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Review Article

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Impact of Using Brilliant Cresyl Blue Stain on Oocyte and Embryo Selection

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THE SUCCESS of in vitro embryo production is ultimately dependent on the number and quality of cumulus-oocyte complexes harvested from ovaries. The morphological criteria are routinely used for oocytes selection in most laboratories. There is brilliant cresyl blue stain (BCB) test allowing the selection of developmentally competent oocytes which is a simple, quick, economic and feasible protocol. BCB is known to be a non-invasive methodology that allows the selection of immature oocytes in several species. BCB test determine the intra cellular activity of glucose-6-phosphate dehydrogenase (G6PDH) that gradually decreases its activity as oocytes reach their growth phase. The using of BCB test before IVM is still controversial, so this review describes the use of BCB staining test for oocytes and embryo selection to investigate the impact of application of this stain in in-vitro maturation and embryo production in animals.

Key words: Oocytes quality, brilliant cresyl blue stain, embryo quality, selection, *in vitro* maturation.

Introduction

The in vitro production (IVP) inefficiency of embryos has been related to oocyte quality at the start of maturation [1]. The identification and selection of oocytes with good developmental competence is a critical step for successful embryo technologies [2]. Many reports used brilliant cresyl blue (BCB) staining test as an efficient method to screen and classify oocytes quality for various species as buffalo and ovine [3,4].

Quality of oocytes that developed into viable embryos was still an issue of major interest in assisted reproduction technologies [5]. It was known that good quality oocytes were associated with better maturation rate in vitro. The optimal oocyte developmental potential required synchronization between nuclear and cytoplasmic maturation [6]. Cytoplasmic maturation involved a complex molecular reactions as protein phosphorylation, biological macromolecule synthesis and activation of certain metabolic pathways [7].

Rizos et al. [8] mentioned that the intrinsic oocyte quality determined the blastocyst yield, while post fertilization culture system determined the blastocyst quality**.** Routinely, oocyte quality determinations depended on cytoplasm uniformity and the layer of cumulus cells [9].

Oocyte quality and selection

Oocyte selection in the laboratory was a key factor which determined the proportion of oocytes developing to blastocyst stage in animal species. Oocyte quality affected the ability of oocytes to mature, fertilize, give early embryonic survival, maintenance of pregnancy, fetal development and normal offspring [10,11].

Morphological factors related to the quality of cumulus oocyte complexes (COC) and /or oocytes include COC morphology**,** number of cumulus cell layers [12], oocyte diameter [13] and oocyte coloration [14].In addition, the size of perivitelline space [15], thickness, organization of zona pellucida [16], mitochondrial distribution

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and glucose-6-phosphate dehydrogenase activity measured by BCB staining of immature oocytes [17,18].

There were various factors appeared to be critical to oocyte quality including age of donor animal [19,20], maturation media [21], phase of follicular wave [22], follicular size [23], follicle health [24], environmental factors as season or heat stress [25,26,27], hormonal stimulation [28], nutrition [29], storage temperature [30], lactation status of donor animal [31]and genetic background [32,33].

There was a relationship between oocyte size, follicular diameter and its competence [34]. Follicular size affected oocyte quality, which implicating messenger RNA or protein stores as factors involved in oocyte competence [35]. Crozet et al. [36] observed a higher blastocysts percent (6%, 12%, 26%) using oocytes from small (2-3 mm), medium (3-5 mm), large (>5 mm) follicles, respectively. The efficiency of in vitro techniques was low for prepubertal animals as oocyte donors. Prepubertal oocytes were characterized by abnormal cytoplasmic maturation and lower ability to achieve blastocyst stage than those coming from adult donors [37].

Also, Martino et al. [38] mentioned that the ovaries of prepubertal animals had a higher proportion of antral follicles with a diameter smaller than 3 mm and was difficult to release the cumulus oocyte complexes (COCs) by aspiration method. For this reason, oocytes were obtained by slicing the ovary surface, resulting oocytes with different diameters and heterogeneous morphology. In prepubertal goat and sheep, Anguita et al. [39], Catala et al. [40] stated that oocytes with a diameter larger than 125 µm produced higher percentages of blastocyst after IVF. Thus oocyte diameter was a determinant factor for completion of meiosis and full competence for embryo development.

