BIOLOGY OF REPRODUCTION (2012) **87**(1):15, 1–9
Published online before print 2 May 2012.
DOI 10.1095/biolreprod.112.098855

Equine Cloning: In Vitro and In Vivo Development of Aggregated Embryos[1]

Andrés Gambini,[3,4] Javier Jarazo,[3] Ramiro Olivera,[3] and Daniel F. Salamone[2,3,4]

[3]*Laboratory of Animal Biotechnology, Agriculture Faculty, University of Buenos Aires, Buenos Aires, Argentina*
[4]*National Institute of Scientific and Technological Research, Buenos Aires, Argentina*

ABSTRACT

The production of cloned equine embryos remains highly inefficient. Embryo aggregation has not yet been tested in the equine, and it might represent an interesting strategy to improve embryo development. This study evaluated the effect of cloned embryo aggregation on in vitro and in vivo equine embryo development. Zona-free reconstructed embryos were individually cultured in microwells (nonaggregated group) or as 2- or 3-embryo aggregates (aggregated groups). For in vitro development, they were cultured until blastocyst stage and then either fixed for Oct-4 immunocytochemical staining or maintained in in vitro culture where blastocyst expansion was measured daily until Day 17 or the day on which they collapsed. For in vivo assays, Day 7–8 blastocysts were transferred to synchronized mares and resultant vesicles, and cloned embryos were measured by ultrasonography. Embryo aggregation improved blastocyst rates on a per well basis, and aggregation did not imply additional oocytes to obtain blastocysts. Embryo aggregation improved embryo quality, nevertheless it did not affect Day 8 and Day 16 blastocyst Oct-4 expression patterns. Equine cloned blastocysts expanded and increased their cell numbers when they were maintained in in vitro culture, describing a particular pattern of embryo growth that was unexpectedly independent of embryo aggregation, as all embryos reached similar size after Day 7. Early pregnancy rates were higher using blastocysts derived from aggregated embryos, and advanced pregnancies as live healthy foals also resulted from aggregated embryos. These results indicate that the strategy of aggregating embryos can improve their development, supporting the establishment of equine cloned pregnancies.

embryo aggregation, equine cloning, Oct-4, pregnancy, zona-free embryo culture

INTRODUCTION

Cloning represents the only way to rescue the genetics of orchidectomized or highly valuable horses, including those recently deceased. Compared to cloning of other domestic animals, cloning in the horse has been less studied, perhaps as a result of the scarcity of oocyte availability, which is associated with the particular reproductive anatomy-physiology of mares and the general lack of horse slaughterhouses. The first cloned equines were obtained in 2003 [1, 2], and subsequently, additional cloned live foal births have been reported [3–7].

One significant limitation in the initial attempts to clone a horse was the low fusion rate observed between equine cytoplasts and nuclear donor cells [8, 9]. Injecting a cell into an enucleated oocyte as reported by Hinrichs et al. [5] or using zona-free (ZF) cloning as described by Lagutina et al. [4] helped to overcome this problem. Micromanipulation is reduced to a minimum in this latter technique; however, a special in vitro embryo culture with microwells is needed.

A major barrier to the efficient production of nuclear transfer (NT) foals has been the poor in vitro development obtained for cloned equine embryos [6]. In vitro culture reduces embryo development in the equine as higher blastocyst rates were obtained when early intracytoplasmic sperm injection (ICSI) embryos were transferred into oviducts compared to those maintained in in vitro culture [10]. With the exception of a blastocyst rate of 25% reported by Galli et al. [11], the in vitro blastocyst rates for cloned embryos are lower (1%–15% [3, 7]) than those with ICSI embryos (25%–30% [11, 12]). It would seem that the poor development of cloned equine blastocysts might be the result of an inefficient in vitro culture system or of incomplete epigenetic reprogramming of the cell.

It has been stated that embryos produced by NT are epigenetically unique because variations in gene expression among and within somatic cell clones have been observed [13, 14]. In addition, ooplasm derived from donor females of different ages differed in its developmental capacity [15]. Furthermore, different fibroblast lines recovered from the same tissue had different capacities to produce cloned blastocysts [16]. Consequently, producing an embryo with epigenetically different cells by the aggregation of genetically identical cloned embryos but with different ooplasm might have some positive effects for its in vitro and in vivo development.

In the mouse, cloned embryo aggregation increased blastocyst cell numbers and promoted higher in vivo development rates [17]. In the bovine, higher numbers of aggregate structures resulted in an increase in cleavage and blastocyst rates and in the number of cells per embryo [18, 19]; early cloned pregnancy rates were also improved [20].

Recently, in vivo and in vitro equine embryo Oct-4 expression patterns were described [21], and some aberrations in the expression of this marker were noted. An improved expression of the Oct-4 transcription factor (essential for peri-implantation development and embryonic pluripotency) has been reported in aggregated murine cloned embryos along with higher rates of fetal and postnatal development [17].

This study was designed to determine whether embryo aggregation improves in vitro and in vivo cloning efficiency in the equine through an assessment of the quantity and quality, the Oct-4 level expression and growth of embryos, the establishment of pregnancies, and the birth of foals following embryo transfer.

MATERIALS AND METHODS

Reagents

All chemicals were obtained from Sigma Chemical Company (St. Louis, MO), unless otherwise stated.

[1]Supported in part by Biosidus and Sidus Company funds.
[2]Correspondence: E-mail: salamone@agro.uba.ar

Received: 3 January 2012.
First decision: 19 January 2012.
Accepted: 26 April 2012.

eISSN: 1529-7268 http://www.biolreprod.org
ISSN: 0006-3363

Care and Use of Research Animals

Research protocols followed the guidelines stated in the *Guide for the Care and Use of Agricultural Animals in Agricultural Research and Teaching*. The Comision Nacional de Biotecnologia Argentina approved the animal confinement conditions.

