Front. Agr. Sci. Eng. 2019, **6**(1): 61–65
https://doi.org/10.15302/J-FASE-2018227
Available online at http://journal.hep.com.cn/fase

RESEARCH ARTICLE

Effects of enucleation method on *in vitro* and *in vivo* development rate of cloned pig embryos

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Abstract Enucleation is a crucial procedure for mammalian somatic cell nuclear transfer (SCNT), especially for domestic animal cloning. Oocytes of domestic animals such as pigs and cattle contain dark lipid droplets that hinder localization and removal of the nucleus. Using an oocyte enucleation technique that can obtain a high enucleation rate but has minimal negative effects on the reprogramming potential of oocyte for cloning is beneficial for enhancing the outcome of SCNT. In this study, we compared the pig cloning efficiency resulting from blind aspiration-based (BA-B) enucleation and spindle imaging system-assisted (SIS-A) enucleation, and compared the pig SCNT success rate associated with BA-B enucleation and blind aspiration plus post-enucleation staining-based (BAPPS-B) enucleation. SIS-A enucleation achieved a significantly higher oocyte enucleation success rate and tended to obtain a higher *in vivo* full term development rate of SCNT embryos than BA-B enucleation. BAPPS-B enucleation also obtained significantly higher *in vitro* as well as *in vivo* full term development efficiency of cloned porcine embryos than BA-B enucleation. These data indicate that SIS-A and BAPPS-B enucleation are better approaches for pig SCNT than BA-B enucleation.

Keywords cloning, enucleation, pig, SCNT

1 Introduction

Enucleation is a key step for successful somatic cell nuclear transfer (SCNT). It is important especially in the cloning of domestic animals such as pigs and cattle, because the oocytes of these species contain dark lipid droplets, which hamper nucleus identification and removal. Increasing the enucleation rate and minimizing the detrimental effects of enucleation on the reprogramming ability of enucleated oocytes can improve the success rate of cloning.

Blind aspiration-based (BA-B) enucleation is a simple mechanical technique commonly used in SCNT. This method performs enucleation by removing a small volume of cytoplasm that presumably contains the nucleus adjacent to the first polar body[1,2]. This approach results in an enucleation rate that varies widely among laboratories (40%–90%) depending on the skill and experience of the operators; it is usually difficult to reach a high enucleation rate due to "blind" localization of the nucleus[2–5].

To increase enucleation efficiency, spindle imaging system-assisted (SIS-A) enucleation has been used for oocyte enucleation in pig, cattle and other species[6–13]. This method employs a polarized light microscope to visualize the meiotic spindle-containing oocyte nucleus. The SIS-A approach can achieve a high oocyte enucleation rate (88%–100%) in several mammalian species[8,10,12,13] and improve the *in vitro* development efficiency of cloned embryos in pigs and cattle[6,7].

Fluorescence dye staining has also been used to localize clearly the oocyte nucleus before enucleation[14,15]. This method can be referred to as pre-enucleation staining-assisted enucleation. Using this approach, oocytes can be precisely and completely enucleated; however, prior to enucleation, they suffer from prolonged ultraviolet (UV) light exposure, which negatively affects the reprogramming competence of enucleated oocytes[6,16–18]. To minimize the damaging effect of UV light irradiation, oocytes can be stained with fluorochrome after BA-B

Received December 7, 2017; accepted February 24, 2018

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enucleation and then examined under UV light to quickly discard non-enucleated or incompletely enucleated oocytes. This method could be called BA plus post-enucleation staining-based (BAPPS-B) enucleation. It not only minimizes the detrimental effect of UV light by shortening the exposure time of oocytes but also ensures the identification of 100% enucleated oocytes for subsequent nuclear transfer.

Thus far, no study has compared the effects of the BA-B, SIS-A and BAPPS-B enucleation on *in vitro* and *in vivo* development rate of cloned porcine embryos. In this study, pig SCNT embryos were produced through BA-B, SIS-A and BAPPS-B enucleation, and the *in vitro* as well as *in vivo* full term development efficiency between BA-B-produced and SIS-A-produced and between BA-B-produced and BAPPS-B-produced pig SCNT embryos were compared.

2 Materials and methods

2.1 BA-B enucleation

Matured oocyte was sucked firmly onto a holding pipette (outer diameter = 100–120 μm, inner diameter = 20–30 μm) to ensure immobility. The enucleation pipette (inner diameter = 15 μm) was inserted through the zona pellucida. The first polar body and adjacent cytoplasm that presumably contains all the chromosomes were aspirated into the enucleation pipette, which was then withdrawn from the oocyte.

