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Technical note

Post-maturation zona perforation improves porcine parthenogenetic trophoblast culture

I.M. Saadeldin [a,c], S.J. Kim [a], Y.B. Choi [a], B.C. Lee [a,b,*]

[a] Department of Theriogenology and Biotechnology, College of Veterinary Medicine and The Research Institute for Veterinary Science, Seoul National University, Seoul 151-742, Republic of Korea
[b] Institute of Green Bio Science and Technology, Seoul National University, Pyeong Chang, Kangwon do, Republic of Korea
[c] Department of Physiology, Faculty of Veterinary Medicine, Zagazig University, Zagazig 44519, Egypt

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ABSTRACT

This study was designed to optimize a method to improve porcine parthenogenetic embryo hatching and trophoblast culture. Mature oocytes (D0PPA) and day 6 blastocysts (D6PPA) were perforated with a 20 μm diameter needle for assisted hatching. The two groups showed a significant difference in hatching rate and blastocyst cell doubling when compared to a non-perforated control group. D0PPA blastocysts were able to form tertiary trophoblast colonies but D6PPA and control groups were not able to grow beyond primary colonies. Quantitative real-time PCR analysis showed significant differences in *BAX*, *BAX*/*BCL2L1* and *HSP70-2* mRNA expression between the experimental groups.

1. Introduction

Many reports have been published about porcine embryonic stem-like cell lines, however cell line validation was not achieved, despite numerous trials for isolation and propagation of putative cell lines, especially those from the *in vitro* produced embryos [1–4]. One of the factors responsible for this failure is the big difference in cell numbers between the *in vivo* and *in vitro* derived embryos [5,6], and particularly those of parthenotes [6] because of cellular apoptosis. An additional barrier is the presence of the zona pellucida (ZP) which can trap blastocysts and prevent them from hatching due to its thickness [7,8]. Hatching *in vitro* occurs at a thin, focal part of the ZP and is mainly caused by blastocyst expansion after embryo compaction [9,10]. Because of the ZP obstacle, the embryonic cells (trophoblast cells and inner cell mass) cannot dramatically double as they normally would in hatched embryos, and instead undergo apoptosis and fragmentation [11,12]. Apoptosis can be reduced by the heat shock proteins (HSPs) family, which attenuates the caspase pathway and through pro-apoptotic factors (e.g. BAX and P53) and the anti-apoptotic BCL-2 family proteins which target the mitochondria-dependent pathways of apoptosis [13,14]. In addition, the HSPs are important for protecting cells from stress [15]. To overcome the problem with hatching, assisted hatching (AH) has been introduced *via* several methods [16]. In the pig, ZP incision method was reported early on *in vivo* derived embryos, but it was invasive and adversely affected embryonic development [17,18]. In other approaches, some attempted to totally remove the ZP enzymatically with pronase but this also affected the developmental competence of embryos [8]. Therefore, in the current study we attempted to optimize a method for improving porcine parthenogenetic embryo-derived trophoblast cell lines using a simple zona pellucida puncture of mature oocytes.

2. Methods

Ovaries were obtained from sows at a local slaughterhouse. The follicular fluids including cumulus-oocyte complexes (COCs) were aspirated from 3 to 6 mm diameter antral follicles. COCs with a compact multi-layered cumulus mass and dark, evenly granulated ooplasm were selected for *in vitro* maturation in four-well dishes in basic maturation medium according to our previous report [19]. Mature oocytes were either directly activated or were perforated before parthenogenetic activation as mentioned below. Parthenogenetic activation was done following our previous report [20]. Blastocysts on days 7 and 8 of the experimental groups were stained with 25 μg mL^{-1} bisbenzamide for cell counting. Blastocyst total RNA extraction, reverse transcription and real-time PCR were done using Applied Biosystems StepOnePlus™ Real-Time PCR Systems (Applied Biosystems, Forest City, CA) according to our previous report [19]. In brief a total 20 μL PCR reaction was made by adding 100 ng cDNA, 1 μM forward primer, 1 μM reverse primer, 10 μL SYBR Premix Ex Taq with ROX reference (Takara Bio Inc. Shiga, Japan) and 6 μL of Nuclease-free water (Ambion Inc., Austin, TX, USA). Each target transcript was relatively quantified in 3 replicates *via* calculating the $2^{-\Delta\Delta Ct}$ method [21] for comparison of relative gene expression patterns in embryos normalized to the housekeeping gene *GAPDH*.

