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Effects of Delayed Activation on In Vitro and In Vivo Developmental Efficiency of Porcine Cloned Embryos (Postprint)

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Abstract

To improve the efficiency of pig cloning and obtain more cloned pigs, this study investigated the effects of delayed activation on the in vitro and in vivo development of porcine somatic cell nuclear transfer (SCNT) embryos. The results showed that compared with the synchronous fusion-activation method, delayed activation reduced the fusion rate of reconstructed embryos ($P > 0.05$), but significantly increased the cleavage rate ($P < 0.01$) and blastocyst rate ($P < 0.05$) of cloned embryos. When reconstructed embryos were subjected to delayed activation with CB-assisted activation for 4 h, the blastocyst rate was significantly higher than that of the group without CB ($P < 0.01$). After transferring cloned embryos into 126 recipient sows, the farrowing rate of the delayed activation group was significantly higher than that of the synchronous activation group ($P < 0.05$). Although no significant differences were observed in litter size, number of live piglets per litter, or cloning efficiency, the delayed activation group clearly obtained more cloned piglets. These results indicate that the delayed activation method can enhance the in vitro and in vivo developmental efficiency of porcine cloned embryos.

Full Text

Preamble

Delayed Activation Improves In Vitro and In Vivo Developmental Efficiency of Porcine Cloned Embryos

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Abstract

To improve the efficiency of porcine somatic cell nuclear transfer (SCNT) and increase the yield of cloned pigs, this study investigated the effects of delayed activation on the in vitro and in vivo development of porcine cloned embryos. Compared with synchronous fusion-activation methods, delayed activation significantly improved the cleavage rate ($P < 0.01$) and blastocyst rate ($P < 0.05$) of cloned embryos, although it slightly reduced the fusion rate of reconstructed embryos ($P > 0.05$). When delayed activation was combined with 4-hour cytochalasin B (CB) treatment, the blastocyst rate was extremely significantly higher than in the group without CB ($P < 0.01$). Following embryo transfer into 126 recipient sows, the farrowing rate in the delayed activation group was significantly higher than in the synchronous activation group ($P < 0.05$). Although no significant differences were observed in average litter size, average live births, or overall cloning efficiency, the delayed activation group produced substantially more cloned piglets. These results demonstrate that delayed activation can enhance both the in vitro and in vivo developmental competence of porcine cloned embryos.

Keywords: Delayed activation; Cloning; Pig

Introduction

Since the first report of cloned pigs in 2000 [1, 2], numerous studies have documented the birth of cloned pigs [3-5]. However, despite more than a decade of research, the production efficiency of cloned pigs remains low [6]. Porcine somatic cell nuclear transfer is a complex technology influenced by multiple technical factors, including donor cell type and source, oocyte quality, fusion, activation, embryo culture, and transfer procedures [7]. Among these, fusion and activation are critical steps that largely determine the developmental capacity of cloned embryos [8].

Following transfer of a somatic cell nucleus into an oocyte, a series of morphological changes occurs, including nuclear membrane breakdown (NEBD), premature chromatin condensation (PCC), and pseudo pronucleus (PPN) formation, collectively representing nuclear remodeling [9]. These events are closely associated with maturation promoting factor (MPF) activity in the ooplasm [10]. When donor nuclei are transferred into pre-activated cytoplasm, NEBD and PCC are not induced, likely due to reduced MPF activity in the activated cytoplasm. If donor nuclei undergo NEBD and PCC, the accessibility of reprogramming factors increases [11]. Chromosomal analysis has shown that the ploidy of reconstructed embryos is closely related to the number of pronuclei formed after

activation and the extrusion of pseudo-polar bodies [12]. The number of PPNs in reconstructed embryos affects ploidy, developmental competence, and cytoskeletal modification. Agents such as demecolcine and cytochalasin B (CB) can improve porcine cloned embryo development by inhibiting pseudo-polar body extrusion and inducing single pronucleus formation [13].

