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Embryo aggregation does not improve the development of interspecies somatic cell nuclear transfer embryos in the horse

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ABSTRACT

The low efficiency of interspecies somatic cell nuclear transfer (iSCNT) makes it necessary to investigate new strategies to improve embryonic developmental competence. Embryo aggregation has been successfully applied to improve cloning efficiency in mammals, but it remains unclear whether it could also be beneficial for iSCNT. In this study, we first compared the effect of embryo aggregation over *in vitro* development and blastocyst quality of porcine, bovine, and feline zona-free (ZF) parthenogenetic (PA) embryos to test the effects of embryo aggregation on species that were later used as enucleated oocytes donors in our iSCNT study. We then assessed whether embryo aggregation could improve the *in vitro* development of ZF equine iSCNT embryos after reconstruction with porcine, bovine, and feline ooplasm. Bovine- and porcine-aggregated PA blastocysts had significantly larger diameters compared with nonaggregated embryos. On the other hand, feline- and bovine-aggregated PA embryos had higher blastocyst cell number. Embryo aggregation of equine-equine SCNT was found to be beneficial for embryo development as we have previously reported, but the aggregation of three ZF reconstructed embryos did not improve embryo developmental rates on iSCNT. *In vitro* embryo development of nonaggregated iSCNT was predominantly arrested around the stage when transcriptional activation of the embryonic genome is reported to start on the embryo of the donor species. Nevertheless, independent of embryo aggregation, equine blastocyst-like structures could be obtained in our study using domestic feline-enucleated oocytes. Taken together, these results reported that embryo aggregation enhance *in vitro* PA embryo development and embryo quality but effects vary depending on the species. Embryo aggregation also improves, as expected, the *in vitro* embryo development of equine-equine SCNT embryos; however, we did not observe positive effects on equine iSCNT embryo development. Among oocytes from domestic animals tested in our study, the feline ooplasm might be the most appropriate recipient to partially allow preimplantation embryo development of iSCNT equine embryos.

1. Introduction

Differentiated somatic cells can be reprogrammed to the totipotent state when transferred into enucleated oocytes by means of somatic cell nuclear transfer (SCNT) [1]. Interspecies somatic cell nuclear transfer (iSCNT) is

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achieved by transferring a donor cell into the cytoplasm of an enucleated oocyte from another different species/family/order/class. Interspecies somatic cell nuclear transfer can be used to investigate the interactions between the somatic cell and the ooplasm. Furthermore, iSCNT can be used to produce viable embryos, pregnancies, and deliveries [2–8]. In recent years, iSCNT has been used to understand the mechanisms involved after the fusion of a somatic cell with an enucleated oocyte (reviewed by Long et al. [9]). Nevertheless, producing a viable embryo after activation of reconstructed cloned embryo remains a complex phenomenon.

Stem cell and cell reprogramming research could benefit from using iSCNT and oocytes from species that are more accessible and abundant. In particular, iSCNT may facilitate obtaining pluripotent stem cells by producing interspecies cloned embryos [10,11] and the study of mitochondrial/genomic DNA compatibility [12]. A few studies using iSCNT with mouse- [13], cattle- [14,15], and sheep- [5] enucleated oocytes fused with equine cells have reported low-blastocysts rates. However, iSCNT remains an exciting tool for species with limited availability of oocytes, such as the horse, and for endangered species in which assisted reproduction is needed. Moreover, the genus Equus has the unusual characteristic of being able to produce viable offspring when crossing individuals of different species, even with different phenotypic and karyotypic characteristics [16,17]. This characteristic makes the equine an interesting model for iSCNT studies.

To overcome the low efficiency of iSCNT, it is necessary to investigate new strategies to improve its embryo developmental competence. Embryo aggregation has been successfully applied to improve cloning efficiency in several mammals [18–24], but it remains unclear whether it could also be beneficial for iSCNT. Therefore, we sought to evaluate the effects of embryo aggregation on the *in vitro* embryo development efficiency of iSCNT in the equine. We first investigated the effects of embryo aggregation on *in vitro* development and blastocyst quality of porcine, bovine, and feline ZF parthenogenetic (PA) embryos. We then tested if embryo aggregation improved *in vitro* development of equine-cloned embryos generated by iSCNT with enucleated oocytes from porcine, bovine, and domestic feline.

