

In Vitro Development of Horse Oocytes Reconstructed with the Nuclei of Fetal and Adult Cells¹

Xihe Li, Lee H.-A. Morris, and W.R. Allen²

University of Cambridge, Department of Clinical Veterinary Medicine, Equine Fertility Unit, Mertoun Paddocks, Newmarket, Suffolk CB8 9BH, United Kingdom

ABSTRACT

This study investigated the basic conditions required for the production of horse embryos by the transfer of the nuclei of fetal and adult fibroblast cells to enucleated oocytes. Cumulus-oocyte complexes were recovered from abattoir ovaries and matured in vitro in groups of 20–30 for 28–30 h in tissue culture medium 199 containing 20% v:v fetal bovine serum in coculture with equine oviduct epithelial cells. Fetal fibroblast cells (FFC) were derived from a 32-day-old Thoroughbred × Pony fetus, and adult skin fibroblast cells (SFC) were obtained from subdermal biopsies recovered from a 4-yr-old female Pony. The rates of fusion between the recipient cytoplasm with either FFC or SFC were significantly greater when the cells were treated with a combination of direct current (DC) pulses and Sendai virus rather than with DC pulses alone (81%–82% vs. 49%–57%, $P < 0.05$). There were no differences in the rates of nuclear reprogramming between FFC and SFC (88% vs. 84%), but the rate of cleavage of the resulting embryos to the 2-cell stage was higher when FFC were used (53%) than when SFC were used (35%). Blastocysts were obtained from oocytes reconstructed with both types of donor cells and after culture in vitro for 6–7 days, but the overall proportion of blastocysts produced was very low in both cases (FFC, 4%; SFC, 7%). These results demonstrate a very limited potential for in vitro development of horse embryos after nuclear reprogramming following the transfer of nuclei from either fetal or adult fibroblasts into recipient enucleated oocytes.

developmental biology, early development, embryo, meiosis, oocyte development

INTRODUCTION

Since the development of embryo transfer techniques in the horse [1], a variety of new breeding technologies such as in vitro fertilization (IVF), intracytoplasmic sperm injection (ICSI), and nuclear transfer have been applied to horse breeding both in the laboratory and on the studfarm [2–7]. However, the low efficiency has limited the commercial application of these technologies in this species. The reasons for this are numerous and include the restricted availability of oocytes, practical problems with in vitro oocyte maturation and fertilization, and the need to develop an effective in vitro culture system for early embryonic development.

Nuclear transfer using blastomeres from early embryos has been applied to many animal species during the past

two decades [8–14] to investigate functional regulation of the relationship between the nucleus and cytoplasm and to produce cloned animals [15–19]. More recently, cloned offspring have been produced using somatic cells in sheep [20], mice [21–23], cattle [24–26], goats [27], and pigs [28, 29]. These studies have demonstrated that the nucleus of a differentiated adult mammalian somatic cell can be reprogrammed and have the potential to develop into a new animal.

Many aspects of reproduction in equids are unique to this genus, and basic methodologies developed in rodents and other domestics species, including IVF, ICSI, and nuclear transfer, may not be automatically applicable to the mare. There have been a few reports of attempted nuclear transfer in the horse using the basic techniques used in cattle and sheep. However, irrespective of the rates of cell fusion achieved, the subsequent rates of cleavage and development of the reconstructed oocytes have been very low when compared with the results of nuclear transfer in ruminants [6, 30–32].

In the present study, we investigated the basic conditions required for nuclear transfer in the horse using both fetal and adult fibroblasts as donor cells. We also studied the rates of cell fusion when using a combination of direct current (DC) electrical impulses of varying intensity and Sendai virus, and we related these factors to the rates of nuclear reprogramming, cleavage, and subsequent development of the reconstructed oocytes when cultured in vitro.