Oocytes Collection and In Vitro Maturation

Equine ovaries were obtained during the breeding season from a slaughterhouse and transported to the laboratory within 4–7 h after slaughter, at 27°C–29°C. Cumulus-oocyte complexes (COCs) were recovered by a combination of scraping and washing of all visible follicles, using an 18-gauge needle and a syringe filled with Dulbecco modified Eagle Medium (DMEM; product no. 11885; Gibco, Grand Island, NY) supplemented with 1 mM sodium pyruvate (product no. P2256), and 15 IU/ml heparin (product no. H3149-50KU). Maturation of COCs was conducted for 24 to 26 h in 100-µl droplets of bicarbonate-buffered Tissue culture medium-199 (31100-035; Gibco) containing 10% fetal bovine serum (FBS; product no. 10499-044; Gibco), 1 µl/ml insulin-transferrin-selenium (ITS; product no. 51300-044; Gibco), 1 mM sodium pyruvate (product no. P2256), 100 mM cysteamine (product no. M-9768), 0.1 mg/mL follicle-stimulating hormone (Folltropin; Bioniche, Belleville, ON, Canada), and 2% antibiotic-antimycotic (ATB; product no. 15240-096; Gibco) under mineral oil (product no. M8410) in 6.5% CO_2 in humidified air at 39°C.

Preparation of Oocytes

Removal of cumulus and zona pellucida. The cumulus was removed by pipetting oocytes in 0.05% trypsin-EDTA (product no. 25300; Gibco) and then vortexing them for 2 min in hyaluronidase ([product no. H-4272]1 mg/mL in Hepes-buffered Tyrode medium containing albumin, lactate, and pyruvate; TALP-H [22]). After being rinsed in TALP-H, oocytes were individually observed with stereoscopic microscopy for the presence of the first polar body, which confirmed nuclear maturation.

Metaphase II oocytes were incubated in 1.5 mg/ml pronase (product no. P-8811) in TALP-H for 3–6 min on a warm plate to remove the zona pellucida. ZF oocytes were washed in TALP-H and placed in a microdrop of synthetic oviductal fluid (SOF) [23, 24] supplemented with 2.5% FBS and 1% ATB until enucleation. Some matured oocytes were left with the ZP intact to be used later for parthenogenic activation.

Enucleation. Prior to enucleation, ZF oocytes were incubated for 5 min in a microdrop of SOF containing 1 µg/mL Hoechst bisbenzimide 33342 dye (product no. H33342) and 0.5 µg/ml of cytochalasin B (product no. C6762). The metaphase plate was aspirated using a blunt pipette under UV light, and a closed holding pipette was used to support the oocyte during the enucleation. ZF enucleated oocytes were kept in a SOF microdrop until NT. Successful enucleation was assessed by the observation under UV light of the entire metaphase plate inside the pipette.

Somatic Cell Culture

Two sources of donor somatic cells (male A and male B) were used at different times. Adult fibroblasts were obtained through culture of minced tissue from neck or tail biopsies of Criollo equine skin. Fibroblasts were cultured in DMEM with 10% FBS, 1% ATB, and 1 µl/ml ITS in 5% CO_2 in humidified air at 39°C. After the primary culture was established, fibroblasts were either subcultured every 4–6 days or expanded and frozen in DMEM with 20% FBS and 10% dimethyl sulfoxide and stored in liquid nitrogen. Quiescence of donor cells was induced by growth to confluency for 3–5 days prior to NT. Populations of cells were prepared by trypsinization 30 min before NT and then washed and resuspended in DMEM.

Nuclear Transfer and Embryo Construction

ZF enucleated oocytes were individually transferred to 50-µl drops of 1 mg/ml phytohemagglutinin (product no. L8754) dissolved in TCM-199. After a few seconds, oocytes were quickly dropped over a single donor cell resting on the bottom of a 100-µl TALP-H drop; consequently, these two structures were paired. Afterward, the couplets were placed in fusion medium (0.3 M mannitol, 0.1 mM $MgSO_4$, 0.05 mM $CaCl_2$, and 1 mg/ml polyvinyl alcohol) for 2–3 min and then removed to a fusion chamber containing 2 ml of fusion medium at 30°C. Fusion was performed with a double-direct current pulse of 1.2 kV/cm, in which each pulse lasted for 30 µsec and were 0.1 sec apart. Later, couplets were individually placed in a 10-µl droplet of SOF medium supplemented with 2.5 % FBS and incubated under mineral oil at 39°C in 5% CO_2 in air. At 30–50 min after the pulse, each droplet was assessed. Couplets were considered fused when the donor cell was not observed in the droplet. Nonfused couplets were re-fused. Nonfused couplets after re-fusion were discarded. At 2 h after fusion, ZF reconstructed embryos (ZFREs) were activated.

Activation

To activate the ZFREs, we treated them with 8.7 mM ionomycin (product no. I24222; Invitrogen, Carlsbad, CA) in TALP-H for 4 min, followed by individual culture for 4 h in a combination of 1 mM 6-dimethylaminopurine (product no. D2629) and 5 mg/ml cycloheximide (product no. C7698) in a 5-µl drop of SOF. Some matured oocytes were parthenogenetically activated with the same protocol.

Embryo Aggregation and In Vitro Embryo Culture

A slightly modified Well of the Well (WOW) system [25] was used to culture ZFREs. These microwells were produced using a heated glass capillary slightly pressed to the bottom of a 35- × 10-mm Petri dish. Microwells were covered with a 50-µl microdrops of SOF medium (16 microwells per microdrop) and then different numbers of ZFREs were randomly introduced into each microwell (Day 0). The act of placing more than one ZFRE per well was considered ‘‘embryo aggregation.’’ Three different experimental groups were constructed: group 1X, one ZFRE per microwell (nonaggregated embryos; control group); group 2X, two ZFREs per microwell; and group 3X, three ZFREs per microwell. The number of ZFREs per microdrop was similar among groups.

Embryos were cultured in a humidified gas mixture (5% CO_2, 5% O_2, 90% N_2) at 38.5°C. Half of the medium was renewed on Day 3 with DMEM/F-12 Ham medium (DMEM/F12; product no. D8062) containing 10% FBS and 1% ATB. Cleavage was assessed 72 h after activation, and blastocyst formation and blastocyst diameter rates were recorded on Day 8 when the embryos were either fixed or transferred to synchronized mares.

