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Selection of developmentally competent sheep zygotes using the Brilliant Cresyl Blue (BCB) test, after IVF

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ABSTRACT

Selecting oocytes and competent zygotes likely to develop, is crucial for in vitro sheep embryo production. Ovine oocyte and zygote selection, based on their morphology, is often influenced by personal preference and the lack of universal standards. Therefore, the objective of this study was to identify a colorimetric assay associated in evaluating zygote developmental competence – with selection based on the use of the Brilliant Cresyl Blue (BCB) test. The BCB test allows researchers to determine the activity of glucose-6-phosphate dehydrogenase (G6PDH), an enzyme synthesized by fertilized oocytes. After in vitro fertilization (IVF), presumptive zygotes were exposed to BCB, diluted in mDPBS (DPBS with 0.4% BSA) for 10 min at 38.5 °C in a humidified air atmosphere. Zygotes were categorized into 3 categories, according to whether they were stained (category 1), moderately stained (category 2) or unstained (category 3). Category 1, 2 and 3 zygotes were considered to have a low, mid or high G6PDH activity, respectively. Control zygotes were incubated directly after IVF (without exposure to BCB). After BCB categorization, zygotes were maintained in the culture medium for 7 days. The category 3 zygotes yielded a higher blastocyst developmental rate (31.1%), than the control (not tested; 18.8%; $P<0.01$), category 2 (17.1%; $P<0.01$) and category 1 (11.6%; $P<0.01$) zygotes. In addition, the total number of cells per blastocyst obtained from the category 3 zygotes was higher, compared to the other groups. In conclusion, it would seem as if the BCB staining, facilitates sheep zygote categorization for high developmental potential.

1. Introduction

One of the main problems confronting IVF laboratories is the need for oocyte and zygote selection, after in vitro fertilization (IVF). The relatively low level of efficiency achieved using in vitro embryo production (IVEP), has been manifested by the frequent failure of recovered oocytes and obtained zygotes to reach the blastocyst stage (Wittemer et al., 2000). This is related generally to the quality of the oocytes at the onset of IVM or zygotes at the end of the fertilization process (Rodriguez-Gonzalez et al., 2002; Ebner et al., 2003). Mammalian immature oocytes are routinely selected for IVF on the basis of visual assessment of the morphological features, such as thickness and compactness of the cumulus investment, and the homogeneity of the ooplasm (Gordon, 2003). Similar to the oocyte, morphological criteria, namely the position of second polar body, the pronuclear morphology, and the nuclear morphology have been employed to correlate the developmental capacity of zygotes (Gianaroli et al., 2003). Morphologically the best oocytes do not necessarily have the highest developmental competence (Blondin and Sirard, 1995). Those oocytes with a better cumulus expansion during maturation may however also not necessarily show the highest blastocyst development rate (Dessie et al., 2007). In addition, oocyte and zygote selection, based on their

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morphology is influenced mainly by subjective judgments and lacks specific universal standards. Moreover, no standard zygote grading systems are available for assisted reproduction laboratories (Depa-Martynow et al., 2007). Therefore, investigating other non-invasive and non-perturbing techniques is necessary to predict and select competent zygotes to ultimately increase the efficiency of in vitro embryo production programs (Dessie et al., 2007).

A relationship between the glucose metabolism of the bovine fertilized oocytes (zygotes) and their developmental competence has been recorded (Comizzoli et al., 2003). Glucose metabolism occurs through a number of pathways (Dumollard et al., 2007), and the pentose phosphate pathway (PPP) has been demonstrated to be activated shortly after sperm penetration into the oocyte in mice (Urner and Sakkas, 2005), the cow (Comizzoli et al., 2003) and sea urchins (Swezey and Epel, 1986). The glucose metabolism through the PPP has also been shown to be necessary for successful fertilization in the murine (Urner and Sakkas, 1999, 2005) and bovine zygotes (Comizzoli et al., 2003). Both spermatozoa and oocytes metabolize glucose through the PPP, and NADPH appears essential for gamete fusion in mice (Urner and Sakkas, 1996, 2005) Zygotes and embryos have a unique property during glucose metabolism: glucose-6-phosphate dehydrogenase (G6PDH), a rate controlling enzyme in the PPP, gradually increases as the zygotes (Urner and Sakkas, 1999) and embryos develop in mice (Dumollard et al., 2007). On the other hand, after sperm–oocyte fusion, an increase in glucose uptake by the fertilized oocytes has been observed, but not before the formation of the male and female pronuclei (Urner and Sakkas, 2005). When glucose metabolism was measured following IVF, both glycolysis and the PPP activities were dramatically increased in fertilized oocytes (Swezey and Epel, 1986; Urner and Sakkas, 1999). Previous studies in mice have indicated that, metabolic activation during pronuclear formation is induced by specific sperm factors that may promote the translation of mRNA coding for glucose metabolic enzymes, such as G6PDH (Travis et al., 2001). In addition, the level of glucose metabolism through the PPP during male pronucleus formation determines successful embryonic development up to the blastocyst stage (Comizzoli et al., 2003).