* Corresponding author. Department of Theriogenology and Biotechnology, College of Veterinary Medicine, Seoul National University, Gwanak-ro, Gwanak-gu, Seoul 151-742, Republic of Korea. Tel.: +82 2 880 1269; fax: +82 2 873 1269.
E-mail address: bclee@snu.ac.kr (B.C. Lee).

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Table 1
Embryonic development after zona perforation on day 0 (D0PPA) or on day 6 (D6PPA).

	D0PPA	Control	D6PPA
Activated oocytes	528	677	
Cleavage (%)	480 (90.9)	617 (91.13)	
Day 6 blastocyst (%)	211 (39.96)	244 (36.04)	
Total BL for comparison	140	110	110
Hatching (%)	104 (74.28)[a]	12 (10.9)[b]	74 (67.27)[a]
Day 7 cell count	59 ± 6.32[a]	37.5 ± 3.1[b]	52 ± 10.53[a]
Day 8 cell count	98 ± 10.4[a]	61.6 ± 3.05[b]	68.3 ± 9.07[b]
Hatched BL on feeder cells	60	12	60
Attached and TE growth (%)	13 (21.66)[a]	1 (8.33)[b]	4 (6.66)[b]
RQ of mRNA expression[d]			
BAX	0.42 ± 0.02[a]	1[b]	3.05 ± 0.13[c]
BCL2L1	0.93 ± 0.07[a]	1[b]	3.33 ± 0.61[b]
BAX/BCL2L1	0.42 ± 0.02[a]	1[b]	0.91 ± 0.05[b]
HSP70-2	1.55 ± 0.06[a]	1[b]	0.31 ± 0.03[c]
P53	0.88 ± 0.03[a]	1[a]	0.78 ± 0.07[a]

TE; trophectoderm.
RQ; relative quantitation of mRNA expression. The values represent mean ± SD.
Values carrying different superscripts in the same row are significantly different ($P < 0.05$).
[d] mRNAs expression was normalized to *GAPDH* expression and compared to the arbitrary control group.

Primer sequences, annealing temperatures and approximate size of the amplified fragments are listed in Supplemental Table 1.

Day 8 hatched blastocysts from the three experimental groups were randomly chosen and seeded on mouse embryonic fibroblasts, which had been mitotically inactivated with mitomycin C, as feeder cells [22] for trophoblast culture.

Denuded mature oocytes (D0PPA) or day 6 blastocyst (D6PPA) were perforated using a 20 μm diameter injection needle beveled to a 30° angle for minimal invasiveness. To examine whether this perforation can activate oocytes or not; we punctured 50 oocytes with the same needle and subjected them to the same steps of parthenogenesis but without electrical activation. Another control group ($n = 50$) was handled similarly but without puncture. No blastocysts were formed, indicating that zona perforation is not a possible factor for parthenogenesis.

The data were analyzed using Pearson's chi-square test (for the ratios) and Student's *t*-test (for the means). Statistical significance was considered when the *P* value was less than 0.05.

3. Results and discussion

Assisted hatching methods include mechanical incision of the zona; chemical zona drilling with acidic medium; chemical zona thinning; laser-assisted hatching and piezo technology [16]. These methods are mostly applied at the blastocyst stage or immediately prior to embryo transfer [23]. In the current study we attempted by puncturing mature oocytes to increase the proportion of porcine parthenogenetic hatched blastocysts able to attach and form primary trophoblast outgrowths under feeder-dependent culture conditions. We compared the effect of oocyte (D0PPA) *vs.* blastocyst (D6PPA) zona perforation on 1) embryo morphology, 2) embryo development and trophoblast outgrowths, and 3) apoptosis and stress related genes mRNA expression.

In the D6PPA group, it was noticed that blastocysts collapsed within 15–60 s after perforation, then expanded again within 1–2 h. However, in the D0PPA treatment, there was no apparent effect on the ooplasm of punctured oocytes or on day 6 when compared with the non perforated control group. It is known that AH in the blastocyst stage makes the blastocele collapse, causing what is called "artificial shrinkage (AS)" which features alternating contraction–expansion cycles and cytoplasmic extensions of the trophectoderm however, the mechanism of blastocele collapse and recovery of their spheroidal shape is unclear [24].

In the D6PPA and control groups, the protrusion of embryonic cells (hatching) occurred on day 7 while in the D0PPA group hatching occurred earlier, by the end of day 5.

