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Delayed blastocyst formation or an extra day culture increases apoptosis in pig blastocysts

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ABSTRACT

In the present study, the timing was examined of blastocyst collection/formation or of how the duration of post-blastulation culture affected the quality and developmental competence of *in vitro*-produced pig parthenogenetic embryos. The earliest apoptotic signals were observed at the morula stage while the earliest cytoplasmic fragmentation was observed before the 4- to 8-cell stage of embryo development. Nuclear condensation was detected in morulae and blastocysts, but not all condensed nuclei were positive for the apoptotic signal (TUNEL staining). The mean blastocyst diameter increased with delayed blastocyst collection or extended post-blastulation culture, but decreased with delayed blastocyst formation. Delayed blastocyst collection/formation or an additional day of post-blastulation culture increased the frequencies of apoptosis, condensed nuclei, and low quality blastocysts (those showing a nuclear destruction that negated counting of the nuclei); increased the expression of the pro-apoptotic *BAX* gene; and reduced the ratio of ICM (inner cell mass) cells to TE (trophectoderm) cells. In addition, delayed blastocyst formation decreased *POU5F1* gene expression. These results suggest that a delay in blastocyst collection/formation or an additional day of culture could increase the incidence of apoptosis, decrease the ICM:TE cell ratio, and influence the gene expression and diameter of blastocysts derived from *in vitro*-produced pig embryos. These findings provide a useful reference for improving the quality of *in vitro*-produced embryos.

1. Introduction

Although early stage (*i.e.*, one-cell stage) embryo could be ideal for embryo transfer by minimizing the exposure to an *in vitro* environment, the developmental capacity of the embryo may not be evaluated prior to the transfer and the embryo could be transferred into oviducts surgically. Embryo transfer of blastocyst-stage embryos derived from *in vitro* culture has been shown to produce live piglets (Li et al., 2009; Schmidt et al., 2010; Lee et al., 2013). During the development of pre-implantation embryos, however, it is important to assess developmental potential *in vitro* at the blastocyst stage. Because *in vitro* culture cannot fully mimic the natural conditions, development of *in vitro*-produced pig embryos can be disrupted to a greater extent than *in vivo*-derived counterparts even though there has been considerable research to enhance quality of *in vitro*-produced embryos (Dang-Nguyen et al., 2011). The use of high quality blastocysts (*i.e.*, those with typical morphology, numerous nuclei and a lesser apoptotic index) for

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embryo transfer can improve the likelihood of a successful pregnancy. It is important, therefore, to compare blastocysts before transfer to surrogates.

In vitro-produced pig blastocysts are usually collected after a certain number of days in culture, and are described on this basis [*e.g.*, Day 6 (D6), Day 7 (D7) or Day 8 (D8) blastocysts]. Day 5 (early-collected) blastocysts reportedly yield a greater quality of human *in vitro*-fertilized (IVF) embryos that are more desirable for embryo transfer compared with D6 (late-collected) blastocysts (Shapiro et al., 2008). In pigs, D6 SCNT blastocysts reportedly have fewer TUNEL-positive nuclei than D7 or D8 blastocysts (Hao et al., 2003). These results suggest that blastocyst quality could decrease and apoptosis frequency may increase if there is a longer period of blastocyst development before collection, but the mechanism underlying this correlation has not been entirely elucidated.

Because *in vitro* culture is performed on a large number of embryos, there is the potential for non-synchronized embryonic development, with some embryos developing to blastocysts earlier than others. For example, some embryos form blastocysts early on day 6 of culture [termed formation 6 (F6) blastocysts], while others form blastocysts late on day 7 or 8 of culture [termed formation 7 (F7) or formation 8 (F8) blastocysts]. Previous studies showed that in many mammals, the timing of the first cleavage greatly affects the potential of an embryo to develop into a blastocyst (Mateusen et al., 2005; Booth et al., 2007; Dang-Nguyen et al., 2010). Extensive studies have shown that early cleaving embryos have a greater rate of blastocyst formation than later cleaving embryos (Kobayashi et al., 2004; Mateusen et al., 2005; Booth et al., 2007; Dang-Nguyen et al., 2010; Bohrer et al., 2015). To our knowledge, however, no previous study has examined whether the use of early or late formed blastocysts could influence blastocyst quality in pigs.

