Effect of presence of corpora lutea on cumulus expansion of in vitro matured bovine oocytes selected by trypan blue and brilliant cresyl blue tests

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ABSTRACT
Trypan Blue (TB) and Brilliant Cresyl Blue (BCB) are used to select and determine viability and competence of oocytes, however, the effects of corpora lutea on cumulus expansion during in vitro maturation are still undetermined. Cumulus-oocytes complexes (COCs) from ovaries with ipsilateral (ICL), contralateral (CCL) and without corpora lutea in either ovary (OCL) were selected by visual examination, TB and BCB staining, and matured in vitro to evaluate cumulus expansion. The overall percentage of visually selected COCs was similar in ICL, CCL and OCL (P > 0.05). Proportions of live and mature COCs were similar in all groups (P > 0.05). The overall percentage of BCB+ COCs was 70.1%. BCB+ ICL COCs had less cumulus expansion (60.5%) than BCB+ CCL and OCL COCs (75.7 and 71.4%, respectively; P < 0.01). BCB– ICL COCs had less cumulus expansion (20.0%) than BCB– CCL and OCL (39.7 and 46.1%; P < 0.01). BCB+ CCL and OCL COCs showed the highest cumulus expansion index (P < 0.01). Presence of corpus luteum in the ovary affects negatively cumulus cells expansion. TB and BCB staining facilitate the selection of oocytes with higher degrees of cumulus expansion.

1. Introduction
In vitro production of bovine embryos is an essential part of embryo technology nowadays. The process involves three main stages, (1) in vitro maturation of oocytes, (2) sperm capacitation and in vitro fertilization, and (3) in vitro culture of embryos, which are usually developed in approximate times of 24 h, 48 h and 9 d, respectively, after the recovery and selection of the oocytes (Rehman et al. 2001). During the process of in vitro-derived embryos, one of the most important factors that influence the blastocyst rate outcome is maturation of the oocytes, which involves both nuclear and cytoplasmic events, including changes in the plasma membrane and nuclear and cytoplasmic maturation. This in vitro process includes the attainment of metaphase 2 stage of the oocyte in a period of 18–24 h (Landínez et al. 2010). Therefore, selection is primordial in order to obtain cumulus-oocytes complexes (COCs) that are capable of being fertilized effectively. Several methods have been used for this purpose over the years, including criteria related to the visualization of morphological aspects such as cytoplasmic homogeneity and pigmentation as well as the observable amount of cumulus cells (Stojkovic et al. 2001; Nagano et al. 2006). Additionally, the use of Trypan Blue (TB) and Brilliant Cresyl Blue (BCB) staining has been reported as a means to determine the viability and developmental competence of the oocyte, without negative effects on the viability or health of the oocytes and their granulosa cells (Alcoba et al. 2016).

Stain of TB is commonly used as a supravital method that determines viability of cells, and it is used for this purpose in the oocyte selection process, as nonviable (dead) oocytes take up this dye and stain blue (Jian-Min et al. 2011; Filippiak and Larocca 2012b; Santos et al. 2016). On the other hand, the BCB test has been established as a non-invasive and non-perturbing mean for selecting more homogeneous and more competent oocytes (Pujol et al. 2004; Alm et al. 2005), as it determines the activity of glucose-6-phosphate dehydrogenase (G6PDH), an enzyme synthesized in the growing oocytes but inactive in the oocytes that have finished their growing phase that converts BCB stain from blue to colourless (Alm et al. 2005). Thus, the oocytes that have completed their growth phase stain blue, while the growing oocytes remain colourless (Opiela and Kątska-Książkiewicz 2013).

Proper maturation is needed for the acquisition of developmental competence of the oocyte once it is fertilized (Krishner 2004), and the surrounding somatic (cumulus) cells of the oocyte play a fundamental role in this process (Thompson et al. 2007; Lolicato et al. 2015). Expansion of cumulus cells takes place in the follicle shortly before ovulation in vivo, as well as during maturation in vitro and is based on synthesis of hyaluronic acid into the extracellular space, where it plays a role as the structural component of expanded cells and signals molecule regulating oocyte maturation, therefore, a sufficient amount of cumulus cell layers followed by a large
expansion is essential for successful oocyte maturation, fertilization and embryo development (Nevoral et al. 2014). In fact, one of the first morphological indicators of the successful completion of oocyte maturation is expansion of the cumulus mass away from the oocyte (Regassa et al. 2011). Considering this, the degree of expansion of cumulus cells has been assessed by several authors according to a subjective scale and a cumulus expansion index (CEI), which has been formulated in order to assign a quantifiable numerical value to the observed expansion (Fagbohun and Downs 1990; Pandey et al. 2010; Gomez et al. 2012).