MATERIALS AND METHODS

Culture Media

Tissue culture medium 199 (TCM199, 22340-012; Gibco BRL, Grand Island, NY) and Dulbecco modified Eagle medium (DMEM, 22320-014; Gibco BRL) were used as the basic media for oocyte maturation and culture of the reconstructed oocytes after nuclear transfer. A monolayer of horse oviduct epithelial cells (OECs), generated as required from a frozen culture line of horse OECs, was used in coculture with the oocytes during their maturation and during the development of the reconstructed oocytes. For oocyte maturation, TCM199 was supplemented with 20% v:v heat-inactivated fetal bovine serum (FBS; Gibco BRL), 10 $\mu\text{g/ml}$ FSH (Sigma Chemical Co., St. Louis, MO), 5 $\mu\text{g/ml}$ LH (Sigma), and 1 $\mu\text{g/ml}$ estradiol (Sigma). Culture drops (500 μl) containing 20–30 cumulus-oocyte complexes (COCs) were made under mineral oil (M8410; Sigma) with the monolayer of OECs in four-well culture plates (176740; Nunc, Roskilde, Denmark). For development, 500- μl culture drops of DMEM were supplemented with 10% v:v FBS and, under mineral oil, were used to culture groups of 20–30 reconstructed oocytes, again on the monolayer of OECs in a four-well Petri culture plate.

Oocyte Collection and Maturation

Horse ovaries were obtained from two abattoirs and transported to the laboratory in PBS containing 125 IU/ml penicillin and 35 IU/ml streptomycin over 20 h while being maintained at a temperature of 10–20°C. COCs were recovered from the ovaries by scraping the walls of follicles 0.5–3.0 cm in diameter, and groups of 20–30 COCs were matured in vitro by coculture with OECs for 28–30 h at 38°C in 5% CO₂ in air [33].

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²Correspondence: W.R. Allen, TBA Equine Fertility Unit, Mertoun Paddocks, Woodditton Rd., Newmarket, Suffolk CB8 9BH, U.K. FAX: 44 1638 667207; e-mail: vetart@ah.org.uk

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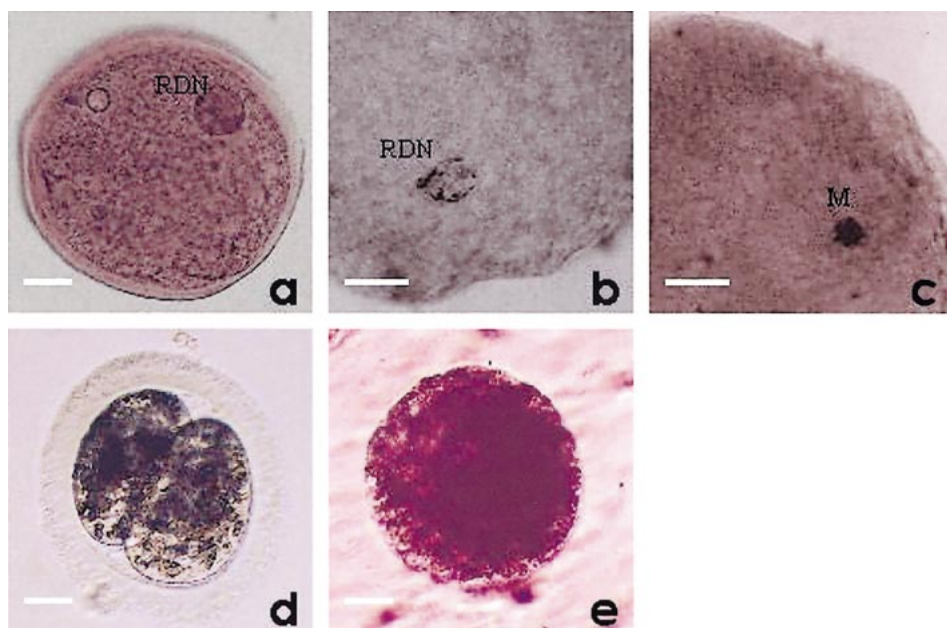


FIG. 1. Nuclear reprogramming, cleavage, and in vitro development of horse oocytes reconstructed using adult skin fibroblast cells. **a, b** Reprogrammed donor nucleus (RDN) 12–18 h after cell fusion. **c** Metaphase stage (M) of the reconstructed oocyte during the first cell cycle 18 h after cell fusion. **d** A 2-cell stage embryo 30 h after cell fusion. **e** An early blastocyst obtained from a reconstructed oocyte cultured in vitro for 6 days. Bar = 30 μ m.

