Delayed activation methods and development of pig somatic cell nuclear transfer embryos: a meta-analysis
Running Head: Delayed activation affects development of pig SCNT

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SUMMARY
For pig somatic cell nuclear transfer (SCNT), artificial activation to trigger development can improve efficiency. However, because timing is key to activation, delayed activation is controversial. Two oocyte activation procedures are available: those that occur immediately after fusion and those that take place several hours after fusion. Both methods are successful for generating SCNT fetuses. We studied whether delayed activation is associated with pig SCNT embryo development. We searched the literature for publications from 2000 to 2018 and identified 1109 studies, 8 of which we included in a meta-analysis. A Higgins statistic was used to measure heterogeneity. Delayed activation enhanced embryo cleavage (OR = 0.73, 95% CI 0.61-0.87) and blastocyst formation (OR = 0.79, 95% CI 0.65-0.97) significantly and offered the same quality pig SCNT embryo (OR = -0.03, 95% CI -3.12–3.05). The Egger method and Begg's test were used to estimate publication bias, and there was none (Pr > |z| = 1). The most commonly used pig embryo media are NCSU-23 and PZM. Studies suggest that each offers unique advantages. Culture medium influences cleavage heterogeneity, and NCSU-23 can increase cleavage. Delayed time causes blastocyst heterogeneity; if the delay exceeds 1 h of activation, blastocyst formation improves. If the delay does not exceed 30 min, blastocyst formation decreases. Donor nuclei and recipient oocyte cytoplasm require a period of adaptation for chromatin remodeling and reprogramming. The timing of these events depends on maternal factors in the recipient oocyte cytoplasm. Thus, recipient oocyte cytoplasm requires time to mix with donor cytoplasm. Then, maternal factors can be added after donor nuclei are added. New nuclei may shuttle mRNA to the cytoplasm, and this can cause chromatin remodeling and reprogramming. Thus, future SCNT studies should focus on delay of activation timing.

KEY WORDS
Blastocyst cell number; Delayed activation; Meta-analysis; Pig; SCNT.

INTRODUCTION

For somatic cell nuclear transfer (SCNT), the lack of sperm-induced fertilization requires artificial activation for embryonic development[1]. Two kinds of procedures are available: those with simultaneous fusion and activation (SA)[2,3] and those with delayed activation (DA) occurring several hours after fusion[4]. Both methods are successful for generating SCNT fetuses.

DA and SA differ with respect to how donor nuclei and recipient oocyte cytoplasm are timed to adapt prior to activation[4]. After receptor oocyte nuclear removal, mature cytoplasm degrades over time[5]. Thus, although more time is better for adaptation, prolonged delays in activation allow cytoplasm degeneration. Therefore, DA is controversial. The purpose of this meta-analysis was, therefore, to determine whether DA is associated with pig SCNT embryo development.

MATERIALS AND METHODS

Database and data extraction

Two authors (ZHG and LL) performed literature searches in PubMed and ScienceDirect for articles published from 2000 to 2018. Submitted terms were used to search all articles in the following databases: ("swine"[MeSH Terms] OR "swine"[All Fields]) OR ("swine"[MeSH Terms] OR "swine"[All Fields] OR "porcine"[All Fields]) OR ("swine"[MeSH Terms] OR "swine"[All Fields] OR "pig"[All Fields]) AND (activation[All Fields] OR active[All Fields]) AND (SCNT[All Fields] OR ("clone cells"[MeSH Terms] OR ("clone"[All Fields] AND "cells"[All Fields]) OR "clone cells"[All Fields] OR "clone"[All Fields])) AND ("2000/01/01"[PDAT]: "2018/ 01/01"[PDAT] (PubMed); and pub-date > 2000 and pub-date <2018 and TITLE-ABSTR-KEY ((swine OR porcine OR pig) AND (SCNT OR clone)) (ScienceDirect). Criteria for inclusion are presented in Table 1.

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Table 1 - Inclusion and exclusion criteria.