Since ooplasmic factors mediate reprogramming [14], synchronous activation methods may not fully exploit the potential of cytoplasmic factors, whereas delayed activation introduces a fusion-activation interval that allows cytoplasm with high MPF levels to act on donor nuclei more effectively. However, the impact of synchronous versus delayed activation on cloning efficiency in porcine SCNT has been inconsistent across studies [15-18], and clear evidence regarding the relationship between activation methods and embryo developmental capacity remains elusive. Moreover, previous reports have focused exclusively on in vitro developmental efficiency, necessitating validation of whether delayed activation can truly improve cloning efficiency through birth outcomes. Large-scale transplantation experiments examining the effects of delayed activation on in vivo embryo development are currently lacking. Therefore, this study evaluated the effects of synchronous and delayed activation on both in vitro and in vivo development of porcine cloned embryos.

Materials and Methods

1.1 Reagents and Culture Media

TCM-199 powder and Dulbecco' s phosphate-buffered saline (DPBS) powder were purchased from Gibco. All other chemicals and reagent-grade water were from Sigma unless otherwise specified. Maturation medium consisted of modified TCM-199 supplemented with 10% porcine follicular fluid. Oocyte washing medium was DPBS with PVA. Electrofusion medium contained 0.25 mol/L mannitol and 0.1 mmol/L MgCl . Electrical activation medium contained 0.25 mol/L mannitol, 0.1 mmol/L CaCl , and 0.1 mmol/L MgCl . Embryo culture medium was PZM-3. CB supplementation medium contained 5 g/mL CB in embryo culture medium.

1.2 Donor Cell Isolation and Culture

Donor cells were derived from ear tissue of elite boars from Guangdong Wens Group. Ear skin samples were collected and stored in DMEM at 4°C for transport to the laboratory. Tissue fragments were minced, washed with DMEM, resuspended in fetal bovine serum, and transferred to culture dishes for incubation at 37°C with 5% CO in saturated humidity. After 5-7 hours, DMEM containing 10% fetal bovine serum was added. Cells were passaged upon reaching 90% confluence, and contact-inhibited ear fibroblasts at passages 3-5 were used as nuclear donor cells.

1.3 Oocyte Collection and Maturation

Ovaries were obtained from slaughtered sows and transported to the laboratory in 37°C saline. After three washes with antibiotic-supplemented saline, follicles (2-6 mm) were aspirated using a 10 mL syringe with an 18G needle. Cumulus-oocyte complexes (COCs) were collected under a stereomicroscope using a hand-made pipette, washed three times in washing medium and twice in maturation medium, then cultured in pre-equilibrated maturation medium for 44 hours at 39°C with 5% CO in saturated humidity. Matured COCs were treated with 0.1% hyaluronidase and pipetted repeatedly to remove cumulus cells. Oocytes with a distinct perivitelline space, uniform cytoplasm, and a clearly extruded first polar body were selected for cloned embryo construction.

1.4 Cloned Embryo Construction

Mature oocytes were enucleated by removing the polar body and approximately 15% of surrounding cytoplasm using a micromanipulator. A single somatic cell was injected into the perivitelline space to complete reconstruction. Two fusion-activation methods were employed:

Synchronous fusion-activation: Reconstructed oocytes were placed in activation medium-filled fusion chambers, oriented so that the donor cell-recipient oocyte membrane contact plane was parallel to the electrodes. Fusion and simultaneous activation were induced with two DC pulses (80 V/mm, 100 s). Fused embryos were washed in embryo culture medium and cultured for 4 hours in pre-equilibrated medium containing 5 g/mL CB, then transferred to fresh embryo culture medium until blastocyst stage.

Delayed activation: Reconstructed embryos were placed in electrofusion medium and fused using different parameters, then incubated in embryo culture medium for 1 hour. Fused embryos were selected and activated with different DC pulse parameters, then either cultured directly in embryo culture medium or first cultured for 4 hours in medium containing 5 g/mL CB before continued culture to blastocyst stage.

1.5 Embryo Transfer

Naturally cycling Large White sows were used as recipients. Surgical transfer was performed 48 hours after the onset of standing estrus. Recipients were anesthetized and the oviducts were exposed via laparotomy. Embryos were transferred equally into both oviducts before incision closure. Pregnancy was diagnosed by B-ultrasound on day 28 post-transfer.

1.6 Statistical Analysis

To minimize experimental error, all trials were conducted during the same season using oocytes from the same source. Data are presented as mean ± standard error. Statistical analysis was performed using SPSS software. Fusion rate,

cleavage rate, blastocyst rate, pregnancy rate, and farrowing rate were analyzed using chi-square tests. Total blastocyst cell numbers, average litter size, and average live births were analyzed using one-way ANOVA.