Preparation of Donor Cells

Fetal fibroblast cells (FFC) and adult skin fibroblast cells (SFC) were used as donor cells after they had undergone 3–7 passages. The FFC originated from a 32-day-old Thoroughbred \times Pony fetus, and the SFC came from a 4-yr-old female pony. To prepare the FFC, the head and viscera of the fetus were discarded, and the remaining tissues were sliced finely before being digested enzymatically in 0.5% v:v trypsin-EDTA (Sigma) in PBS for 30 min at 30°C. The digested fragments were washed twice in PBS by centrifugation at 700 \times g for 10 min, and the resulting pellet of cells was resuspended in DMEM supplemented with 10% v:v FCS and cultured at 37°C in 5% CO₂ in air for 14–20 days. After the cells had been passaged 2 to 4 times, samples were taken from the new growing line and frozen for future use. For preparation of the SFC, a small piece of subdermal tissue collected under local anesthesia from the neck of the mare was also sliced finely, washed, and cultured as described previously for the FFC and then passaged 2–4 times before the cells were harvested and frozen.

To prepare FFC and SFC as donor cells, they were thawed and then subjected to starvation culture for 3–5 days in DMEM containing 0.5% v:v FBS to regulate their cycles to the G₀/G₁ phases. Analysis of these cells by flow cytometry showed that the nuclei of more than 90% of both the FFC and the SFC were at the G₀/G₁ phase of the cycle after such treatment.

Nuclear Transfer

The nuclear transfer procedure was performed as described previously by Li et al. [31] using a micromanipulator (Transferman; Eppendorf, Hamburg, Germany) attached to an inverted microscope (Olympus IMT-2; Olympus, Tokyo, Japan). All manipulations were performed in a basic medium of Earle balanced salt solution (EBSS)-20% v:v FBS on a heated stage (CO 102; Linkam, Tadworth, U.K.) that provided a working temperature of 30°C.

After 28–30 h of in vitro maturation, metaphase II (MII) oocytes were selected after removing their cumulus cells by suspension in 200 IU/ml hyaluronidase (Sigma) in EBSS-20% FBS for 5 min followed by gentle pipetting. Before enucleation, MII oocytes were placed for 10 min in EBSS-20% FBS with 5 μ g/ml cytochalasin B (CCB; Sigma) and 5 μ g/ml Hoechst 33342 (Sigma). They were enucleated in EBSS-20% FBS with 5 μ g/ml CCB, and only those oocytes in which removal of both the polar body and the MII nucleus was confirmed by observation under UV light were included in the study. The monolayer of starved FFC or SFC was immersed in 0.05% trypsin in 0.02% EDTA-EBSS solution (Gibco BRL) for 10 min at 38°C to separate the cells from the culture plate. These were then washed once and centrifuged at 300 \times g for 5 min. The resulting cell pellet was resuspended and cultured in EBSS supplemented with 10% v:v polyvinylpyrrolidone (Sigma) for 1–3 h before a cell was selected and injected into the perivitelline space of the recipient enucleated oocyte,

alone or in combination with a small volume (2–3 times the oocyte volume) of inactivated Sendai virus (VR-907, 1–3 \times 10³/ml hemagglutinating activity units; LGC, Middlesex, U.K.).

Cell Fusion and Activation Treatment of the Reconstructed Oocytes

The fibroblast-cytoplasm couplets produced from FFC and SFC were aligned manually in 0.28 M mannitol fusion buffer in a 1.0-mm fusion chamber and subjected to two DC pulses of 220–250 kV/cm for 30 μ sec delivered by an ECM830 Electro Square Porator (BTX, San Diego, CA). Fibroblast-cytoplasm couplets that fused were then activated chemically by transfer to PBS containing 5 μ M ionomycin for 5 min, followed by culture for 2, 4, or 6 h in TCM199 containing 5 μ g/ml CCB and 10 μ g/ml cycloheximide (Sigma). Successful cell fusion was considered to have occurred when the donor cell had become fully incorporated into the cytoplasm of the enucleated oocyte.