Inclusion	Exclusion
Species not limited but must include pigs	Does not mention pigs on examination
Language must be English	Other language
Immediate activation versus delayed activation method for pig SCNT embryo	Insufficient data
SCNT blastocyst rate or cell number data are available	Insufficient data
Electrical fusion must be involved	No electrical fusion

Meta-analysis

The inverse-variance method was used to compare DA (control) data and SA (experimental) data. A Higgins statistic was used to measure heterogeneity. The I^2 ranged from 0 to 100%; $I^2 > 50\%$ indicated heterogeneity, but $I^2 < 50\%$ indicated homogeneity[6]. Publication bias was assessed visually with Christmas tree plots. For all applicable articles, we converted standard errors of the mean (SEM) to standard deviations (SD). All calculations were carried out with Review Manager (RevMan for Windows, Version 5.3, The Nordic Cochrane Centre, The Cochrane Collaboration, Copenhagen, Denmark). Funnel plots were constructed to test reporting bias. Egger's method[7] and Begg's test were used to assess publication bias using Stata 12.0 (Stata Corp, College Station, TX).

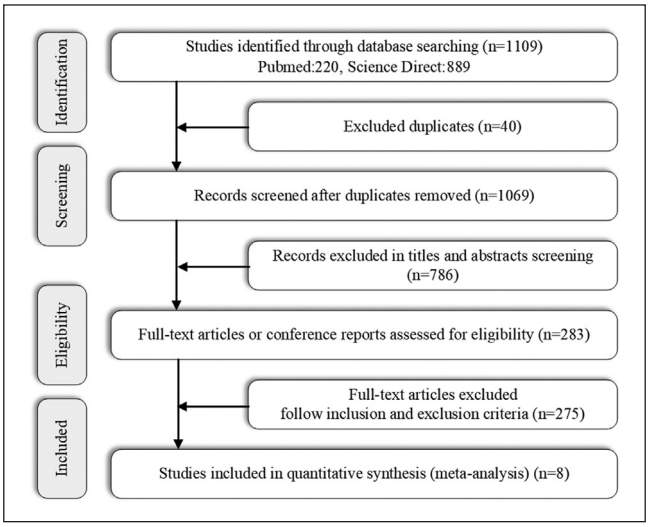

Figure 1 - Summary of study selection.

RESULTS

A total of 1109 studies were identified as shown in Fig. 1. Eight studies were included after the final review[2,3,8-13]. Background information and study characteristics from all studies are summarized in Table 2.

We analyzed eight studies that contrasted DA and SA and their effects on embryonic cleavage. Fig. 2 shows that DA increases cleavage (OR = 0.73, 95% CI 0.61-0.87). There was high heterogeneity among studies ($I^2 = 53\%$; $p = 0.04$), so a subgroup analysis of IVC medium, delay time, and donor cell source was performed (Table 2). IVC medium was the source of heterogeneity. Specifically, NCSU-23 increased cleavage, but PZM-3 did not. The Egger method and Begg's test were used to estimate publication bias, and there was none ($Pr > |z| = 1$).

We also found that (Fig. 3) DA can improve blastocyst formation (OR = 0.79, 95% CI 0.65-0.97), and again the studies were heterogeneous ($I^2 = 59\%$; $p = 0.02$). Subgroup analysis (Table 2) showed that heterogeneity was due to a delay time of 1 h. Publication bias was not found.

Finally, we analyzed pig SCNT blastocyst number, and DA and SA did not affect this. There was no publication bias or study heterogeneity for this factor (OR = -0.03, 95% CI -3.12 to 3.05; $I^2 = 27\%$; $p = 0.24$) (Fig. 4).

DISCUSSION

Main finding

The data show that embryo cleavage and blastocyst formation were significantly increased with DA. Blastocyst numbers were the same for both approaches.

Studies show that DA can lead to bovine SCNT embryonic abnormal chromatin configurations and within 2.5 h after fusion can improve blastocyst formation[14]. However, DA is reported not to affect the developmental ability of bovine SCNT embryos[15]. In pig research, DA and SA both produce piglets[8]. In seven included papers, only Bang et al. directly studied the differences between DA and SA in the production of offspring. Their results showed that DA-cloned piglet births were 1.5 times more efficient than those which used SA[8]. A very high efficiency of SCNT piglet production using a delayed activation method has also been reported[16]. That study also reported the effects of delayed activation depending on the various activation times after cell-oocyte fusion in pig SCNT[3].

Table 2 - Characteristics of studies included in the systematic review.