Blastocysts were classified into grades according to morphology, following the method of McKinnon and Squires [26]: grade I consisted of expanded blastocysts with certain differentiation among the trophoectoderm and inner cell mass, grade II blastocysts showed mild expansion, and grade III blastocysts were nonexpanded.

In Vitro Embryo Culture Beyond Day 7 and Cloned Embryo Explants

Some blastocysts from male B cell line were kept in in vitro culture from Day 7 until Days 16–17, unless they collapsed earlier. On Day 12, blastocysts were placed in a new microdrop of DMEM/F12 medium with 15% FBS and ATB. An increase in blastocyst size was determined every 24 h by using a millimeter eyepiece.

On Days 16–17, five aggregated embryos that were found collapsed on that day were picked up using 18-gauge needles and then cultured as tissue samples in DMEM/F12 medium containing 10% FBS, 1% ATB, and 1 µl/ml ITS in 5% CO_2 in humidified air at 39°C. Explants were obtained from the five embryos and grown for 7 to 10 days and then fixed using the protocol below.

Embryo Fixing and Oct-4 Staining

Blastocysts were fixed in 4% formaldehyde (product no. F1635) in Dulbecco phosphate buffered saline (DPBS; product no. 14287-072; Gibco, Grand Island, NY) for 20 min, rinsed in DPBS with 0.4% albumin from bovine serum (product no. A7906) for 20 min and then permeabilized in 0.1% Triton-X (product no. T-9284) in PBS for 15 min. Nonspecific immunoreactions were blocked by incubation for 30 min in blocking solution consisting of 3% FBS and 0.2% Tween 20 (product no. H5152; Promega, Madison, WI) in DPBS. Incubation with the primary antibody against Oct-4 (goat polyclonal immunoglobulin G [IgG]; code SC-8628; Santa Cruz Biotechnology, Santa Cruz, CA) diluted 1:100 in blocking solution was performed for 1 h. Then, embryos were rinsed in blocking solution for 15 min. Incubation with the secondary antibody (Alexa 488-donkey anti-goat IgG; code A11055; Molecular Probes Inc., Eugene, OR) diluted 1:1000 in blocking solution was performed for 45 min in darkness. Then, embryos were rinsed in blocking solution and counterstained with 0.01 mg/ml propidium iodide (product no. P-4864) in blocking solution for 10 min in darkness. For negative controls, blastocysts were not exposed to primary antibody. All stages were performed at room temperature. Embryos were mounted under coverslips in a drop of cooled 70% glycerol (product no. G9012) in DPBS, using wax posts to prevent their collapse.

TABLE 1. Effects of equine ZF cloned embryo aggregation on in vitro development to blastocyst stage at Day 8.

Experimental group	No. of recovered oocytes	No. of matured oocyte (%)	No. of ZFREs (%)[d]	No. of embryos (well)	No. of cleaved (%)	Blastocyst production		
						N	Per well (%)	Per ZFREs (%)
A cell line								
1X	281	131 (46.6)	111 (39.5)	111	91 (81.0)[a]	10	9.0[a]	9.0[a]
2X	350	175 (50.0)	164 (46.9)	81	74 (91.4)[a]	13	16.0[a]	7.9[a]
3X	395	205 (51.9)	186 (47.0)	62	62 (100.0)[c]	23	37.1[b]	12.5[a]
Total	1026	511 (49.8)	459 (44.7)	254	227 (89.4)	46	18.1	10
B cell line								
1X	398	203 (51.0)	190 (47.7)	190	151 (79.5)[a]	11	5.8[a]	5.8[a]
2X	372	179 (48.1)	156 (41.9)	78	74 (94.9)[b]	11	14.1[b]	7.1[a]
3X	403	212 (52.6)	195 (48.4)	65	62 (95.4)[b]	19	29.2[c]	9.7[a]
Total	1173	594 (50.6)	541 (46.1)	336	287 (85.4)	41	12.2	7.5

[a,b,c] Values with different superscripts in a cell line row and in a column are significantly different (chi-squared test; $P < 0.05$).
[d] ZFRE, zona-free reconstructed embryos.

Embryos were analyzed with a Nikon Confocal C.1 model scanning laser microscope. An excitation wavelength of 488 nm was selected from an argon-ion laser to excite the Alexa-conjugated secondary antibody and a 544 nm wavelength to excite propidium iodide. Images of serial optical sections were recorded every 5-μm vertical step along the Z-axis of each embryo. Three-dimensional images were constructed using EZ-C1 version 2.20 software. Due to the large number of cells from Day 16 embryos, 10 different areas of each blastocyst were analyzed until approximately 1500 cells were counted.

Embryo Transfer, Pregnancy Monitoring, and Foaling

Blastocyst transfer to recipients was performed during the breeding season. Thirty Criollo mares between 3 and 10 years of age were examined 2–3 times/week by transrectal ultrasonography (5-MHz linear probe; model 500; Aloka) to determine the phase of their estrous cycle. Prostaglandin F2 alfa (Ciclase; Sintex, Buenos Aires, Argentina) and human chorionic gonadotropin (Ovusyn; Sintex, Buenos Aires, Argentina) were used to synchronize the day of ovulation. Transcervical transfer of one to three Day 7–8 blastocysts was performed 5–7 days after ovulation, using an embryo transfer sheath with a side opening. Prior to embryo transfers, mares were introduced into a palpation chute, and the perineal area was carefully washed. Only three embryo transfers were performed using Day 16 expanded blastocysts derived from group 2X. Blastocysts were transported in a 0.5-cc straw containing SOF, and the shipping container was held at 36°C for the 3-h transport interval. Pregnancy was diagnosed by transrectal ultrasonography 15 days after ovulation, and those mares that were pregnant were examined weekly throughout the first trimester. Sizes of vesicles and embryos of different experimental groups were measured. Second and third trimester pregnancies were monitored on a monthly basis until foaling.

In mares with advanced pregnancies, combined uteroplacental thickness (CUPT), aortic diameter, and heart beat were measured by transabdominal ultrasonography. Mares were received daily supplementation with altrenogest (Regu-Mate, San Antonio, TX) until Day 300 of gestation when they were moved to an equine hospital (Kawell Equine Rehabilitation Center, Solís, Argentina), where they were monitored daily until parturition. Foals were healthy at birth and did not need medical assistance.