In Vitro Culture of Reconstructed Oocytes

Groups of 5–10 reconstructed oocytes were cocultured with a monolayer of OECs in 500- μ l drops of development medium (DMEM plus 10% v:v FBS) at 38°C in an atmosphere of 5% CO₂ in air. Nuclear reprogramming (nucleus 2–3 times larger than that of the original donor cell with a nucleolus or some condensed chromatin (Fig. 1, a–c) in the reconstructed oocytes was analyzed 12–18 h after fusion treatment by staining with 5 μ g/ml Hoechst 33342 (Sigma), as described previously by Li et al. [34, 35]. Two-cell stage embryos were cocultured on a monolayer of OECs for 6–7 days, and half the volume of the culture medium was changed every 2 days.

Statistical Analysis

Each experimental group was replicated 3–5 times, and the results were evaluated by chi-square analysis. Differences between groups were considered statistically significant at $P < 0.05$.

RESULTS

Fusion of Fetal and Adult Fibroblast Cells with Recipient Cytoplasm

The effects of method of fusion of the FFC and SFC to recipient oocytes after nuclear transfer are presented in Table 1. The fusion rate of FFC induced by DC pulses alone was significantly lower than that obtained when DC pulses were combined with Sendai virus (57% vs. 82%, $P < 0.05$).

TABLE 1. The effect of DC pulses, alone or in combination with Sendai virus, on the fusion of horse fetal and adult fibroblast cells with enucleated horse oocytes.^a

Donor cells	Fusion treatment		Fusion results	
	DC pulses	Sendai virus	Total no. of oocytes	No. fused (%)
FFC	+	–	44	25 (57 ^c)
FFC	+	+	51	42 (82 ^d)
SFC	+	–	41	20 (49 ^c)
SFC	–	+	32	5 (16 ^b)
SFC	+	+	121	98 (81 ^d)
SFC	–	–	15	0

^a DC, Direct current; FFC, fetal fibroblast cells; SFC, skin fibroblast cells.

^{b-d} Values with different superscripts are significantly different ($P < 0.05$).

Similarly, rates of fusion with SFC were lower when the cell-couplets were stimulated by DC pulses alone compared with the combination of DC pulses and Sendai virus (49% vs. 81%, $P < 0.05$). On the other hand, Sendai virus alone induced fusion of only 16% (5/32) of the cell-couplets in the SFC group.

Nuclear Reprogramming and Cleavage of the Reconstructed Oocytes

The rates of nuclear reprogramming and cleavage of the reconstructed oocytes in the FFC and SFC groups are compared in Table 2. When using FFC for donor nuclei, the rate of reprogramming was increased by lengthening the culture time in CCB and cycloheximide from 2 h to 4 or 6 h (63%, 100%, and 88% respectively; 2 h vs. 4–6 h, $P < 0.05$). Furthermore, higher cleavage rates were obtained after 4–6 h culture (33%–53%). Similar nuclear reprogramming and cleavage rates were obtained when SFC were used as donor cells after 6 h culture in DMEM supplemented with CCB and cycloheximide (84% and 35% respectively).

In Vitro Development of 2-Cell Stage Embryos

The development of 2-cell stage embryos reconstructed from the transfer of either FFC or SFC is summarized in Table 3. There were no significant differences in development rates of 2-cell stage embryos obtained from FFC and SFC ($P > 0.05$). Eight of 26 (31%) of the FFC and 8 of 28 (29%) of the SFC 2-cell stage embryos developed beyond the 8-cell stage, and 7 of 26 FFC (27%) and 4 of 28 SFC (14%) embryos developed to the 16-cell/morula stage. Thereafter, only a very low proportion (4%–7%) of 2-cell embryos derived from both donor cell types developed to blastocysts after 6–7 days of coculture in vitro with a monolayer of OECs (Fig. 1, d and e). Staining of 3 of the blastocysts produced in this manner with Hoechst 33342 confirmed normal division of the nuclei. No differences in morphology were observed in the blastocysts derived from either fetal or adult cell types.