2.2 SIS-A enucleation

Matured oocytes were placed individually in 10 μL microdroplets covered with mineral oil on a glass Petri dish. Meiotic spindle visualization was performed using the Oosight Imaging System (CRI, Woburn, MA, USA). Each oocyte was rotated with the help of two micropipettes to set the meiotic spindle at 90°. Each oocyte was sucked firmly onto a holding pipette (outer diameter = 100–120 μm, inner diameter = 20–30 μm) to ensure immobility. The enucleation pipette (inner diameter = 15 μm) was inserted through the zona pellucida. The second meiotic spindle with a positive signal of a white spot was aspirated into the enucleation pipette under polarized light microscopy and then withdrawn from the oocyte.

2.3 BAPPS-B enucleation

Each batch of oocytes enucleated by the BA-B procedure described above was stained with 1 $g \cdot mL^{-1}$ DNA dye Hoechst 33342 and examined under UV light irradiation for less than 10 s. Non-enucleated or incompletely enucleated oocytes with positive staining signal inside the cytoplasm were immediately discarded.

2.4 Analysis of enucleation rates

Oocytes enucleated by the BA-B or SIS-A method were stained with 1 $mg \cdot mL^{-1}$ DNA dye Hoechst 33342. Non-enucleated and incompletely enucleated oocytes with positive staining signal inside the cytoplasm were counted under UV light for the calculation of enucleation rate.

2.5 SCNT

Porcine ovaries were purchased from a slaughterhouse located in Guangzhou City, Guangdong Province, China. Cumulus-oocyte complexes were aspirated from the ovaries, matured *in vitro* for 42–44 h, then freed from their cumulus cells by repeated pipetting in 0.10% hyaluronidase. Only oocytes with an extruded first polar body were selected for subsequent enucleation. About 500–600 matured oocytes derived from the same batch of ovaries collected from the same slaughterhouse were produced each time. In Experiment 1, half of the mature oocytes were randomly selected and allocated to the BA-B group, and the other half was allocated to the SIS-A group. In Experiment 2, half of the mature oocytes were randomly selected and allocated to the BA-B group, and the other half was allocated to the BAPPS-B group. Matured oocytes were enucleated by the BA-B, SIS-A or BAPPS-B techniques described above. After enucleation, ear fibroblasts derived from a 2-year old adult Duroc boar that has a high breeding value were microinjected into the perivitelline space of the enucleated oocytes. The oocyte-donor cell complexes (for the BAPPS-B group) were cultured in porcine zygote medium 3 (PZM3) at 39°C, 5% CO_2, 5% O_2, 90% N_2 and 100% humidity for 1.5 h. The cell complexes were activated to fuse in a medium containing 250 $mmol \cdot L^{-1}$ mannitol, 0.1 $mmol \cdot L^{-1}$ $CaCl_2 \cdot 2H_2O$, 0.1 $mmol \cdot L^{-1}$ $MgCl_2 \cdot 6H_2O$, 0.5 $mmol \cdot L^{-1}$ HEPES and 0.01% polyvinyl alcohol by two successive DC pulses at 1.2 $kv \cdot cm^{-1}$ for 30 μs using an electrofusion instrument (CF-150/B, Biological Laboratory Equipment Maintenance and Service, Budapest, Hungary). The activated cloned embryos were then cultured in PZM3 containing cytochalasin B (5 $\mu g \cdot mL^{-1}$) for 4 h. After the post-activation treatment, the reconstructed embryos were cultured in PZM3 at 39°C, 5% CO_2, 7% O_2, 88% N_2 and 100% humidity.

Cloned embryos cultured for 22–24 h (at one to two cell stages) were examined to remove dead embryos and abnormally cleaved embryos with broken membrane. The remaining normal cloned embryos were loaded into a transparent transfer tube and kept in a portable incubator (Minitube, Delavan, WI, USA) during transportation to the farm where the recipient sows were housed. Yorkshire sows in parities 2–5 with similar genetic background, raised in a same pig farm under the same conditions and exhibiting a naturally standing estrus within 40–42 h prior to embryo transfer, were used as embryo recipients. The

sows were anesthetized with an anesthetic consisting of ketamine (25 mg·kg^{-1}) and xylazine (1.1 mg·kg^{-1}) for induction and 3% of isoflurane for maintenance. The ovaries and oviducts were exposed by cutting an incision (about 7 cm) along the midline of the sow's abdomen between the last two pairs of teats. The cloned embryos in 0.1 mL culture medium were delivered directly into the recipient oviduct using a 1-mL syringe attached to a transparent transfer tube. The transfer tube was examined subsequently under a microscope to ensure that all the embryos were transferred. Two sows were used as embryo recipients each time. In Experiment 1, one recipient received embryos of the BA-B group and another received embryos of the SIS-A group. In Experiment 2, one recipient received embryos of the BA-B group and another received embryos of the BAPPS-B group. Each recipient sow received 200–230 cloned embryos.