Cloned Foal Confirmation

To confirm that the obtained foals and fetuses were clones, hair samples from each animal and its respective donor animal were used for DNA comparison (Veterinary Genetics Laboratory, University of California, Davis, CA). Fourteen loci were analyzed in each study, and each locus was the same between cloned foals and their donor animals.

Statistical Analysis

Differences among treatments in each experiment were determined using Statistix version 0.8 software. Blastocyst rates and embryo quality were analyzed by chi-squared or Fisher exact test. Differences between Oct-4-positive cell numbers were analyzed for significance by using the proportions test. We assessed the effect of treatment on in vitro and in vivo embryo growth rates by using one-way within-subjects (repeated measures) analysis of variance model. Multiple observations of embryo growth rates from the same experimental units (embryo) along days constituted the within-subject factor. Post hoc pairwise comparisons among mean growing rates between days were performed with Tukey honestly significant differences tests. In order to compare pregnancy rates, we performed a binomial test. Success probability under the null hypothesis of a random pregnancy was $P = 0.14$ (probability of pregnancy of nonaggregated group).

RESULTS

Aggregation Effect on In Vitro-Cloned Blastocyst Production and Quality at Day 8

Approximately two COCs were recovered from each ovary. A total of 1105 of 2199 oocytes (50.25%) were selected for the presence of the first polar body (nuclear maturation rate). In a sample of 223 couplets, fusion and re-fusion rates were 78% and 69%, respectively. A total of 1000 ZFREs were produced (451 from donor animal A, and 549 from B) and cultured in vitro in three different groups. Cleavage and blastocyst rates per well and per ZFRE were recorded for all experimental groups (Table 1). A significant linear improvement of blastocyst rate per well was observed with an increase in the numbers of aggregated structures. Furthermore, aggregation did not involve the use of additional oocytes to obtain blastocysts, as no significant differences were observed in blastocyst rates on a per ZFRE basis.

Diameters and morphological characteristics of Day 8 blastocysts obtained with cell line A and classified in grades are shown in Table 2. Cloned blastocyst quality was reduced when no aggregation was used.

Oct-4 expression from Days 8 and 16 parthenogenetic and cloned embryos of all experimental groups is shown in Table 3. The percentage of Oct-4-positive cells was less in Day 16 cloned equine embryos and in parthenogenetic embryos than in Day 8 cloned equine embryos. Although no differences between aggregated and nonaggregated embryos were observed at Day 8, the Day 16 3X aggregated group showed a smaller proportion of Oct-4-positive cells (Fig. 1).

Aggregation Effects On In Vitro Development of Cloned Blastocysts Derived from B Cell Line Beyond Day 7

The in vitro growth rate of 28 equine cloned blastocysts derived from the B cell line was determined daily. A similar pattern of growth was observed between aggregated and nonaggregated groups. The growth rate per day was not statistically different between Day 7 and Day 11, but it was significantly different between Day 13 and Day 16. Mean embryo size per day ± SD were 112.43 μm ± 32.44 on Day 7; 160.18 μm ± 46.11 on Day 8; 235.32 μm ± 66.28 on Day 9;

TABLE 2. Blastocyst quality classified by morphological characteristics and by diameter at Day 8.

Group	No. of blastocysts*	Morphological classification Grade I (%)	Grade II (%)	Grade III (%)	Blastocyst diameter 80–115 μm (%)	120–170 μm (%)	180–220 μm (%)	230–270 μm (%)	+ 270 μm (%)
1X	10	1 (10.0)	1 (10.0)	8 (80.0)[a]	7 (70.0)[a]	2 (20.0)	0	1 (10.0)	0
2X	11	3 (27.3)	4 (36.4)	4 (36.4)[b]	5 (45.4)[b]	3 (27.3)	0	1 (9.1)	2 (18.2)
3X	23	6 (26.1)	8 (34.8)	9 (39.1)[b]	7 (30.4)[b]	10 (43.5)	4 (17.4)	1 (4.3)	1 (4.3)
Total	44	10 (22.7)	13 (29.5)	21 (47.7)	19 (43.2)	15 (34.1)	4 (9.1)	3 (6.8)	3 (6.8)

* Blastocysts obtained with A cell line.
[a,b] Values with different superscripts in a column are significantly different (chi-squared test; $P < 0.05$).

515.86 μm ± 267.90 on Day 11; 788.82 μm ± 391.91 on Day 12; 1118.50 μm ± 533.74 on Day 13; 1705.20 μm ± 917.93 on Day 14; 2242.20 μm ± 953.44 on Day 15; and 2870.80 μm ± 1178.30 on day 16.

Five explants retrieved from the aggregated group were found collapsed in Day 17 embryos grown in vitro, and a capsule-like structure was found in two of them (Fig. 2). No pregnancies resulted from the three embryo transfers performed with Day 16 embryos derived from group 2X.

Aggregation Effects on In Vivo Development of Cloned Embryos

Embryo transfer, pregnancy, and survival rates for cell lines A and B are given in Table 4. Early pregnancy rates were higher when aggregated embryos were transferred. Significant differences were found in pregnancy rates of nonaggregated embryos compared to those in 3X blastocysts. Only pregnancies derived from aggregated embryos survived beyond the fifth month of gestation, and the two cloned foals were also from aggregated groups (Fig. 3). Lengths of gestation were 330 and 350 days. Different effects of embryo aggregation on pregnancy rates were observed within the cell lines used in this study.

The sizes of in vivo embryos derived from cell line A were measured in transrectal ultrasonography images between Day 25 and Day 40 of gestation. Apparently embryo aggregation did not affect in vivo embryo development. Mean size ± SD of embryos (n = 4) on Day 25 was 0.92 mm ± 0.17; 1.75 mm ± 0.310 on Day 30; and 2.50 ± 0.18 on Day 40.

The dynamics of in vivo vesicle growth were also recorded by ultrasonography between Days 14 and 60 of gestation. Embryo aggregation did not change the in vivo vesicle growing pattern. Mean size ± SD (N) of vesicles on Day 14 was 14.97 mm ± 3.98 (7); 25.11 mm ± 2.75 on Day 20 (7); 35.29 mm ± 3.251 on Day 30 (6); 52.60 mm ± 10.90 on Day 40 (5); 67.00 mm ± 11.77 on Day 50 (5); and 85.00 mm ± 8.66 on Day 60 (3).