DISCUSSION

To be able to apply the technique of nuclear transfer to horse breeding, it is necessary to establish the criteria for cell fusion and subsequent embryonic development. The requirements for initiation of cell fusion appear to differ among species. For example, use of inactivated Sendai virus has been shown to be effective for nuclear transfer in rodents and sheep [9, 13] but not in the cow [12, 14]. Al-

TABLE 2. Nuclear reprogramming and cleavage of reconstructed horse oocytes after nuclear transfer using fetal and adult fibroblast cells.^a

Donor cells	Culture time in CCB + cycloheximide (h)	No. of fused oocytes	No. reprogrammed (%)	No. developed to 2-cell stage (%)
FFC	2	16	10 (63 ^b)	2 (13 ^b)
	4	18	18 (100 ^c)	6 (33 ^{b,c})
	6	34	30 (88 ^c)	18 (53 ^c)
SFC	6	81	68 (84 ^c)	28 (35 ^{b,c})

^a CCB, Cytochalasin B; FFC, fetal fibroblast cells; SFC, skin fibroblast cells.

^{b-d} Values with different superscripts are significantly different ($P < 0.05$).

ternatively, DC electrical pulses have been widely used to produce hybridomas and for other cell fusion applications, including nuclear transfer in many species of laboratory and domestic animals [13, 36, 37]. The rates of fusion between a donor cell and recipient oocyte after electrical stimulation have been 60%–90% in sheep, cattle, and pigs when using embryonic, fetal, or adult donor cells [20, 24, 28]. Moreover, embryonic and fetal donor cells have exhibited higher fusion rates than adult somatic cells under similar conditions [20, 24, 28].

By comparison, the few reports describing attempted nuclear transfer by electrical stimulation in the horse have achieved fusion rates of only 30%–70% when using either fetal or adult donor cells [6, 30, 32]. In the present study, we investigated the efficacy of combining Sendai virus with electrical stimulation to improve fusion rates with fetal and adult cells and, indeed, these rates increased significantly from 57% and 49%, respectively, when using only electrical stimulation to 82% and 81%, respectively, with the addition of Sendai virus to the stimulation process. Furthermore, treatment with Sendai virus alone induced fusion in only 16% of cell-couplets after nuclear transfer. Thus, our results suggest that, at least in the horse, a useful synergy exists between electrical stimulation and treatment with Sendai virus for the induction of cell fusion. The fusion of two cells in an electrical field is the result of 2 successive processes—membrane fusion and the subsequent “spherizing” of the 2 cells [13]. Therefore, we speculate that Sendai virus facilitates the process of adhesion between the donor cell and recipient cytoplasm and thereby improves the fusion rate after stimulation with the DC pulses. However, the mechanisms underlying the relationship between Sendai virus and electrical pulses require further investigation.

The G₀/G₁ stage of the cell cycle is generally required for donor cell nuclei in sheep and cattle nuclear transfer [17, 20, 24], and in our preliminary study, we noted that more than 90% of both the FFC and SFC were at the G₀/G₁ phase of the cycle after starvation culture for 3–5 days. After nuclear transfer, 63%–100% of the nuclei of the fused cells had become reprogrammed (enlarged nuclei with or without recondensed chromosomes) in the recipient cytoplasm after 2–6 h in culture in CCB and cycloheximide

TABLE 3. In vitro development of 2-cell horse embryos obtained from oocytes reconstructed by nuclear transfer of fetal or adult fibroblast cells.^a

Donor cell	No. 2-cell embryos	No. (%) 2-cell embryos that developed to			
		<8-cell	>8-cell	>16-cell/morula	Blastocyst
FFC	26	10 (38)	8 (31)	7 (27)	1 (4)
SFC	28	14 (50)	8 (29)	4 (14)	2 (7)

^a FFC, Fetal fibroblast cells; SFC, skin fibroblast cells.