2.6 Analysis of the *in vitro* development indexes of cloned embryos

Activated cloned embryos were cultured in PZM3 at 39°C, 5% CO_2, 7% O_2, 88% N_2 and 100% humidity. The time of embryo activation was set as 0 h. The cleavage and blastocyst rates of cultured embryos were assessed 24 and 168 h after activation, respectively. The total number of cells of blastocysts was counted at 168 h by staining the embryos with 1 μg·mL^{-1} DNA dye Hoechst 33342 and viewing the cell nuclei under a fluorescence microscope.

2.7 Diagnosis of recipient pregnancy and delivery of cloned piglets

The pregnancy status of the recipient sows was examined by a B-mode ultrasound scanner (Wuxi Biomedical Technology Co., Ltd., Wuxi, China) one month after embryo transfer. If spontaneous farrowing did not occur until gestation day 116, then the recipient sows were injected with a prostaglandin analog (cloprostenol, 200 μg per recipient) to induce delivery. If the recipients still did not start to farrow 24 h after the injection, then Caesarean section was performed to deliver the cloned piglets. The total number of born cloned piglets was recorded.

2.8 Statistical analysis

To analyze the enucleation, cleavage, blastocyst, pregnancy and delivery rates of recipients and the development rate of transferred cloned embryos, chi-square analysis and Fisher's exact test were performed to determine the differences between the experimental groups. To analyze the total number of cells per blastocyst between two means (±SEM), *t*-test was used. Statistical significance was determined when the $P < 0.05$. All the data were analyzed by using SPSS software version 17 (IBM Corp, Armonk, NY, USA).

3 Results

The SIS-A enucleation resulted in a significantly higher oocyte enucleation success rate than BA-B enucleation (95.0 vs. 83.1, $P < 0.01$; Table 1). However, cloned porcine embryos produced by SIS-A and BA-B enucleation had similar *in vitro* development indexes, including cleavage rates (74.6% vs. 76.3%), blastocyst rates (13.3% vs. 18.3%) and total numbers of cells per blastocyst (47±4.10 vs. 40±3.34; Table 2). Sows that received BA-B-generated SCNT embryos exhibited the same pregnancy rates (69.70% vs. 69.70%) and farrowing rates (42.42% vs. 42.42%) as sows that received SIS-A-generated SCNT embryos (Table 3). The *in vivo* full term development efficiency (total number of born cloned piglets/total number of transferred cloned embryos) was not significantly different between the BA-B and SIS-A embryos, but there was an increased trend for SIS-A embryos (0.86% vs. 1.11%, $P = 0.13$).

Table 1 Comparison of the success rates of BA-B and SIS-A enucleation

Enucleation method	Number of manipulated/successfully enucleated oocytes	Enucleation rate/%
BA-B	384/319	83.07**
SIS-A	322/306	95.03**

Note: ** Significantly different at $P < 0.01$.

Although BAPPS-B and BA-B embryos had similar cleavage rates (72.3% vs. 76.0%) and blastocyst rates (17.8% vs. 21.9%) in *in vitro* culture, the former has a significantly higher total number of cells per blastocyst than the latter (41±1.69 vs. 36±1.77, $P < 0.05$; Table 4). Sows that received BAPPS-B embryos also had a significantly higher farrowing rate than sows that received BA-B embryos (48.4% vs. 22.6%, $P < 0.05$). However, these two groups of recipient sows did not exhibit a significant difference in pregnancy rates (67.7% vs. 58.1%). The *in vivo* full term development rate (total number of born cloned piglets/total number of transferred cloned embryos) of BAPPS-B embryos was significantly

Table 2 Comparison of *in vitro* development rate of pig SCNT embryos generated by BA-B and SIS-A enucleation

Enucleation method	SCNT embryos	Cleaved/%	Blastocysts/%	Total number of cells per blastocyst
BA-B	240	183 (76.25)	44 (18.33)	40±3.34
SIS-A	279	208 (74.55)	37 (13.26)	47±4.10