Although reports on the effects of the presence of corpora lutea on the developmental potential of oocytes are still limited, an interaction between the development of both a corpus luteum (CL) and present follicles has been reported previously (Pfeifer et al. 2009). The presence of a CL affects in a negative way estradiol concentration as well as quantity and quality of follicles (Penitente-Filho et al. 2014). Additionally, several authors point out the negative effect of the presence of a CL (Hajarian et al. 2016) or progesterone (Silva and Knight 2000; Shimada and Terada 2002) on the competence of oocytes, when a CL is also present in the ovary. Results from other studies indicate that the presence or absence of CL does not influence in a significant manner the cleavage rate of the first blastocysts, when compared with oocytes from ovaries without CL or in the absence of progesterone. On the other hand, Hendriksen et al. (2004) indicate that oocytes from subordinate follicles show reduced developmental competence when a dominant follicle is present (effect of inhibin) and suggest a beneficial effect of progesterone on the quality of oocytes, when a CL is also present in the ovary. Results from other studies indicate that the presence or absence of CL does not influence in a significant manner the cleavage rate and blastocyst development (Chian et al. 2002; Sugulle et al. 2008). The objective of the present study was to determine and evaluate the effects of the presence of corpora lutea on the expansion of the cumulus cells of bovine oocytes obtained from slaughtered cows and selected through TB and BCB stain tests.

2. Materials and methods

2.1. Ethical standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national and institutional guides on the care and use of laboratory animals in Mexico (NOM-051-ZOO-1995: Humanitarian care of animals during mobilization, NOM-033-ZOO-1995: Humanitarian slaughter of domestic and wild animals and the institutional regulation of Bioethics Committee of Universidad Autónoma de Ciudad Juárez).

2.2. Chemicals

Except when stated otherwise, all media were prepared with media from Sigma-Aldrich Chemical Company (St. Louis, MO, USA). Additionally, follicular fluid was obtained from >8 mm follicles of ovaries of previously slaughtered healthy cows, centrifuged and inactivated by a 30 min water bath at 56°C following the methodology described by Filipiak and Larocca (2012a).

2.3. Harvesting of ovaries and COCs

A total of 171 ovaries that showed activity by the presence of graafian follicles or corpora lutea were collected at a local abattoir (located in Ciudad Juárez, México) approximately 20 min after the non-pregnant cows or heifers (bos taurus) had been slaughtered. Ovaries were collected and placed in one of three single containers, according to the presence of a visible matured CL (ipsilateral CL; ICL group, n = 68), absence of a CL but coming from a reproductive tract with a CL in the adjacent ovary (contra lateral CL; CCL group, n = 77) or absence of a CL in either ovary from the female (OCL group, n = 26). Afterwards, the containers were filled with 0.9% sodium chloride solution at 35°C and within 3 h of collection, ovaries were transported to the laboratory. After arrival, the temperature was checked to confirm that it was within the range 31–35°C and the ovaries were washed with sterile 0.9% sodium chloride solution at a temperature of 35°C. All follicles of 2–8 mm in diameter in all ovaries were aspirated using a 10-ml syringe and an 18-gauge hypodermic sterile needle. Aspirate content from ovaries of ICL, CCL and OCL groups was collected into previously warmed gridded search dishes. A total of 1056 COCs were obtained (ICL n = 422, CCL n = 476 and OCL n = 158) and only those with homogeneous or homogeneous with few areas with irregular pigmentation cytoplasm and ≥5 layer of compact cumulus cells around the oocyte (scoring categories 1 or 2; as described by Stojkovic et al. 2001) were selected in each group for the present study.