Only embryos resulting from aggregation achieved advanced stages of pregnancy with placental development. With the exception of one mare, CUPT measurements of cloned pregnancies derived from aggregated embryos ranged from 0.6 cm to 0.9 cm between months 4 and 6, from 0.8 cm to 1.0 cm between months 6 and 9, and from 0.9 cm to 1.3 cm in the last 3 months.

DISCUSSION

This work addressed embryo aggregation as a strategy to support cloned embryo development, which remains low in the equine compared to that in other large domestic animals like sheep (30% [4]), pigs (40% [27]), and cows (22% [19]). The present study is the first to describe the effects of aggregation on cloned equine embryo development.

In Vitro Embryo Development of Aggregated Embryos to Days 7–8

In our study, cloned embryo aggregation at the zygote stage significantly improved the rate of blastocyst production on a per well basis and may also have decreased the amount of ZFREs needed to produce blastocysts, as blastocyst rate per ZFREs of the 3X experimental group tended to be higher than that of the control ($P = 0.0840$). The improved blastocyst rate per well was linear with the number of aggregated structures used to achieve aggregation. Regarding embryo quality, smaller and more grade III Day 8 blastocysts were obtained when no aggregation was performed. These positive effects of embryo aggregation could be due to an epigenetic compensation, an increase in embryo cell numbers at the beginning of their development, or a combination of both situations.

It has been stated that ooplasm contains factors involved in cell reprogramming. However, each cell might be reprogrammed differently [13, 14]. Therefore, cellular and ooplast factors vary between each reconstructed embryo. Thus, cloned embryos generated by the aggregation of 2 or 3 genetically

TABLE 3. Evaluation of Oct-4 expression in equine parthenogenetic, aggregated, and nonaggregated cloned blastocysts at Day 8 and Day 16.

Group	n	Blastocysts with Oct-4 + cells	Total counted cells of all blastocysts	Oct-4 + cells	Percentage of Oct-4/total cells
Day 7 blastocysts					
Parthenogenetic	2	2	324	150	46[a]
1X	2	2	410	362	88[b]
2X	2	2	437	363	84[b]
3X	2	2	377	328	87[b]
Day 16 blastocysts					
1X	3	3	4485	974	22[c]
2X	2	2	3119	676	22[c]
3X	3	3	4303	540	13[d]

[a,b,c,d] Values with different superscripts in a column are significantly different (difference of proportions test; $P < 0.05$).