Results

2.1 Effects of Delayed Activation on Fusion Rate and In Vitro Development

Two fusion-activation methods were compared for their effects on in vitro developmental efficiency. Delayed activation employed two different parameter sets. Parameter set 1 used identical parameters to synchronous activation, while parameter set 2 used fusion parameters of 85 V/mm, 60 s, 2 pulses and activation parameters of 80 V/mm, 80 s, 2 pulses.

For parameter set 1, 209 reconstructed embryos were used for synchronous activation and 213 for delayed activation across six replicates . Compared with synchronous activation, delayed activation significantly reduced fusion rate but showed no significant differences in cleavage rate, blastocyst rate, or total blastocyst cell number. Although delayed activation with identical parameters did not improve blastocyst rate, it enhanced blastocyst quality. However, the significant reduction in fusion rate decreased the final embryo yield, necessitating parameter optimization.

For parameter set 2, 394 reconstructed embryos were used for synchronous activation and 530 for delayed activation across six replicates. Delayed activation yielded lower fusion rates than synchronous activation, but the difference was not significant. Regarding in vitro developmental efficiency, delayed activation significantly improved cleavage rate ($P < 0.01$) and blastocyst rate ($P < 0.05$), with total blastocyst cell numbers also higher ($P = 0.07$). These results demonstrate that delayed activation with optimized parameters can improve in vitro developmental efficiency of porcine cloned embryos, and this parameter set was used for all subsequent delayed activation experiments [Figure 2: see original paper].

2.2 Effects of CB Treatment After Delayed Activation on In Vitro Development

The effect of CB treatment following delayed activation was evaluated by comparing two groups: one cultured for 4 hours in medium containing 5 g/mL CB before continued culture to blastocyst stage, and another cultured directly in embryo culture medium. The experiment used 214 embryos in the non-CB group and 226 embryos in the CB group across six replicates . CB treatment for 4 hours significantly improved blastocyst rate ($P < 0.01$) compared with the untreated group, while also increasing cleavage rate and total blastocyst cell numbers. Thus, CB treatment after delayed activation enhances blastocyst formation in porcine cloned embryos [Figure 3: see original paper].

2.3 Effects of Activation Method on In Vivo Developmental Efficiency

Reconstructed embryos prepared on the same day (using identical donor cells, oocyte batches, and enucleation/injection methods) were activated using different methods, treated with CB for 4 hours, and then transferred into recipient sows. After 114 days, farrowing outcomes were recorded including 28-day pregnancy rate, farrowing rate, average litter size, average live births, total piglets per recipient, and cloning efficiency [TABLE:1, TABLE:2].

As shown in Table 1, the farrowing rate following embryo transfer was significantly higher in the delayed activation group than in the synchronous activation group ($P < 0.05$). Table 2 reveals that the delayed activation group exhibited higher values for average total piglets per litter, total piglets per recipient, and overall cloning efficiency, with only average live births per litter being lower than the synchronous group, though none of these differences reached statistical significance.

Discussion

Incomplete donor nuclear reprogramming is the primary cause of low cloning efficiency, while NEBD and PCC represent initial events in donor nuclear morphological remodeling. High MPF activity and glutathione levels in oocytes affect nuclear remodeling of reconstructed embryos, and only non-activated oocytes maintain high MPF activity [19]. Therefore, adequate interaction between donor nucleus and recipient cytoplasm before activation is crucial for promoting donor nuclear reprogramming and subsequent embryo development. Insufficient exposure of donor nuclei to cytoplasmic factors before activation may lead to incomplete reprogramming and poor embryo development [20]. Yin et al. [21] demonstrated via immunofluorescence that donor nuclei in delayed activation embryos divided into two chromosome clusters by 3 hours post-activation, whereas in synchronous activation embryos, the donor nuclear membrane ruptured quickly but formed a large polar body-like structure only after 6 hours. Moreover, the proportion of delayed activation embryos forming two nuclei was significantly higher, indicating the impact of delayed activation on donor nuclear remodeling. Although some cloning studies continue to use synchronous activation [22, 23], our results show that delayed activation yields superior both in vitro and in vivo developmental efficiency.