2.4. TB and BCB staining and in vitro maturation

Following morphologic selection, COCs were washed twice in phosphate-buffered saline solution (PBS) containing 136.9 mM (0.8%) NaCl, 2.7 mM (0.02%) KCl, 10.1 mM (0.1%) Na₂HPO₄, 1.8 mM (0.02%) KH₂PO₄, 5.3% fetal bovine serum (FBS; Gibco, Grand Island, NY, USA) and 50 µg/ml gentamycin. After being washed, COCs in each dish were stained with 4.5 mM (0.4%) TB for 10 min at 38.5°C in a humidified atmosphere with 5% CO₂ and washed again in PBS for viability determination. Although some cumulus cells appeared to be stained, only COCs with unstained oocytes were considered live and continued in the experimental procedures. COCs with stained oocytes with or without stained cumulus cells were discarded from the study. After viability determination, COCs of all groups were stained for cytoplasmic maturity by exposing them to 22 µM (0.85%) BCB (Hycel, Ciudad de México, México) diluted in maturation medium (mTCM-199) containing 84.8% TCM-199 with Earle’s salts, 5% FBS, 10% follicular fluid, 0.2% sodium piruvate (Gibco, Grand Island, NY, USA) and 50 µg/ml gentamycin and incubated for 90 min at 38.5°C in a humidified atmosphere with 5% CO₂. After the incubation time, COCs were washed in mTCM-199 and classified according to BCB staining as mature (BCB+; COCs that included oocytes with dark blue cytoplasm) or immature (BCB--; COCs that included oocytes with colourless cytoplasm), as described by Rodriguez-Villamil et al. (2016). Afterwards, they were placed in the same medium (20–30 COCs/100 µl) in separate wells by ovary group (ICL, CCL and OCL) and maturity classification (BCB+ and BCB–) and
covered with mineral oil for a subsequent maturation process at the same incubation conditions for 22 h.

2.5. Assessment and qualification of cumulus expansion

All visual evaluations were carried out by the same technician. Expansion of cumulus cells was considered when at least few morphological changes in the distribution of the cells of each COC after the in vitro maturation process were observed (Zhang et al. 2010; Caixeta et al. 2013). Additionally, a cumulus expansion numerical index (CEI) was formulated by calculating the average expansion scoring value for each group of COCs (Fagbohun and Downs 1990; Pandey et al. 2010). Score 0 indicated no expansion, which was considered when a detachment of cumulus cells was observed, showing a partially or fully denuded oocyte; a score of 1 was considered when no expansion was observed but the spherical cumulus cells remained compacted around the oocyte; a score of 2 was considered when only the outermost layers of cumulus cells were expanded; a score of 3 was assigned to those COCs that had all cumulus cells layers prominently expanded, except the corona radiata (cells most proximal to the oocyte); and a score of 4 was assigned to those COCs that showed the maximum degree of expansion of cumulus cells, including the corona radiata (Gomez et al. 2012).

2.6. Statistical analysis

All the statistical analysis was performed using SAS software (9.0; Statistical Analysis System Institute Inc. Cary, NC, USA). The proportions of COCs selected for the study (morphologic categories 1 or 2), live COCs (as considered by TB staining), mature COCs (as considered by BCB staining) and COCs with expanded cumulus cells in ICL, CCL and OCL groups were compared with a chi-squared test using the PROC LOGISTIC. Additionally, comparison of numerical values of CEI of COCs in each group was performed under a completely randomized design using the PROC GLM. Comparisons of means were performed with Tukey tests. Differences were considered as statistically significant at the \( P \leq 0.05 \) level.

3. Results

Results regarding COCs selection, live and mature COCs, as well as expansion of cumulus cells of BCB+ and BCB− COCs in ICL, CCL and OCL groups are shown in Table 1. The overall percentage of selected COCs (according to morphologic categories 1 and 2), regardless of the experimental groups was 73.4% (776 out of 1056). The proportion of visually-selected COCs was similar in ICL, CCL and OCL groups (72.0, 75.4 and 71.5%, respectively; \( P > 0.05 \)). Also, the proportion of live COCs, as determined by TB staining, was similar in all groups (77.9, 81.1 and 78.8% for ICL, CCL and OCL, respectively; \( P > 0.05 \)), as well as the proportion of mature COCs, as determined by the use of BCB staining (66.2, 74.9 and 70.8% for ICL, CCL and OCL, respectively; \( P > 0.05 \)). The overall percentage of BCB+ COCs, regardless of the experimental groups was 70.1% (438 out of 617). BCB+ COCs in ICL group showed a lower rate of expansion of cumulus cells (60.5%), when compared to BCB+ COCs in CCL and OCL groups (75.7 and 71.4%, respectively; \( P < 0.01 \)). BCB− COCs in ICL group also showed a lower rate of expansion of cumulus cells (20.0%), when compared to BCB− in CCL and OCL groups (39.7 and 46.1%, respectively; \( P < 0.01 \)).