Brilliant Cresyl Blue (BCB) is an electron acceptor that can be used to semi-quantitate the level of G6PDH activity in live embryos, by the modification of a visual color change (Tian et al., 1998). The BCB test is based on the ability of G6PDH to convert the BCB stain from blue to colorless (Alm et al., 2005). BCB has previously been used to select more competent bovine and porcine oocytes prior to in vitro maturation (IVM) (Roca et al., 1998; Pujol et al., 2004) and to determine the sex of murine embryos (Williams, 1986) and bovine embryos (Iwata et al., 2002). In addition, Wongsrikeao et al. (2006), reported that the selection of oocytes using the BCB test before IVM, improved the rate of nuclear maturation, monospermic fertilization in porcine oocytes, and the subsequent embryonic development after IVF – as well as the enhancement of embryo quality (increasing the total number of cells per blastocyst).

However, a zygote scoring system, as well as other embryonic development markers, may be useful in determining the number and quality of embryos to be transferred. However, the application of the BCB test in the screening of developmentally competent sheep zygotes has not been documented. Hence, this experiment was conducted to select more competent zygotes to further enhance the IVEP of sheep embryos.

2. Materials and methods

All plastic ware i.e. culture vessels and dishes used in these experiments were obtained from Falcon (USA), while all chemicals and media were purchased from Sigma (USA).

2.1. Oocyte collection

Ovine ovaries were obtained at a local abattoir and transported to the laboratory in sterile saline containing 50 IU/mL penicillin and 50 μg/mL streptomycin at 37 °C, within 3 h of slaughter. Ovaries were washed three times in warm saline and the extraneous tissue removed. Cumulus–oocyte complexes (COC's) were aspirated from follicles (2–8 mm in diameter), using an 21-gauge needle, fixed to a 10 mL disposable syringe. The medium used for recovery of oocytes was TCM199 with 25 mM HEPES, supplemented with 50 IU/mL heparin, 50 μg/mL gentamycin and 4 mg/mL Bovine Serum Albumin (BSA). Subsequently, only COC's with a compact cumulus mass of more than three layers and homogeneous cytoplasm were used.

2.2. In vitro maturation (IVM)

The medium used for IVM was TCM-199, supplemented with 0.23 mmol/L sodium pyruvate, 0.02 IU/mL p-FSH, 1 μg/mL 17β estradiol, 50 ng/mL epidermal growth factor (EGF), 10% (v/v) FCS and 50 μg/mL gentamycin. The COC's were washed three times in the maturation medium, and then 10–12 COC's in a group transferred into 50 μL droplets of maturation medium in a 35 mm petri dish, under 3 mL mineral oil. Maturation lasted for 24 h, at 38.5 °C in an environment of 5% CO_2, in humidified air.

2.3. Sperm preparation and in vitro fertilization (IVF)

Sheep sperm was extracted from the cauda epididymis, into 2 mL sperm TALP medium (Parrish et al., 1988). Sperm motility was evaluated under a stereo zoom microscope, and motile sperm fraction separated using the swim-up procedure (Wolf et al., 2008). The top 1.5 mL of the medium was then collected after incubation (45 min at 38.5 °C and 5% CO_2 at a 45° angle) and centrifuged at 700 × *g* for 5 min. The supernatant was discarded and the process repeated. The final sperm pellet was dissolved in fertilization medium and the sperm concentration was adjusted to 10×10^6 sperm/mL, before inseminating the oocytes.

Before transfer to the fertilization droplets, the oocytes were washed three times in fertilization medium. Oocytes were then transferred in groups of 10–12 into 45 μL of the fertilization medium (TALP fertilization) (Parrish et al., 1988). Insemination of the oocytes was carried out by adding 2×10^6 sperm/mL, 2 μg/mL heparin, and PHE (penicillamine, 20 μmol/L; hypotaurin, 10 μmol/L; epinephrine, 1 μmol/L). Oocytes were co-incubated with the sperm for 22 h at 38.5 °C, and 5% CO_2 in humidified air.

2.4. Brilliant Cresyl Blue (BCB) test

After 22–24 h of IVF, the cumulus cells and residual sperm around the presumptive zygotes were removed by repeated pipetting in TCM–HEPES medium. Cumulus free presumptive zygotes were classified either as a control group, which were placed immediately into culture without exposure to BCB stain, or treatment group, which were stained with BCB before culture. To perform the BCB test, the denuded zygotes were washed three times in Dulbecco's PBS, modified by the addition of 0.4% BSA. The zygotes were then exposed to 26 μM of BCB, diluted in mDPBS for 10 min at 38.5 °C, in humidified air. This concentration and the duration of BCB staining were used as preliminary studies had shown that 10–15 min was sufficient for G6PDH to reduce the BCB to a colorless compound in the embryos