Study	Culture Time	IVC Medium	Delay time	Donor cell
Bang 2013	6 days	NCSU-23	5 h	Fetuses
De Sousa 2002	7 days	NCSU-23	2 h	Fetuses
Hyun 2003	7 days	NCSU-23	2 h	Fetuses
Kim 2016	6 days	PZM-3	30 min	piPSCs (piPSN-1)
Mizobe 2010	7 days	PZM-3	1 h	Fetuses
Samiec 2012	6-7 days	NCSU-23	1-2 h	Fetuses
Skrzyszowska 2008	6-7 days	NCSU-23	1.5-2 h	Fetuses
You 2010	7 days	NCSU-23	2 h	Newborn

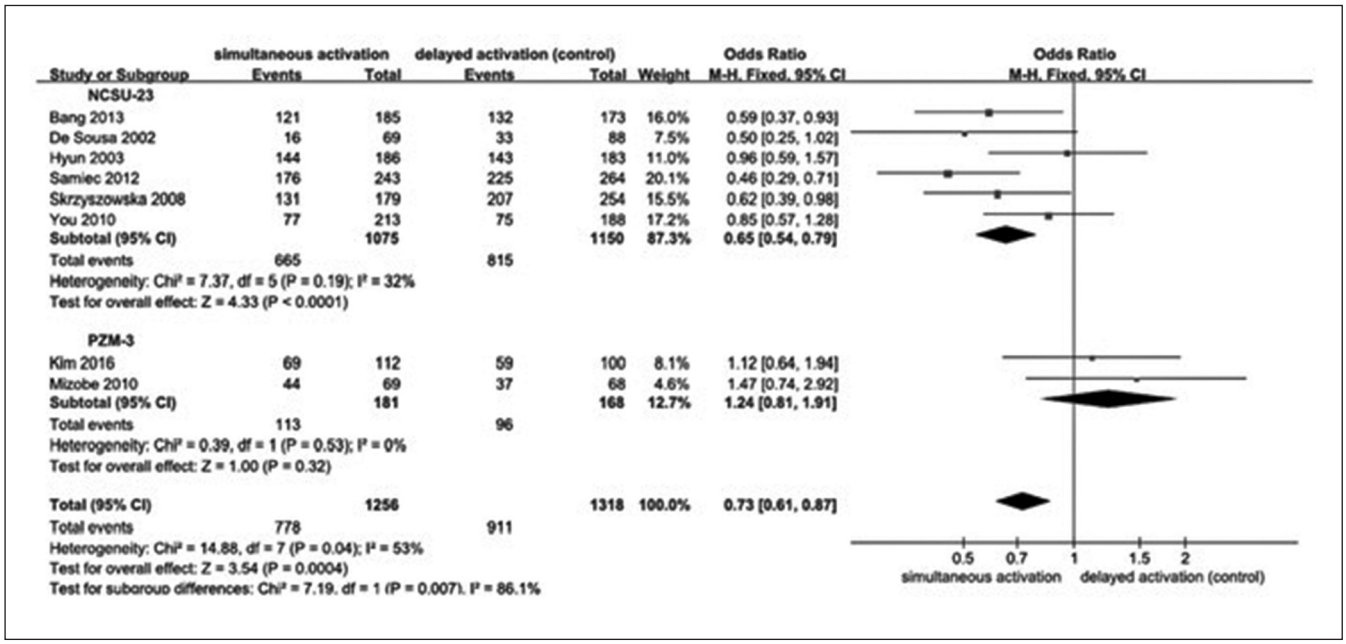

Figure 2 - Forest plot of embryo cleavage with different activations. Delayed activation was the control, and simultaneous activation was the experimental group. CI = 95% confidence interval.