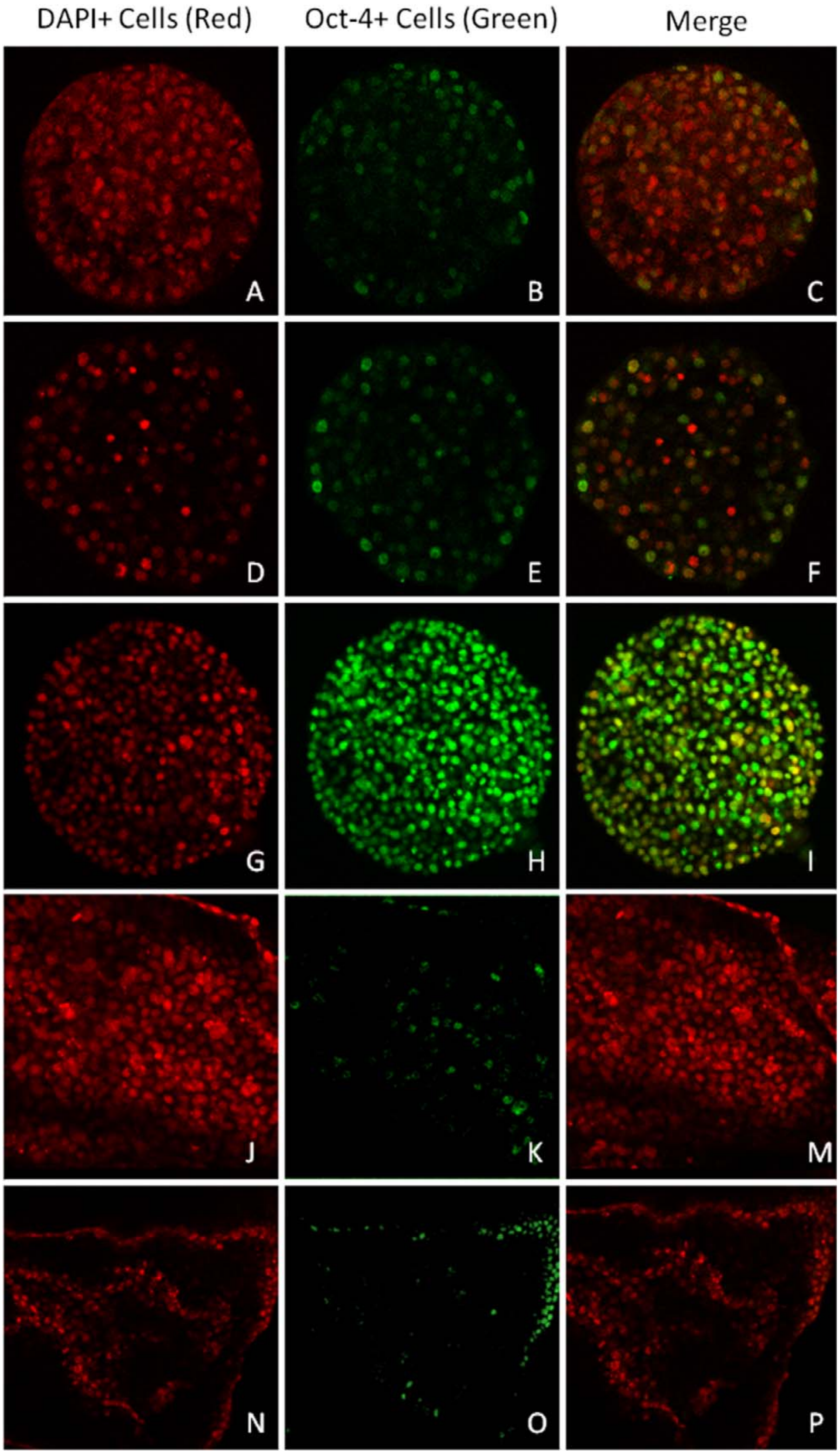

FIG. 1. Photomicrographs of equine cloned embryo expression of Oct-4. **A**, **B**, and **C**) Day 8 nonaggregated parthenogenetic embryo. **D**, **E**, and **F**) Day 8 nonaggregated cloned embryo. **G**, **H,** and **I**) Day 8 group 2X aggregated cloned embryo. **J**, **K**, and **M**) Day 16 nonaggregated cloned embryo. **N**, **O**, and **P**) Day 16 group 3X aggregated cloned embryo. All ZF cloned embryos examined expressed Oct-4 throughout the entire embryo without any special localization of this marker. Original magnification ×40 (**A**–**I**) and ×20 (**J**–**P**).

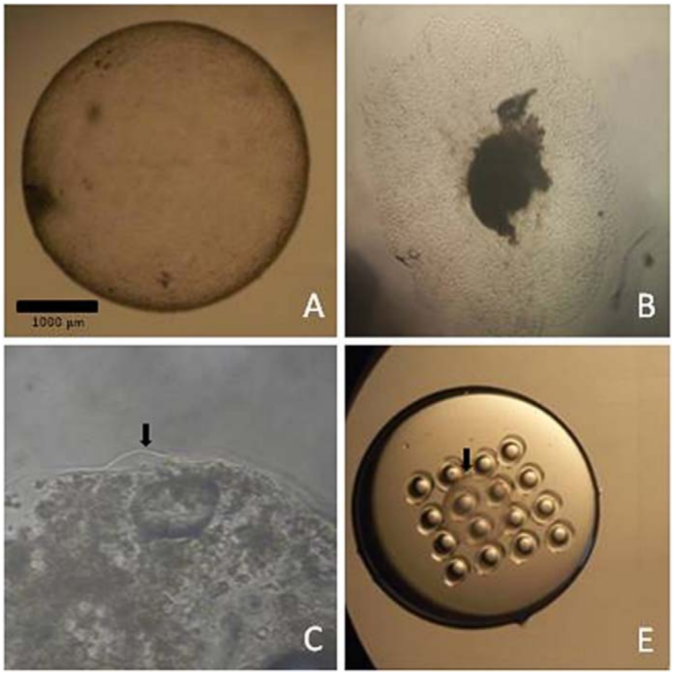

FIG. 2. Photographs of equine cloned embryos and explants. **A**) Day 16 equine cloned aggregated embryo derived from group 2X. **B**) Explants showing embryo cells growing in vitro from an equine cloned embryo derived from group 3X. **C**) A capsule-like structure that was found in one of the five explants (arrow). **D**) Day 14 equine cloned aggregated embryo (Arrow) derived from group 3X in a 100 µl microdrop with the well of the well system. Bar = 1000 µm.

identical donor cells, each one fused with different ooplasm, could be more competent. Different cell reprogramming and compensation for epigenetic cell-autonomous defects might support some of the reasons for the improved developmental competence of aggregated embryos [28]. In addition, mitochondria also influence embryo development [29, 30]. As embryo aggregation may use ooplasm derived from different animals, a combination of blastomeres with different mitochondrial populations coexist during aggregated embryo development.

Previously, it was demonstrated that the initial number of cells influences embryo development, as techniques such as embryo section or blastomere isolation affect embryo quality and development [31–33]. Embryo aggregation at the zygote stage represents a way to increase embryo cell numbers at the beginning of development. Even though the effects of embryo aggregation could not be related to the participation of all the aggregated embryos, a previous report demonstrated that aggregation of cloned ZF blastomeres can result in the development of chimeric blastocysts [34]. Aggregated clones in the mouse have dramatically improved spatial and temporal gene expression as well as developmental potential [17]. In the bovine, the aggregation of clones changes gene expression patterns at the preimplantation stage [35], promotes in vitro development of the resulting preimplantation embryos, doubles blastocyst cell numbers [18], and improves blastocyst growth rates, even when aggregation is performed using embryos reconstructed with one or the fusion of two hemioocytes [19]. Aggregation is considered by Ribeiro et al. [19] as a way to circumvent the unfavorable effects of heteroplasmy caused by fusion during handmade cloning. In the present work, enucleation was performed by micromanipulation. As only a small amount of ooplasm was removed, the fusion of hemioocytes was unnecessary, avoiding the heteroplasmy. Positive effects of aggregation were also observed in in vitro fertilized embryos [36]. Results presented in this study support observations made in other species.

In Vitro Embryo Development Beyond Day 7

A surprising increase in cell numbers and size of all blastocysts obtained was observed during in vitro culture after Day 7, as an indicator of their viability. Another study reported a similar pattern of growth of in vivo-recovered equine embryos subjected to in vitro culture; in that study blastocysts reached an average ± SD diameter of 2052 ± 290 µm on Day 17 [37]. The use of a different culture medium in our study probably explains why larger cloned embryos were seen. To our knowledge, there are no previous reports of in vitro embryo development beyond Day 10 for cloned equine blastocysts. Moreover, it was reported that vivid expansion of the blastocoele does not occur in vitro in the horse [38]. Despite the fact that aggregated embryos started development with more cells, in vitro blastocyst growth showed the same pattern among groups. Thus, we believe that after the blastocyst stage was reached, these initial differences are compensated for and are not reflected in in vitro blastocyst growth dynamics.

We did not see a clear embryo capsule in any of the experimental groups. Similar observations have been reported for in vivo embryos cultured in vitro [37] and for in vitro-produced equine embryos [39]. However, a structure morphologically similar to an equine embryo capsule was found in two of the five explants derived from a Day 17 aggregated cloned embryo (Fig. 2). As no pregnancies were achieved when the capsule was removed [40], we suggest that equine cloned

TABLE 4. Effects of equine cloned embryo aggregation on in vivo development.