Fusion and activation of reconstructed embryos are influenced by numerous factors including electric field strength, pulse duration, pulse number, fusion medium composition, and chamber structure [24]. The effects of different electrical parameters on synchronous activation efficiency have been extensively reported [13, 25, 26]. The parameters used for synchronous activation in this study were optimized through multiple trials in our laboratory (unpublished data). When the same parameters were applied to delayed activation, although in vitro development efficiency was not significantly different and blastocyst quality improved, fusion rate decreased significantly, reducing the number of

embryos obtained. After parameter modification, delayed activation achieved comparable fusion rates to synchronous activation while significantly improving in vitro developmental efficiency. The principle of electrofusion-activation involves electric pulses inducing micropore formation in cell membranes to mediate donor cell-oocyte fusion while simultaneously allowing extracellular Ca^2 influx to elevate intracellular Ca^2 concentration and activate the reconstructed embryo. Excessive pulse duration can negatively impact embryo development [27].

Since oocyte activation is triggered by Ca^2 influx from the activation medium, the presence or absence of Ca^2 in fusion medium during electrofusion is considered a determinant of oocyte activation. When Ca^2 is absent from fusion medium, oocytes are not activated, whereas Ca^2 presence leads to simultaneous fusion and activation [17]. To ensure delayed activation, our study omitted Ca^2 from the fusion medium. However, Ca^2 is not only important for oocyte activation but also plays a crucial role in blastomere compaction [28]. Absence of Ca^2 in fusion medium reduces contact between somatic cells and enucleated oocytes or delays micropore recovery [29], thereby decreasing electrofusion efficiency [30], which explains the lower fusion rates observed in our delayed activation group.

The fusion-activation interval in delayed activation also affects cloning efficiency. Studies in pigs [19], cattle [11], and goats [31] have shown that intervals that are too short insufficiently expose donor nuclei to ooplasmic reprogramming factors [31], while excessively long intervals increase chromosomal aneuploidy and reduce developmental efficiency [19]. Our approach using a 1-hour interval between fusion and activation yielded favorable results.

During fertilization, oocytes extrude half their chromosomes as the second polar body to maintain normal ploidy and form the male pronucleus [13]. Similarly, reconstructed embryos extrude a portion of donor chromosomes to form a pseudo-polar body [32], potentially causing chromosomal aneuploidy and reducing developmental capacity [33]. Maintaining diploidy in reconstructed embryos is an effective and necessary approach to improve SCNT efficiency. CB, which depolymerizes actin filaments [34], is widely used in parthenogenetic activation and SCNT embryos to inhibit polar body extrusion [13]. Sugimura et al. [35] reported that CB treatment of porcine cloned embryos increased the proportion of embryos with single pronuclei and diploid chromosomes, thereby improving blastocyst formation. Additionally, electrical activation can cause severe fragmentation by affecting actin filament distribution, reducing blastocyst rates, while CB treatment can decrease fragmentation [36]. Liu [31] demonstrated that CB treatment improved both in vitro and in vivo developmental efficiency in goat cloned embryos by reducing fragmentation. Our findings that CB treatment enhances porcine cloned embryo development are consistent with reports in mice [37] and cattle [38].

Although blastocyst development in vitro is used to predict in vivo success [39], environmental differences between in vitro and in vivo conditions mean that some treatments improving in vitro efficiency do not significantly affect transfer

outcomes [12, 40]. For practical pig cloning, a method' s utility ultimately depends on whether it improves cloning efficiency (born cloned piglets/transferred embryos) and increases the number of born piglets. Therefore, we conducted comparative embryo transfer experiments. To ensure consistency, both groups were transferred on the same day using the same donor cell and oocyte batches. To minimize recipient effects, 63 sows were used per group. Given the difficulty and cost of pig cloning, this recipient number is substantial compared with literature reports, ensuring reliable results. Since pigs are polytocous animals requiring implantation of at least four embryos to maintain pregnancy, the higher pregnancy rate (65.08% vs. 50.79%) and greater number of born piglets (163 vs. 115) in the delayed activation group confirm improved embryo quality. The cloning efficiency of 1.73% in our delayed activation group exceeds other literature reports [6]. However, average litter size was not high, with a relatively high proportion of stillbirths and low proportion of healthy piglets among live births, consistent with other reports [41], indicating that further research is needed to improve cloning efficiency.

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