Communication between oocyte and its surrounding cumulus cells was also important for the development of competent oocyte at ovulation. Moor and Dai[41]stated that pig oocytes depended on the presence of follicle cells to generate specific cellular signals that coordinate oocyte growth and maturation.

Metabolism might play a critical role in oocyte quality because glycolytic activity in mature oocytes was related with increased embryonic development. Oocytes matured in vitro often had altered energy metabolism lead to reduced developmental potential. This might reflect a deficiency in maturation medium or its intrinsic ability or both [42].

Embryo quality

Embryo quality is an important determinant for successful practical embryo transfer to improve the pregnancy rates. Blastocyst development was the main step for production of live offspring [43]. Comparing the in vivo and in vitro counter parts produced embryos, in vitro produced embryos tend to have darker cytoplasm and lower density [44] as a consequence of their higher lipid content [45], more fragile zona pellucid [46], reduced expression of inter cellular communication devices [47], differences in metabolism [48] and a higher incidence of a chromosomal abnormalities [49].

Several methods for embryo evaluation had been developed as measurement of enzyme activity [50], glucose uptake [51], live-dead stains [52]and morphological evaluations [53]. Two parameters commonly used to evaluate embryo quality include non-invasive and invasive method. The non-invasive method involved embryo color, shape, number, compactness of cells, size of perivitelline space, and number of degenerated cells [53,54]. While, the invasive techniques involved total cell number determination by nucleus staining using propidium iodide (PI) or Hoechst stain [55], evaluation the inner cell mass (ICM) differentiation, trophectoderm (TE) by differential staining [56], cryotolerance [57] and detection of apoptotic cells using different staining techniques [58].

Using of brilliant cresyl blue stain in oocytes and embryos selection

Many reports used brilliant cresyl blue staining as a vital stain to detect the quality oocytes in various species such as buffaloes [3, 59,60], sheep [61], cattle [62,17,18,63], pig [64,65], prepubertal goat [66,67,68], dog [69] and mice [70]. But, the using of this test for embryo selection still remains an open issue.

It was known that immature oocytes synthesized several proteins, including glucose-6-phosphate dehydrogenase during their growth course [71]. Ericsson *et al.* [72] suggested a simple test for the selection of porcine oocytes which had good developmental competence depending on measurement of glucose-6-phosphate dehydrogenase (G6PDH) activity.

A function of G6PDH enzyme was to produce ribose sugars for nucleic acids synthesis through the pentose shunt [17]. Another important function of G6PDH enzyme was responsible for production of NADPH for lipid synthesis [73]. The G6PDH enzyme converted the dye into a colorless form. Oocytes which stained blue (BCB+, low G6PDH activity) were distinguished by higher developmental competence (good quality), while the colorless oocytes (BCB^-, high activity of G6PDH) were characterized by low developmental competence (low quality) [72].

There was species-specific frequency to the response of different concentration of BCB. Many studies reported that the rate of maturation, cleavage and development into blastocyst of selected oocytes, using 26 µM BCB were higher than in oocytes selected with 13 µM, 39 µM and 52 µM BCB in different species as in buffalo [3], goat [68], cattle [17,73], mice [74], sheep [4,75] and bitch [69].

The proportion of BCB+: BCB^- oocytes were related to the species specific frequency of positive coloration of oocyte before oocyte maturation process [2]. In cattle and buffalo, BCB+: BCB^- oocyte was 60:40 [17,18,3], in prepubertal goat was 30:70 [76] and in pig was 84:16 [77].

BCB staining was not only used for oocytes, but also used for zygote treatment. Mirshamsi et al. [78] stained bovine zygote by BCB for 10 min after maturation and fertilization in vitro. They categorized the zygote into three classes, high stained (ZBCB++: low competent zygotes), moderate stained (ZBCB+: moderate competent zygotes) and unstained ($ZBCB^-$: more competent zygotes). The same authors added that selection of oocyte and zygote by BCB test improved the selecting efficiency of high quality embryos, compared to single BCB test.