Group	No. of recipients	Pregnant recipients (%)	Pregnancy No. (%) 8 Months	5 Months	1 Month	No. of offspring (%)
A cell line						
1X	3	1 (33.3)[a]	1 (100.0)	0	0	0
2X	5	2 (40.0)[a]	1 (50.0)	1 (50.0)	1 (50.0)	1 (50.0)
3X	4	3 (75.0)[b]	2 (66.6)	2 (66.6)	0	0
Total	12	6	4	3	1	1
B cell line						
1X	4	0 (0)[a]	0	0	0	0
2X	4	1 (33.3)[a]	1 (100.0)	0	0	0
3X	4	1 (33.3)[a]	1 (100.0)	1 (100.0)	1 (100.0)	1 (100.0)
Total	12	2	2	1	1	1

[a,b] Values with different superscripts in a cell line row are significantly different (binomial test; $P < 0.05$).

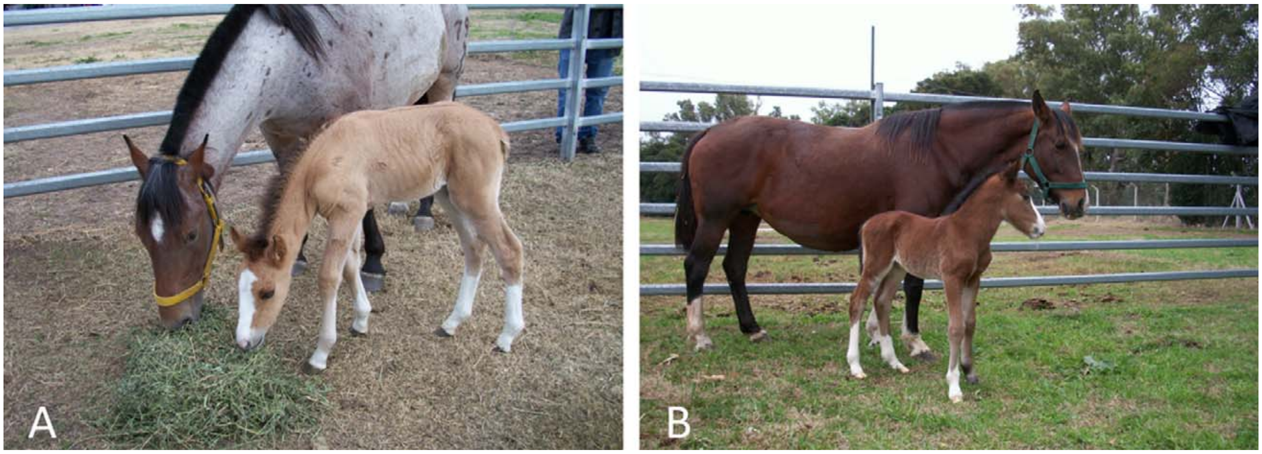

FIG. 3. Photographs of equine cloned foals. **A**) First equine cloned foal derived from 2X group of A cell line, born on the fifth of August, 2010. **B**) Equine cloned foal derived from the 3X group of B cell line, born on 15 August 2011.

embryos produce capsule material, even though it is not visible with light microscopy.

Oct-4 Expression of ZF Aggregated Cloned Equine Embryos

All ZF cloned embryos expressed Oct-4 throughout the entire embryo without any special localization of this marker. In comparison, in in vivo-recovered Day 7 equine blastocysts, Oct-4 expression appeared to be restricted to the inner cell mass [41]. Even though aggregation improves developmental potential in murine and bovine embryos [17, 18], no effects of embryo aggregation on Oct-4 expression, as recorded by immunostaining, were observed in Day 8 equine cloned blastocysts. Day 16 embryos derived from group 3X showed the lowest proportion of Oct-4 cells, indicating that major suppression of this factor might be due to an advanced stage of in vitro development of 3X embryos. These findings partially support the idea described by Choi et al. [21] that a uterine environment is required for local Oct-4 suppression and the differentiation of the trophectoderm in the horse.

In Vivo Embryo Development of Aggregated Embryos

Aggregated 3X equine ZF cloned embryos produced the highest early pregnancy rates, indicating that these embryos have a greater capacity to produce the signals necessary to allow early in vivo embryo development. We consider this to be an important benefit of embryo aggregation.

Transfer of cloned embryos is associated with losses throughout gestation and in early postnatal life [42, 43]. Considering the unique physiology of pregnancy recognition in mares, in which an embryo with a capsule must move through the uterus [44–46], transfers with more than one embryo per mare could improve maternal recognition. However, development of twin pregnancies is undesirable because mares can rarely tolerate this phenomenon [47]. Thus, blastocysts produced by aggregation with a greater capacity to develop, especially during early pregnancy, is an interesting strategy to use in this species.

The in vivo dynamics of embryo and vesicle growth were similar among groups. This situation confirms a developmental compensation of aggregated embryos after the blastocyst stage. Some differences were observed when these results were compared with findings in reports of in vivo-derived embryo and vesicle growth patterns [46]. Apparently, cloned embryos grow more slowly than in vivo embryos, but embryo vesicle sizes seem not to differ between the two types of embryos. More pregnancies need to be established in order to clarify the effect of embryo aggregation in late gestation. Bovine aggregated cloned embryos yielded higher initial pregnancy rates than single embryos. Moreover, all pregnancies remaining on Day 63 were derived from aggregated embryos [20]. Conversely, Tecirlioglu et al. [48] observed no differences in pregnancy rates between aggregated and nonaggregated cloned bovine embryos. Aggregated mice clones also developed to midgestation, yielding more fetuses than nonaggregated clones (12% vs. 3%), and a reduced decidual response was observed in the latter group [17].

Previous reports show that placenta development of cloned pregnancies suffers from several problems, which can be expressed as failure of placentation and abnormal expression of different genes [49–52]. Mares have a particularly late placentation, which makes them an interesting model for placenta research. Pregnancies were able to develop to term, and only one of the three aborted mares had an increase of the CUPT of more than 1.0 cm. The placenta was clearly thickened on the day of abortion, and the fetus had poor hair development and signs of fetal stress. The other fetus recovered was normal for the age of gestation, with no specific attributable cause of the loss. This subject needs further investigation, as normal or altered CUPTs have been observed during cloned pregnancies with normal foaling [53, 54].

