Animal Reproduction Science 126 (2011) 179–186

Contents lists available at ScienceDirect

Animal Reproduction Science

journal homepage: www.elsevier.com/locate/anireprosci

Nuclear transfer procedures in the ovine can induce early embryo fragmentation and compromise cloned embryo development

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ARTICLE INFO

Article history:
Received 6 February 2011
Received in revised form 14 May 2011
Accepted 30 May 2011
Available online 6 June 2011

Keywords:
Cytoplasmic fragmentation
DC pulse
Ionomycin
In vitro development
In vivo development

ABSTRACT

Cytoplasmic fragmentations are frequently observed in early mammalian embryos, and especially in the human. In our research we have observed subtle clues that the occurrence of fragmentation was most likely a result of somatic cell nuclear transfer (NT) protocols, and in particular, the in vitro culture system. In this study we examined various putative factors that might induce early embryo fragmentation in the ovine. The results indicate that nuclear transfer protocols such as the fusion parameter, activation treatment, and especially the choice of culture medium affected embryo cleavage rates and resulted in a higher incidence of fragmented embryos. Upon using the same fusion parameter, activation parameters that were based upon amino acids containing synthetic oviduct fluids (SOFaa) culture system resulted in significantly lower fragmentation rates than when utilizing a Charles Rosenkrans 1 (CR1aa) culture system. Fragmented embryos typically exhibited irregular numbers of blastomeres with the majority of blastomeres devoid of chromatin. Factors such as fusion DC pulse, activation treatment and culture system led to higher fragmentation and also affected in vitro and in vivo embryo development. The SOFaa based culture system produced a higher number of quality NT embryos resulting in higher pregnancy rates and the birth of live lambs as compared to the CR1aa based system ($P < 0.05$). We conclude that early embryo fragmentation in the ovine is caused by suboptimal cloning protocols, and NT embryo development is especially affected by the culture system used.

1. Introduction

Subjective morphological assessment of embryo quality and the use of various non-invasive evaluations to determine developmental competence are commonly used prior to transferring embryos to host recipients. Relevant characteristics most often used are developmental stage to pertinent time, blastomere number, size and shape of embryo, and the extent of cellular fragments within the embryo proper (Giorgetti et al., 1995).

Cytoplasmic fragmentation is frequently observed in early-stage mammalian embryos, and especially in human embryos (Van Blerkom et al., 2001; Hardy et al., 2003). Cytoplasmic fragmentation can be caused by an interruption in the cytoskeleton network, abnormal cytokinetic events (Antczak and Van Blerkom, 1999), or under suboptimal culture conditions and culture system (Winston and Johnson, 1992; Van Blerkom et al., 2001; Hao et al., 2003). Cytoplasmic fragmentation in the human has been associated with reduced blastocyst formation and lower blastocyst cell numbers, specifically in the trophecto-

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doi:10.1016/j.anireprosci.2011.05.015

derm (Hardy et al., 2003). Although a small amount of fragmentation (up to 10–15%) can be tolerated by an embryo, extensive fragmentation rapidly declines blastocyst formation (Hardy et al., 2003). Fragmentation associated abnormalities can cause abnormal pre- and post-implantation development (Dozortsev et al., 1998; Alikani et al., 1999, 2005; Ebner et al., 2001).

Previous studies showed that reconstruction method during somatic cell nuclear transfer (NT) caused cytoplasmic fragmentation. The reconstituted mouse embryos were with a little high fragmentation rate when the cells were directly injected into enucleated cytoplasts in comparison with the electrofusion method (Hosaka et al., 2000). In porcine, Im et al. (2005) reported that medium supplementations such as sorbitol or sucrose suppressed cytoplasmic fragmentation and supported the development of electrically activated oocytes and NT embryos. Activation method resulted changes in actin filament distribution in activated oocytes and caused embryo fragmentation in pigs (Kawahara et al., 2002; Yamanaka et al., 2007). Optimization of activation procedure such as ionomycin combined with cycloheximide can reduce the frequency of fragmentation in pig NT embryos (Yamanaka et al., 2007).