In some experimental scenarios, blastocysts might form on day 6 of culture (F6 blastocysts) but be collected on day 7 (one extra day of post-blastulation, F6 + E1 blastocysts) or day 8 (two additional days of post-blastulation, F6 + E2 blastocysts), or blastocyst might form on day 7 (F7 blastocysts) and be collected on day 8 of culture (one additional day of post-blastulation, F7 + E1 blastocysts). Indeed, with the present experimental conditions, the blastocysts collected on D7 included F7 and F6 + E1 blastocysts, while D8 blastocysts included F8, F6 + E2 and F7 + E1 blastocysts (see Fig. 1). It was, therefore, questioned whether blastocysts that have an additional day of culture after blastulation might exhibit differences in blastocyst quality for *in vitro* embryo production, and whether blastocysts of each type have different effects on the mixed blastocysts.

The aim of present study was to examined whether the timing of blastocyst collection, formation or the duration of post-blastulation culture affected the quality of *in vitro*-produced pig embryos.

2. Materials and methods

2.1. Chemicals

All chemicals were purchased from Sigma (St. Louis, MO, USA) unless otherwise indicated.

2.2. Collection of cumulus-oocyte complexes (COCs) and in vitro maturation

All animal procedures were approved by the Institutional Animal Care and Use Committee of Chungnam National University (CNU-00373). Pig ovaries were collected from pre-pubertal gilts at a local abattoir, maintained at 30–35 °C, and transported to the laboratory within 2 h of removal from the ovaries. Each ovary was washed three times with phosphate buffer solution (PBS) supplemented with penicillin (100 IU/ml) and streptomycin (100 μg/ml). The COCs and pig follicular fluid were aspirated from small antral follicles (3–6 mm in diameter) on the surface of ovaries, using a 10-ml disposable syringe attached to an 18-gauge needle. The COCs characterized by several layers of cumulus cells and a uniform ooplasm were selected and placed in Ca^{2+}/Mg^{2+}-free PBS (Gibco, Grand Island, NY, USA) supplemented with 0.1% polyvinyl alcohol (PVA). Approximately 50–60 COCs were cultured in 500 μl TCM supplemented with 10% porcine follicular fluid, 10 ng/ml epidermal growth factor (EGF), 10 IU/ml hCG and 10 IU/ml PMSG in each well of a four-well multi dish for 22 h at 38.5 °C in air containing 5% CO_2. After maturation during culturing, the COCs were further cultured in the same medium without hormones for 22 h.

2.3. Electrical activation of pig oocytes and in vitro culture

After 44 h of *in vitro* maturation, the oocytes were subjected to electrical activation. Cumulus-free oocytes were washed and equilibrated in an activation solution containing 0.3 M D-mannitol, 0.1 mM $MgSO_4$, 0.05 mM $CaCl_2$, and 0.01% PVA. Activation was induced with a direct current-pulse of 1.5 kV/cm for 100 μs using an Electro Cell Manipulator 2001 (BTX, San Diego, CA, USA). After activation, oocytes were immediately transferred into PZM-3 containing 4 mg/ml bovine serum albumin (BSA) and 7.5 μg/ml cytochalasin B for 5 h. The embryos were washed three times in PZM-3 plus BSA, transferred to 500 μl of the same culture medium in each well of a four-well multi dish, covered with mineral oil, and incubated at 38.5 °C in a humid 5% CO_2 atmosphere. The day of PA was designated as day 1 of culture.

2.4. Blastocyst collection and definition

For delayed blastocyst collection (DBC), blastocysts were collected early on day 6 of culture (Fig. 1Aa, D6 group), late on day 7 of culture (Fig. 1Ab, D7 group), or late on day 8 of culture (Fig. 1Ac, D8 group). For delayed blastocyst formation (DBF), newly formed blastocysts were collected on day 6 (Fig. 1Ba, F6 group), day 7 (Fig. 1Bb, F7 group) or day 8 (Fig. 1Bc, F8 group) of culture. For the