Results regarding CEI values are presented in Table 2. BCB+ COCs in CCL and OCL groups showed similarly higher CEI values than BCB+ COCs in the ICL group (\( P < 0.01 \)). Also, BCB− COCs in CCL and OCL groups showed similarly higher CEI values, when compared to BCB− COCs in the respective ICL group (\( P < 0.01 \)). When the BCB status (positive or negative) was included in a comparison of ICL, CCL and OCL groups, BCB+ CCL and OCL COCs showed the highest values of expansion (2.74 and 2.76, respectively), followed by COCs BCB+ ICL, BCB− CCL and BCB− OCL (1.49, 1.29 and 1.27, respectively. BCB− ICL COCs showed the lowest CEI value (0.74; \( P < 0.01 \)).

4. Discussion

The overall selection rate of COCs by their morphology, including the amount of cumulus cells and cytoplasmic pigmentation in the present study is higher to that reported by Stojkovic et al. (2001), who found 48.8% of COCs of category 1 and 2. On this regard, it has been well documented that several factors inevitably affect the oocyte quality. Such factors may include age of the female, the stage of the estrous cycle, the hormonal patterns involved, biochemical characteristics of the follicular fluid, atresia grade of the follicle and ovarian morphology, among others (Boni 2012). It is important to note that ovaries used in the present study were considered as cyclic by visual
examination and those with any apparent abnormality were discarded, which probably led to a selection of more ovaries containing COCs of higher quality.

Although TB has been widely used to determine cellular viability, its use in bovine in vitro fertilization procedures seems limited. Didion et al. (1990) obtained 93% of living COCs from porcine ovaries, while other authors report higher percentages of living bovine COCs during spring and summer than autumn and winter (87 and 79%, respectively; Filipiak and Larroca 2012b). Although the effect of season of the year was not considered in the present study, it must be noted that these percentages are numerically similar to the ones observed in ICL, CCL and OCL groups of our experiment, which was carried out during the autumn and winter months.

It has been reported widely that BCB stain can be a helpful tool to determine cytoplasmic maturity of oocytes (Alm et al. 2005; Opiela and Kałtska-Książkiewicz 2013; Santos et al. 2016), however, the effectiveness of the BCB test has been considered as questionable by Opiela et al. (2008) due to the fact that no significant differences in terms of blastocyst rates were observed with the use of the stain. With the use of 22 μM BCB staining, BCB+ COCs in the present study showed blue cytoplasmic colouring, indicating that they had finished their growth process, similarly to 26 μM BCB stain used in earlier studies in goats (Rodríguez-González et al. 2002), pigs (Santos et al. 2016) and cows (Alm et al. 2005; Silva et al. 2011). The overall rate of BCB+ COCs in the present study (70.1%) is higher to the rates obtained by other authors in bovine heifers (62%; Pujol et al. 2004; 65%; Silva et al. 2011) and buffalo (57.2%; Manjunatha et al. 2007), but is lower than the 91% rate value obtained by Roca et al. (1998) and Ericsson et al. (1993) with porcine COCs. The higher developmental competence of BCB+ COCs due to the diminished activity of G6PDH, when compared to BCB− COCs, has been demonstrated widely (Pujol et al. 2004; Alm et al. 2005; Silva et al. 2011). Nevertheless, it has also been reported that BCB+ oocytes that develop to form a blastocyst have a higher number of blastomers compared with BCB− oocytes (Opiela et al. 2010). This may indicate a higher survival function of BCB+ COCs, as they have shown a higher caspase-3 activity as an indicator of adequate completion of growth phase, cell differentiation and adaptation to suboptimal in vitro conditions (Opiela et al. 2010). In the present study, more BCB+ COCs showed expansion of the cumulus cells with higher CEI values than BCB− COCs, which has been recognized as a necessary mechanism in a proper oocyte maturation and its acquisition of developmental competence (Lolicato et al. 2015). On this regard, in a previous study by Alm et al. (2005) it was observed that the proportion of BCB− COCs that reached maturity and developmental competence in terms of metaphase II achievement (58.1%) was lower to those proportions observed in COCs in their BCB+ and control (without BCB staining) groups (72.5 and 77.1%, respectively). These authors attribute this effect as a result of the unfinished growth and development of these oocytes.