During our study in ovine cloning, we observed that a high proportion of cloned embryos fragmented when certain conditions were used. This led us to propose that the occurrence of fragmentation may be inherent to the nuclear transfer protocol. This was particularly noted with the type of in vitro culture system used. We designed this experiment to investigate the effects of various manipulating procedures such as fusion, activation, and the culture system on the incidence of fragmentation in early-stage NT embryos in the ovine. By improving the in vitro culture conditions leading to decreasing the rate of embryo fragmentation, we were able to increase embryo development and the birth of live lambs.

2. Materials and methods

2.1. Chemicals and materials

All chemicals used in this study were purchased from Sigma (St. Louis, MO, USA) unless otherwise indicated.

2.2. Donor cell culture

Primary ovine fibroblast cultures were established from an ear biopsy as described by Wu et al. (2010). Tissues were washed thoroughly, minced, suspended in DMEM/Ham's F12 (1:1) (Hyclone, Logan, UT, USA) supplemented with 15% fetal bovine serum (FBS) (HyClone), and then seeded in 25 cm^2 tissue culture flasks, and cultured at 38 °C in a humidified atmosphere of 5% CO_2 in air for several days. Cells were harvested in tissue culture medium containing 10% DMSO and stored in liquid nitrogen until use in NT. Frozen/thawed cells were grown to 80% confluence, and passages 2–16 were used as nuclear donors.

2.3. Oocyte collection and maturation in vitro (IVM)

Sheep oocyte maturation in vitro was as described (Lee and Campbell, 2008). Ovaries were collected from a local slaughterhouse and transported to the laboratory within 2 h in a thermos containing physiological saline at about 25 °C. Cumulus oocyte complexes (COC) were released by slicing the ovaries. Only COC with evenly granulated cytoplasm and with at least 3 layers of cumulus cells were selected and incubated in maturation medium TCM199 (Gibco BRL, Grand Island, NY, USA) supplemented with 10% FBS, 0.01 IU/mL FSH, 1 IU/mL LH and 0.01 μg/mL 17-β-estradiol. A group of 30–40 COC were then transferred into 500 μL maturation medium pre-warmed in 4-well dishes (Nunc, Denmark) and cultured at 38 °C in 5% CO_2 in air with high humidity for 18 h.

2.4. Nuclear transfer and embryo culture

After maturation, the cumulus cells were removed by vortexing COC in PB1 medium (calcium and magnesium containing PBS (HyClone Laboratories), 0.32 mM sodium pyruvate, 5.55 mM glucose and 3 mg/ml BSA) containing 10 mg/mL hyaluronidase. Oocytes with a first polar body were used as recipient cytoplasts. Enucleation was employed to remove the first polar body and the adjacent small volume of cytoplasm containing the metaphase spindle metaphase plate as our previously described (Li et al., 2004c). A single donor cell was subsequently transferred to the perivitelline space of the enucleated cytoplast. Fusion of the reconstructed couplets was performed in a mannitol fusion medium by two electric DC pulses of 1.4 or 1.8 kV/cm for 40 μs with a 1 s interval. At about IVM 25–26 h, the fused clones were activated by 5 μM ionomycin for 5 min followed by a 5 h incubation in 10 μg/mL cycloheximide (CHX) medium.

After activation, the cloned embryos were cultured in two culture media SOFaa (Wang et al., 1998) and CR1aa (Rosenkrans et al., 1993), respectively, under mineral oil in 50 μL droplets in 5% CO_2 in air at 38 °C under high humidity. Normal cleavage, embryo fragmentation and blastocyst development were examined as described below.

2.5. Preparation of ovine cumulus cell co-culture

Cumulus cell co-cultures were prepared as our previous report (Li et al., 2004a,b). In summary, after vortexing of the matured oocytes the cumulus cells were collected and centrifuged at 800 × *g* for 3 min. The soft pellet was diluted 1:10 by bicarbonate buffered TCM-199 + 20% FCS and seeded into a Petri dish in 30 μL droplets covered by mineral oil and cultured at 38 °C in 5% CO_2 in air. The cells reached about 90% confluence and formed mono-layer in about 48 h. Before transfer of embryos into the droplets, fresh embryo culture medium was replaced.

2.6. Evaluation of fragmentation

After incubation for 50–60 h, or culture for 5–7 days, the embryos were morphologically examined. The embryos were fixed with 3.7% (w/v) paraformaldehyde in PBS