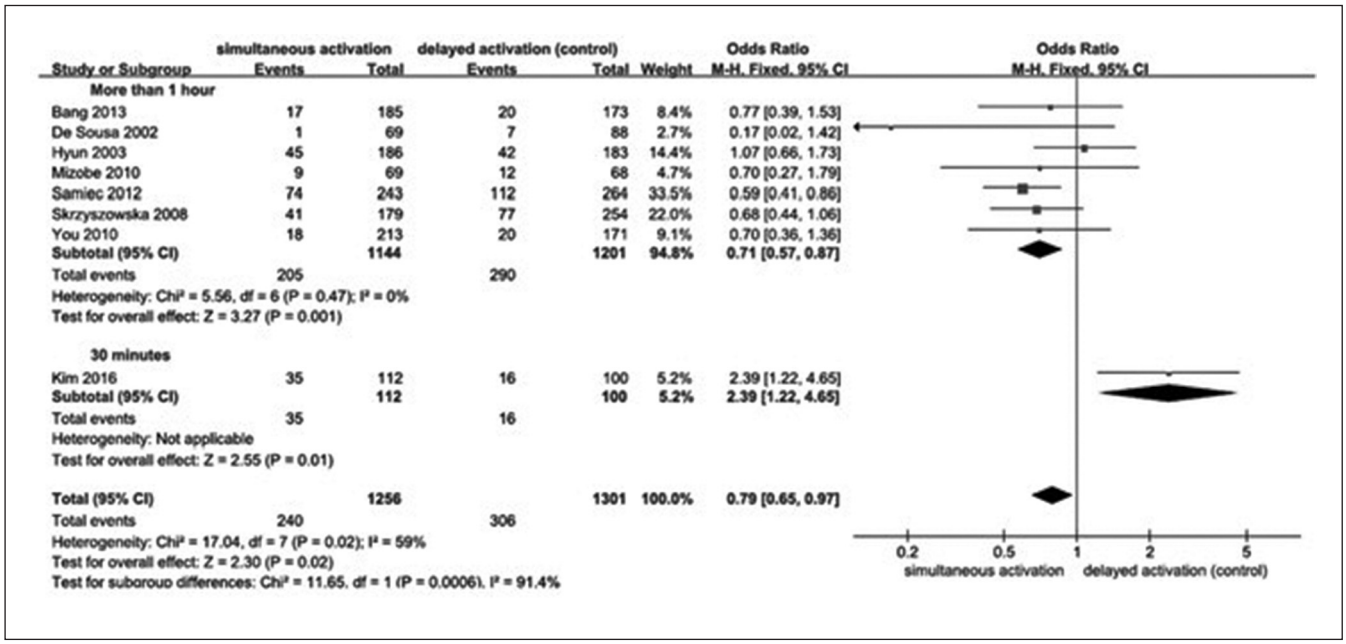

Figure 3 - Forest plot of blastocysts for different activation timings. Delayed activation was the control. CI = 95% confidence interval.

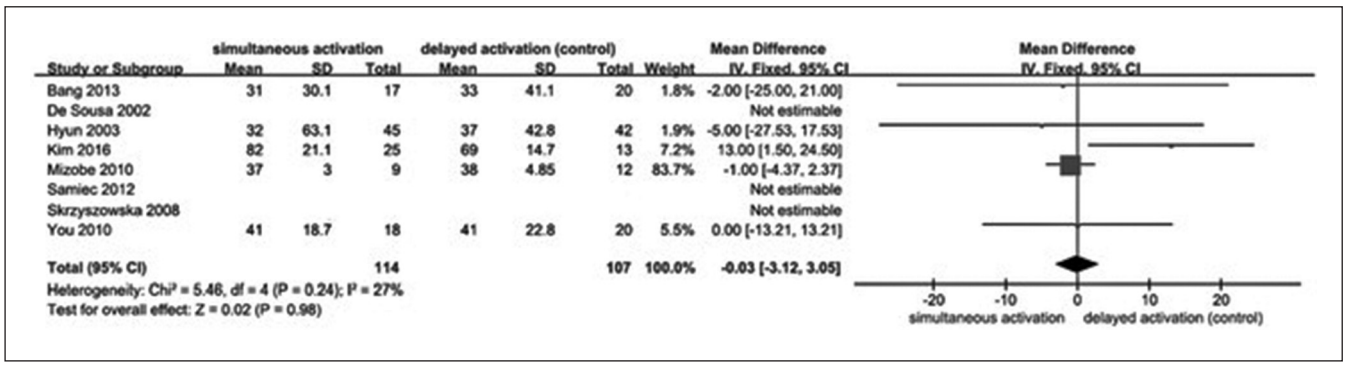

Figure 4 - Forest plot of blastocyst numbers (delayed activation vs simultaneous activation). Simultaneous activation was the experimental group. CI = 95% confidence interval.