Advantage of using BCB

Egerszegi et al. [2] stated that BCB+ porcine oocytes at the beginning of IVM were characterized by high mitochondrial activity in their cytoplasm, the activity was decreased after 22 h of IVM, stayed at the same level up to the final maturation, while BCB^- oocytes population during IVM had a low level of mitochondrial activity without dynamic changes. Moreover, its activity increased at fertilization and early embryonic development as their respiratory activities were positive related with meiotic progress and cumulus cell expansion rate [79].

It was reported that G6PDH activity not only related to cytoplasmic maturation and mitochondrial activity [80], but also related to cytoplasmic lipid content [81]. Castaneda *et al.* [82] demonstrated that the higher lipid content of BCB+ bovine oocytes provided a functional and cellular basis for their greater developmental competence. Cytoplasmic lipid content had been described recently as an essential parameter for oocyte developmental competence and early embryo development in cattle [83] and sheep [75].

In sheep, BCB stain was effective method for oocyte selection and verified this through the relation between stained oocytes, oocyte diameter, GSH content and developmental competence. The diameter of BCB+ oocytes was 163µm, while the diameter of BCB^- oocytes was 159µm. Glutathione content in BCB+ was 6.39 pM, while GSH content in BCB^- was 0.26 pM[61].

Opiela et al. [84] suggested that the advantage of BCB staining included; picking out of oocytes with better developed mitochondria, higher cleavage and blastocyst number for BCB+ stained oocytes, compared to BCB^- oocytes. But, its hardship included lack of significant difference between blastocyst numbers from BCB+ and control oocytes. Also, extension of the overall time for oocyte recovery since the BCB test required extra manipulation and handling. This time requirement would also result in increased cost which affected on overall IVP efficiency [85]. In cases where careful morphological selection was conducted before subjecting oocytes to BCB staining, only 30% of COCs remained colorless. This was the most likely cause of the lack of statistical differences between blastocysts developed from BCB+ and control oocytes [84]. Moreover, there was no report proved that blastocyst rates obtained from BCB+ oocytes higher than routine IVP with morphologically selected oocytes by 30-40% [86].

Disadvantage of using BCB

Scholkamy et al. [87] reported a higher DNA damage in both BCB+ and BCB- oocytes, compared to non-stained control vitrified oocytes. Also, Pawlak *et al.* [88] observed a higher rate of chromosomal abnormalities in porcine oocytes when stained by BCB. Wongsrikeao et al. [89] concluded that BCB staining after in vitro maturation had a negative impact on the cleavage and development of porcine embryos. Opiela et al. [84] indicated that oocytes subjected to BCB

staining had a tendency towards apoptosis as the Bax transcript level in BCB– oocytes was significantly higher (P<0.001) in comparison to non-stained oocytes. In addition, Pawlak et al. [90] do not recommend the application of BCB staining in oocyte selection due to relatively high similarity in in-vitro maturation of BCB+ and control oocytes.

Conclusion

Oocyte selection was a vital step for successful in vitro embryo production process. Many reports were used brilliant cresyl blue stain for oocyte selection. But, recent studies do not recommend to use this stain as a routine work for oocyte selection as it is chemical toxic substance, time consuming and had deleterious effect on embryos.

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تأثير استخدام صبغة كريزيل الزرقاء الزاهية على اختيار البويضات و الأجنة– بحث مرجعى

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يعتمد إنتاج الأجنة أساسا على عدد و جودة البويضات المجمعة من المبايض. و اختيار البويضات يستخدم دائما على أساس الشكل الظاهري. و يعتبر اختبار صبغة كريزيل الزرقاء الزاهية بسيط و سريع و اقتصادي , ويستخدم فى اختيار البويضات فى العديد من السلالات الحيوانية. و يتحدد الاختبار بنشاط إنزيم G6PDH الذي يقل كميته بنمو البويضات. و مازال الاختبار متفاوت فى نتائجه و لذلك تهتم هذه المقالة بوصف ودراسة هذا الاختبار فى اختيار البويضات و الأجنة لمعرفة المدى و الجدوى من استخدامه فى النضج المعملي و إنتاج الأجنة فى الحيوانات.

الكلمات الدالة: جودة البويضات، صبغة كريزيل الزرقاء الزاهية، جودة و اختيار الأجنة، النضج المعملى.