In the present study, a lower proportion of BCB+ COCs was observed in the ICL group, when compared to CCL and OCL groups. These results agree with those observed by Hajarian et al. (2016), who obtained 44.1% of BCB+ COCs from ovaries with a CL and 57.3% of BCB+ COCs from ovaries without a CL, but do not agree to those obtained by Sugulle et al. (2008), who obtained similar rates of BCB+ in both types of ovaries (73.9% from ovaries with a CL and 69.5% from ovaries without a CL). Additionally, COCs from the CCL and OCL groups showed higher rates of expansion of cumulus cells and higher CEI values, when compared to COCs from the ICL group, regardless of the result of BCB test (positive or negative). These results may indicate that a negative effect of the presence of a CL (and progesterone) may occur on the function of COCs coming from ovaries with a CL in a paracrine manner, but not on CCL or OCL COCs. On this regard, it has been reported that oocytes contained in a follicle are potent stimulators of granulosa cell proliferation and are capable of secreting paracrine factors that inhibit granulosa cell Luteinizing hormone (LH) receptor expression and progesterone production, both indicative of a suppression of luteinization (Li et al. 2000; Gilchrist et al. 2008). The impact of progesterone in mammalian oocyte maturation related to expansion of cumulus cells and oocyte quality before or after ovulation has not been fully established. A report from Hajarian et al. (2016) indicates that the presence of a CL in the ovary may have negative effects on developmental competence of ipsilateral oocytes, as COCs from their ICL group yielded a lower percentage of cleavage and blastocyst formation, when compared to the CCL group. Similarly, Islam et al. (2007) also indicates that COCs of superior quality may be obtained from CL-absent ovaries, while Chian et al. (2002) reports similar rates of oocyte maturation and fertilization in COCs from ovaries with or without a CL. The hormonal environment of the follicle will necessarily affect the function of the follicle and the oocyte. The systemic pathway by which progesterone inhibits the growth of the follicle is through the suppression of LH pulse frequency, which mainly affects estradiol synthesis (Santos et al. 2009). Besides the inhibin produced by the follicle, inhibin secreted by the CL leads to the demise of all follicles that reached the recruitment phase except for the dominant follicle (Lonergan 2011). Both the effect of progesterone as well as the one of inhibin appear to be absent in ovaries without CL, which may lead to a more appropriate follicular fluid that provides better conditions of oocyte maturation, when compared to that in ovaries with a CL (Shabankareh et al. 2010; Lonergan 2011), as high progesterone concentrations in the follicular fluid and an active expression of progesterone receptors in the cumulus cells affect the maturation and developmental competence of COCs (Robker et al. 2000; Urrego et al. 2015). Another factor involved in oocyte quality related to the presence of a CL is the ovarian vascularity. When a new CL forms, new blood vessels form to irrigate it, and the newly-formed CL will receive the greatest rate of blood flow compared with other ovarian tissues. This difference of blood flow results in a decreased supply of gonadotropins and other biochemicals and hormonal factors necessary for the development of the follicle and the oocyte which may influence negatively on oocyte quality and maturation (Acosta and Miyamoto 2004).

5. Conclusion

In conclusion, results of the present study indicate that the presence of a CL in the ovary exerts a negative effect on expansion
of cumulus cells of the oocyte. It is also concluded that TB and BCB staining tests facilitate the selection of competent oocytes that will show a higher degree of expansion of their cumulus cells.

**Disclosure statement**

No potential conflict of interest was reported by the authors.

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