2. Materials and methods

2.1. Chemicals

Except otherwise indicated, all chemicals were obtained from Sigma Chemicals Company (St. Louis, MO, USA).

2.2. Experimental design

Two experiments were performed in our study. In experiment one, we produced nonaggregated and aggregated porcine, bovine and feline zona-free PA embryos to test the effects of embryo aggregation on the *in vitro* embryo development and blastocyst quality. In experiment two, tested species in experiment one were used to produce interspecific equine zona-free (ZF)–cloned embryos, and to evaluate the effects of embryo aggregation on the *in vitro* development. In addition, nonaggregated and aggregated homospecific ZF equine–cloned embryos were produced as control.

In experiment one, IVM ZF oocytes from porcine, bovine, and feline species were parthenogenetically activated. For each species, immediately after activation, zona-free parthenogenetic embryos (ZFPE) were culture in a microwell system, placing one (1x-nonaggregated) or three (3x-aggregated) ZFPE per microwell. Therefore, experimental groups for experiment one were: porcine 1x (nonaggregated) and porcine 3x (aggregated); bovine: 1x (nonaggregated) and bovine 3x (aggregated), and feline: 1x (nonaggregated) and feline 3x (aggregated). *In vitro* culture was performed for 8 days, and we measured blastocyst size and cell number (Fig. 1).

In experiment two, IVM ZF oocytes from porcine, bovine, feline, and equine species were enucleated by micromanipulation and then fused in all cases with equine skin fibroblasts to produce zona-free reconstructed embryos (ZFREs). For each species, immediately after activation, ZFREs were culture in a microwell system, placing one (1x-nonaggregated) or three (3x-aggregated) ZFREs per microwell. Therefore, experimental groups for experiment two were: equine-porcine: 1x (nonaggregated) and equine-porcine 3x (aggregated); equine-bovine: 1x (nonaggregated) and equine-bovine 3x (aggregated); equine-feline: 1x (nonaggregated) and equine-feline 3x (aggregated), and equine-equine: 1x (nonaggregated) and equine-equine 3x (aggregated). *In vitro* culture was performed for 8 days, and some obtained blastocysts were used for *in vitro* embryo culture after Day 8 (Fig. 2).

2.3. Oocyte collection and in vitro maturation

2.3.1. Porcine

Ovaries were collected from gilts at a local slaughterhouse and transported to the laboratory at around 25 °C to 30 °C within 3 hours of collection. Cumulus-oocyte complexes (COCs) from 3 to 6-mm follicle diameter were aspirated using an 18 gauge needle attached to a 10-mL disposable syringe. Compact COCs were selected and matured in 100-μL droplets of tissue culture medium bicarbonate-buffered TCM-199 (31100–035; Gibco, Grand Island, NY, USA) under mineral oil (M8410), supplemented with 0.3-mM sodium pyruvate (P2256), 100-mM cysteamine (M9768), 5-μg/mL *myo*-Inositol (I5125), 1-μg/mL insulin-transferrin-selenium (ITS; 51300–044, Gibco) 1% antibiotic-antimycotic (ATB; 15240–096, Gibco), 10% porcine follicular fluid (follicular fluid was obtained from follicles of 3–6 mm of diameter, centrifuged at 1900 × g for 30 minutes at 5 °C, filtered and then aliquoted and stored at −20 °C), 5-ng/mL basic fibroblast growth factor (F3685) and 10 μg/mL of FSH (NIH-FSH-P1, Folltropin, Bioniche, Caufield Junction Caufield North, Victoria, Australia). Maturation was performed at 38.5 °C in a humidified atmosphere of 6.5% CO_2 in 90% air for 42 to 44 hours.

2.3.2. Bovine

Cow ovaries were transported from a local slaughterhouse to the laboratory in a thermo container at 24 °C to