(Fig. 1, a and b, and Table 2), as observed similarly in other species. However, only 13%–53% of these reconstructed oocytes proceeded to the 2-cell stage, whereas the remaining noncleaved, reconstructed oocytes developed only to, or just before, the metaphase stage of mitosis of the first cell cycle. Successful cell division is organized by the mitotic chromosomes and the formation of the metaphase spindle after DNA replication during the normal mitotic cell cycle, and the transition from G₂ to metaphase has been shown to be induced by specific cytoplasmic factors [38]. Hence, we further speculate that the block to the first cell division in many of our reconstructed horse oocytes may have been caused by a deficiency of the cytoplasmic transition factors or failure to activate the recipient cytoplasm. Alternatively, the oocytes may not have been able to form a normal metaphase spindle because of microtubule damage incurred during enucleation.

Mature oocytes are arrested at the MII stage in association with a high level of maturation-promoting factor (MPF) in the cytoplasm, which is inactivated after the induction of intracellular Ca²⁺ oscillation by sperm down-regulating cytosolic factor activity during fertilization [39–41]. Inactivation of MPF releases oocytes from metaphase arrest and allows the resumption of mitotic cycles [42]. A metaphase II stage of oocyte chromatin occurs in young bovine and rabbit oocytes after activation because of recondensation of chromosomes and reentry of activated oocytes into a new metaphase arrest by restoration of MPF levels [43, 44]. Cycloheximide has been used to decrease the intracellular level of MPF, after which oocyte activation can be induced using a combination of chemical stimulants (ionophore or ionomycin) and electrical pulses [24, 32, 45, 46]. In the present experiment, the rates of nuclear reprogramming and cleavage were both increased, from 63% to 100% and from 13% to 53%, respectively, by extending the culture time of the reconstructed oocytes in medium containing CCB and cycloheximide from 2 to 6 h (Table 2). The rates of 2-cell embryo cleavage in this experiment were still lower than those achieved in other species, even when using CCB and cycloheximide to reduce the MPF level. Thus, we speculate that the mechanism of horse nuclear reprogramming might be different, and the discovery of a more effective procedure for oocyte activation should be a key point in future investigations of horse embryo cloning.

In the present study, early blastocysts were obtained from both FFC and SFC donor cell types after coculture with OECs for 6–7 days (Fig. 1e). However, the rate of blastocyst formation was only 4%–7% of the reconstructed oocytes, which is significantly lower than the rates achieved in other species. A previous study involving ICSI with horse oocytes indicated that the developmental competence of sperm-injected oocytes was similar between oocytes obtained from slaughterhouse ovaries that were transported for a longer time (20–24 h) at a lower temperature range (10–20°C) and those transported for a much shorter time (3–4 h) at a higher temperature (30–35°C) [33]. This deficiency of developmental competence in cloned horse embryos might be related to factors such as damage to the cytoskeleton during enucleation or exposure to ultraviolet light. Or it could be due to failure of formation of the “equine blastocyst capsule” in vitro. This capsule is a tough, elastic investment that develops uniquely between the zona pellucida and the trophectoderm in the Day 6.5 late morula/early blastocyst stage equine embryo [47, 48]. It is considered to be essential for survival of the young embryo in the potentially hostile maternal uterus during the

period of embryonic mobility between Days 6 and 17 after ovulation [49]. It provides structural protection from the strong myometrial contractions and aids the accumulation and imbibition of essential nutrient components in the exocrine endometrial gland secretions [50].

In summary, the present experiment demonstrates, first, a synergistic relationship between DC pulses and Sendai virus in the induction of cell fusion in horse nuclear transfer and, second, an equality between fetal and adult somatic donor cells in their ability to achieve cell fusion, nuclear reprogramming, and embryonic development. Improving the in vitro developmental potential of horse embryos derived from such nuclear transfer and achieving full-term development of such cloned horse embryos will be the goals of future research programs.

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