Table 3 Comparison of *in vivo* development rate of pig SCNT embryos generated by the BA-B and SIS-A enucleation

Enucleation method	Total number of transferred SCNT embryos	Total number/pregnant/farrowed recipients	Pregnancy/farrowing rate/%	Total number of cloned piglets born	Development rate/%
BA-B	7469	33/23/14	69.70/42.42	64	0.86
SIS-A	6779	33/23/14	69.70/42.42	75	1.11*

Table 4 Comparison of *in vitro* development rate of pig SCNT embryos generated by the BA-B and BAPPS-B enucleation

Enucleation method	SCNT embryos	Cleaved/%	Blastocysts/%	Total number of cells per blastocyst
BA-B	429	326 (75.99)	94 (21.91)	36±1.77*
BAPPS-B	501	362 (72.26)	89 (17.76)	41±1.69*

Note: * Significantly different at $P < 0.05$.

Table 5 Comparison of *in vivo* development rate of pig SCNT embryos generated by the BA-B an BAPPS-B enucleation

Enucleation method	Number of manipulated /successfully enucleated oocytes	Total number of transferred SCNT embryos	Total number/pregnant /farrowed recipients	Pregnancy/farrowing rate/%	Total number of born cloned piglets	Development rate/%
BA-B	8023/not determined	7038	31/18/7	58.06/22.58*	24	0.34**
BAPPS-B	9049/7234	6735	31/21/15	67.74/48.39*	55	0.82**

Note: * Significantly different at $P < 0.05$; ** Significantly different at $P < 0.01$.

higher than for BA-B embryos (0.82% vs. 0.34%, $P < 0.01$; Table 5).

4 Discussion

Previous studies have shown that SIS-A enucleation has no detrimental effect on the developmental competence of enucleated oocytes[11,19–21] and that this method can obtain a higher number of successfully enucleated pig oocytes and a higher development rate for cloned pig embryos than BA-B enucleation[7,22]. The results of the current study also indicated that SIS-A enucleation achieves a higher number of successfully enucleated oocytes and tends to produce a higher *in vivo* SCNT embryo development rate than BA-B enucleation. These results suggest that SIS-A enucleation is a better approach for pig SCNT than BA-B enucleation.

The findings from this study further demonstrated that *in vitro* development capacity as well as *in vivo* full term development rate of pig SCNT embryos produced via BAPPS-B enucleation are significantly higher than those of pig SCNT embryos produced via BA-B enucleation. This result indicates that BAPPS-B enucleation can enhance pig cloning efficiency more effectively than BA-B enucleation. The difference in the cloning efficiency between BAPPS-B and BA-B enucleation can be attributed to the difference in the oocyte enucleation success rates of these two techniques. BAPPS-B enucleation can ensure the production of 100% enucleated oocytes for subsequent SCNT, which BA-B enucleation cannot achieve. The BAPPS-B enucleation used in this study only requires less than 10 s of UV irradiation of oocytes. Exposure of oocytes under UV light for less than 10–15 s did not affect the development potential of rabbit and bovine reconstructed embryos[23,24], whereas the exposure of rabbit oocytes or mouse zygotes to UV irradiation for 20–30 s reduced their viability[24,25]. Therefore, the short UV exposure time used in BAPPS-B enucleation in this study probably has no negative effect on the development of cloned porcine embryos.

5 Conclusions

In summary, SIS-A enucleation is a better method for pig SCNT than BA-B enucleation because it gives a higher oocyte enucleation success rate and tends to improve *in vivo* development rate of pig SCNT embryos compared to BA-B enucleation. BAPPS-B enucleation is also a better method for pig cloning than BA-B enucleation because it enhances *in vitro* as well as *in vivo* development efficiency of cloned porcine embryos compared with BA-B enucleation.

Acknowledgements This study was supported by the National Natural Science Foundation of China (31772554), the Department of Science and Technology of Guangdong Province, China (2016B020233006, 2015TX01N081 and 2016A020210074) and the team project of Guangdong Agricultural Department (2017LM2148).

Compliance with ethics guidelines Chengcheng Zhao, Junsong Shi, Rong Zhou, Ranbiao Mai, Lvhua Luo, Xiaoyan He, Hongmei Ji, Gengyuan Cai, Dewu Liu, Enqin Zheng, Zhenfang Wu, and Zicong Li declare that they have no conflicts of interest or financial conflicts to disclose.

All applicable institutional and national guidelines for the care and use of animals were followed.

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