Blastocyst formation rate was not significantly different between D0PPA and the control group, $P > 0.05$ (Table 1), suggesting that the current technique with its minimal invasiveness has no damaging effect on embryos. The frequencies of day 8 hatching in the D0PPA and D6PPA groups showed no significant difference, however, they were significantly higher (~6 times) than in the control group, $P < 0.05$ (Table 1). On day 7, the blastocyst cell numbers of D0PPA and D6PPA embryos were significantly higher than that of the control group, while on day 8, the D0PPA blastocyst

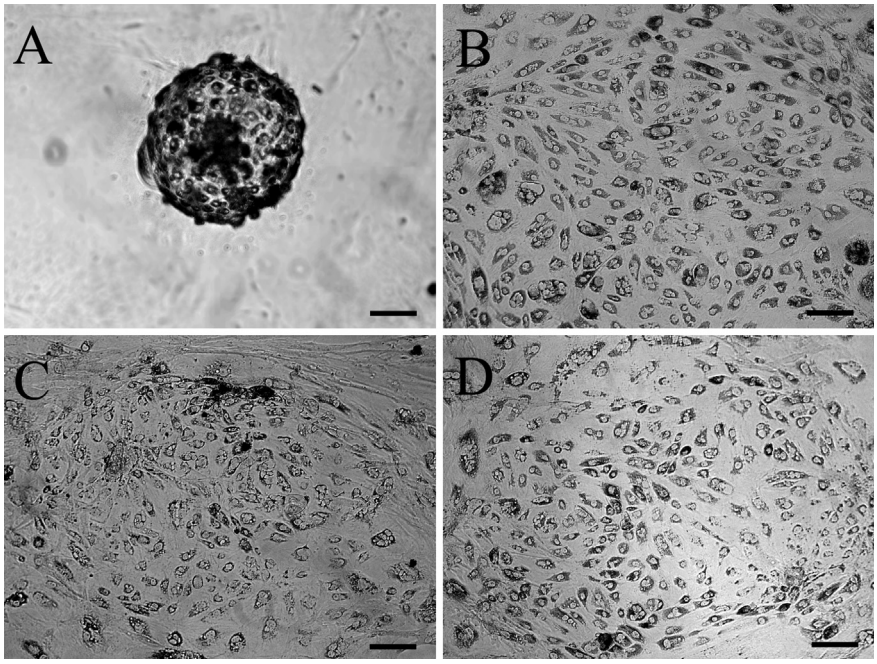

Fig. 1. Porcine parthenogenetic embryo-derived trophoblast culture on mouse embryonic fibroblast feeder cells. Hatched blastocysts (A) were seeded on feeder cells and formed primary trophoblast outgrowths (B) within 6 days. The colony was mechanically transferred to fresh feeder cells and formed a secondary colony (C) within 6 days and the process was repeated for the tertiary colony (D). The scale bar = 100 μm.

total cell count was significantly higher than in the other two groups (Table 1). It is conspicuous that cells of the D0PPA and control groups underwent doubling (1.66-fold and 1.64-fold, respectively) from day 7 to day 8 while D6PPA embryos showed only 1.31-fold cell doubling; this difference might be caused by the stress of the artificial shrinkage and re-expansion of punctured blastocysts. A recent report found that ZP-free embryos had lower apoptosis than intact ones; however, these embryos had lower developmental competence [8].

Furthermore, the rate of blastocyst attachment and trophoblast primary outgrowths on feeder cells was significantly higher in the D0PPA than in the control and D6PPA groups (Table 1), which might be linked to the initial cell number of cultured blastocyst. Moreover, the primary colonies derived from D0PPA embryos were able to establish secondary and tertiary colonies after mechanical passages and culture on new feeder cells (Fig. 1), while outgrowths from the other two groups could not grow beyond primary colonies. Recent attempts failed to obtain more trophoblast passages from parthenogenetic embryos [4], but in the current study, with this technique, we succeeded in establishing tertiary colonies from PA embryos.

The relative quantitation (RQ) of *BAX* (apoptotic gene) and *BCL2L1* (anti-apoptotic gene) mRNAs in D6PPA embryos was significantly higher than in the D0PPA and control groups. The *BAX*/*BCL2L1* ratio, as an indicator of apoptosis, was significantly higher in D6PPA than D0PPA embryos. Stress-related gene *HSP70-2* mRNA expression in D0PPA embryos was significantly higher than in the control group which was significantly higher than D6PPA too. Expression of *P53* showed no significant difference between the three groups. All the perturbed gene expressions might explain the apoptosis and decreased cell numbers in the D6PPA compared to the D0PPA groups.

In conclusion, minimally invasive post-maturation zona perforation of porcine oocytes using a small sized injection needle, as an assisted hatching tool, is preferable than blastocyst puncture because it reduces the stress on blastocysts and permits the blastocyst cell doubling which is a prerequisite for further development of embryo-derived cells *in vitro*.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.placenta.2014.02.003.

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