cytoplasmic maturation of both BCB+ and BCB- oocytes. Torner *et al.* [17] indicated that addition of PRL during IVM prolonged of meiotic maturation of bovine oocytes. The slower meiotic process was accompanied by lower level of histone H, kinase and MAP-kinase (mitogenic activity).

It is possible that PRL acts via cumulus cells; i.e. that can stimulate in cumulus cells the secretion of substance that inhibits oocyte maturation by decreasing the activities of protein kinases [17]. However, PRL can mediate inhibitory activity by releasing intracellular stores of Ca^{++} as decrease of intracellular stored Ca^{++} in the ooplasm of bovine oocytes after PRL treatment during IVM [37]. The mobilization of Ca^{++} from intracellular stores in such cells responsible for mitogenic activity [37] as well as mitogenic activity of this hormone on bovine granulosa cells [38].

The cell number of embryos is an important indicator of the embryonic development and health. It has been suggested that the embryos with large number of cells are more likely to implant and give rise to live off spring [39]. Therefore, the quality of blastocyst assessed in this investigation as number of cells was significantly better for blastocyst derived from BCB+ oocytes in control (100±2.1) and PRL group (112 ± 2.3). On the other hand, the lowest number of nuclei in blastocyst derived from BCB- oocytes in control group (51.3±2.5). Similar results of other studies [1,9] who reported that blastocyst in BCB- group had significantly lower cell number than control. Pujol *et al.* [11] found that BCB staining was accurate for selecting against incompetent heifer oocytes.

In conclusion based on the current study, the selection of bovine oocytes using BCB test improved the embryonic development after IVF, as well as enhancement embryo quality (increasing the number of cells per blastocyst). Furthermore, addition of PRL to culture media enhanced the proportion of the blastocyst rate obtained from oocytes selected by BCB staining.

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