Mammalian oocytes can mature *in vitro,* but oocytes maturing *in vivo* or *in vitro* will reach the metaphase II phase and undergo developmental retardation[17]. Then, only fertilization or activation can promote additional development[18]. This process is mainly regulated by maturation promoting factor (MPF)[18] and cytostatic factor (CSF)[19]. MPF is thought to participate in oocyte maturation of all mammalian research species[20]. High MPF activity can be measured in mammalian oocytes at metaphase stages M-I and -II. When mature oocytes are fertilized or parthenogenetically activated, MPF is inactivated. MPF is a dimer consisting of a catalytic ($P34^{CDC2}$, also known as Cdc2) and a regulatory subunit (cyclin B)[21]. Changes in cyclin B and cdc2 can directly affect the activity of MPF, which is maintained by CSF, which is sensitive to changes in cytoplasmic calcium. When calcium increases in the cytoplasm, CSF activity is reduced, and this decreases MPF activity. Cyclin B is sensitive to oscillations in calcium, which degrade cyclin B during fertilization, and this inactivates MPF[22].

Normally, oocyte activation occurs via sperm entering the oocyte, which causes calcium fluctuations[23,24]. For SCNT technology, it is necessary to reduce MPF during activation to allow normal reconstructed embryo development. Activation methods for SCNT embryos are chemical and physical. Chemical activation can be done with alcohol, calcium ion vectors, cycloheximide, and ionomycin, all of which cause artificial activation of the reconstructed embryo. For a physical method, electrical activation is convenient, efficient, stable, and repeatable[25], and it not only activates the oocyte but also promotes cell fusion.

Donor nuclei and recipient oocyte cytoplasm require a period of adaptation for chromatin remodeling and reprogramming, and the timing of these events depends on maternal factors in the recipient oocyte cytoplasm[5]. Donor nuclei are covered with donor cytoplasm, and this prevents contact of the oocyte cytoplasm with donor nuclei. Delaying activation may allow contact to occur and promotes the development of pig SCNT embryos. Maternal factors not only control reprogramming and drive embryonic growth forward but also block embryonic development. For pig embryos, the initial block occurs at metaphase II, and then it occurs again at the 4-cell stage. MPF has been used to improve SCNT efficiency[21,26]. When calcium increases, MPF and CSF activity are reduced, so many complex signaling pathways also adjust. Donor nuclei mRNA transfer to oocyte cytoplasm is feasible, but this requires more time than required for MPF to contact donor nuclei. After fusion, donor nuclei remain covered by donor cytoplasm. Thus, donor cytoplasm must be mixed with oocyte cytoplasm. This allows donor nuclei to translocate mRNA to the oocyte cytoplasm. These sequential steps require time. Also, maternal factors cannot maintain oocytes awaiting fertilization, so they degenerate.

Other studies have focused on donor nuclei, cell cycle adjustment[27], methylation[28], acetylation[29], and different cell types that offer varied SCNT efficiency[30] due to differences in the cytoplasm. tsRNAs (tRNA-derived small RNAs) can transfer information to the oocyte from sperm[31], and in this way, donor cell cytoplasm may affect SCNT efficacy.

The most common pig embryo media used are NCSU-23, NCSU-37, PZM-3, and PZM-5. Studies suggest that each offers unique advantages[32,33]. One study has shown that SCNT pig blastocysts were significantly more numerous in NCSU-23 than in PZM-5[34]. Our study tried to find the source of the heterogeneity, so subgroup analyses of IVC medium, delay time, and donor cell sources were performed. Finally, we found that the heterogeneity was due to IVC medium.

Implications

DA increases cleavage and blastocyst formation, but it does not change cell numbers, so the embryo quality should be the same as with SA.

CONCLUSION

Recipient oocyte cytoplasm requires time to mix with donor cytoplasm, and donor cell cytoplasm affects SCNT efficacy, but how this occurs is unclear. After mixing, maternal factors are added, and the new donor nuclei can send mRNA to the cytoplasm to cause chromatin remodeling and reprogramming. Thus, DA promotes pig SCNT embryo growth.

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CONTRIBUTIONS

Di Liu designed the project. Zhenhua Guo and Lei Lv performed literature searches. When search conclusions differed, a third author (Bo Fu) helped with the decision.

STATEMENT OF CONFLICT OF INTEREST

None.

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