In conclusion, the data presented in this paper indicate that aggregation of equine ZF-cloned embryos at the day of activation improved blastocyst rates on a per well basis. No extra oocytes were necessary to produce blastocysts, using aggregation. Embryo quality was reduced when aggregation was not performed, and early pregnancy rates were higher following the transfer of blastocysts derived from aggregated groups. Moreover, all advanced pregnancies and live healthy foals obtained in this study were derived from aggregated embryos. ZF equine cloned blastocysts were able to expand and increase in cell number while being maintained in in vitro culture without differences among groups. These results indicate that equine cloning could be improved by using a simple strategy like embryo aggregation.

ACKNOWLEDGMENT

The authors thank Florencia Karlanian and Adrian De Stefano for their assistance with oocyte collection and embryo transfer and Dr. Rafael Fernandez-Martin for reading and discussing the manuscript. Also thanks

to Dr. Elizabeth Crichton and Paola Barboza for English revision and to Hodara Karina for statistical support.

REFERENCES

1. Woods GL, White KL, Vanderwall DK, Li GP, Aston KI, Bunch TD, Meerdo LN, Pate BJ. A mule cloned from fetal cells by nuclear transfer. Science 2003; 301:1063.
2. Galli C, Lagutina I, Crotti G, Colleoni S, Turini P, Ponderato N, Duchi R, Lazzari G. Pregnancy: a cloned horse born to its dam twin [letter]. Nature 2003; 424:635.
3. Lagutina I, Lazzari G, Duchi R, Colleoni S, Ponderato N, Turini P, Crotti G, Galli C. Somatic cell nuclear transfer in horses: effect of oocyte morphology, embryo reconstruction method and donor cell type. Reproduction 2005; 130:559–567.
4. Lagutina I, Lazzari G, Duchi R, Turini P, Tessaro I, Brunetti D, Colleoni S, Crotti G, Galli C. Comparative aspects of somatic cell nuclear transfer with conventional and zona-free method in cattle, horse, pig and sheep. Theriogenology 2007; 67:90–98.
5. Hinrichs K, Choi YH, Love CC, Chung YG, Varner DD. Production of horse foals via direct injection of roscovitine-treated donor cells and activation by injection of sperm extract. Reproduction 2006; 131: 1063–1072.
6. Hinrichs K, Choi YH, Varner DD, Hartman DL. Production of cloned horse foals using roscovitine-treated donor cells and activation with sperm extract and/or ionomycin. Reproduction 2007; 134:319–325.
7. Choi YH, Hartman DL, Fissore RA, Bedford-Guaus SJ, Hinrichs K. Effect of sperm extract injection volume, injection of PLCzeta cRNA, and tissue cell line on efficiency of equine nuclear transfer. Cloning Stem Cells 2009; 11:301–308.
8. Li X, Morris LHA, Allen WR. In vitro development of horse oocytes reconstructed with the nuclei of fetal and adult cells. Biol Reprod 2002; 66:1288–1292.
9. Choi YH, Shin T, Love CC, Johnson CA, Varner DD, Westhusin ME, Hinrichs K. Effect of coculture with theca interna on nuclear maturation of horse oocytes with low meiotic competence, and subsequent fusion and activation rates after nuclear transfer. Theriogenology 2002; 57: 1005–1011.
10. Choi YH, Roasa LM, Love CC, Varner, SPDD Brinsko, Hinrichs K. Blastocyst formation rates in vivo and in vitro of in vitro-matured equine oocytes fertilized by intracytoplasmic sperm injection. Biol Reprod 2004; 70:1231–1238.
11. Galli C, Colleoni S, Duchi R, Lagutina I, Lazzari G. Developmental competence of equine oocytes and embryos obtained by in vitro procedures ranking from in vitro maturation and ICSI to embryo culture, cryopreservation and somatic cell nuclear transfer. Anim Reprod Sci 2007; 98:39–55.
12. Hinrichs K. Update on equine ICSI and cloning. Theriogenology 2005; 64: 535–541.
13. Boiani M, Eckardt S, Schöler HR, McLaughlin KJ. Oct4 distribution and level in mouse clones: consequences for pluripotency. Genes Dev 2002; 16:1209–1219.
14. Park KW, Lai L, Cheong HT, Cabot R, Sun QY, Wu G, Rucker EB, Durtschi D, Bonk A, Samuel M, Rieke A, Day BN, Murphy CN, et al. Mosaic gene expression in nuclear transfer-derived embryos and the production of cloned transgenic pigs from ear-derived fibroblasts. Biol Reprod 2002; 66:1001–1005.
15. Salamone DF, Damiani P, Fissore RA, Robl JM, Duby RT. Biochemical and developmental evidence that ooplasmic maturation of prepubertal bovine oocytes is compromised. Biol Reprod 2001; 64(6):1761–1768.
16. Poehland R, Al-Rostum F, Becker F, Viergutz T, Brunner RM, Kanitz W, Bhojwani S. Donor cell lines considerably affect the outcome of somatic nuclear transfer in the case of bovines. J Reprod Dev 2007; 53:737–748.
17. Boiani M, Eckardt S, Leu NA, Scholer HR, McLaughlin KJ. Pluripotency deficit in clones overcome by clone-clone aggregation: epigenetic complementation? EMBO J 2003; 22:5304–5312.
18. Zhou W, Xiang T, Walker S, Abruzzese RV, Hwang E, Farrar V, Findeisen B, Sadeghieh S, Arenivas F, Chen SH, Polejaeva I. Aggregation of bovine cloned embryos at the four-cell stage stimulated gene expression and in vitro embryo development. Mol Reprod Dev 2008; 75:1281–1289.
19. Ribeiro Ede S, Gerger RP, Ohlweiler LU, Ortigari I Jr, Mezzalira JC, Forell F, Bertolini LR, Rodrigues JL, Ambrósio CE, Miglino MA, Mezzalira A, Bertolini M. Developmental potential of bovine hand-made clone embryos reconstructed by aggregation or fusion with distinct cytoplasmic volumes. Cloning Stem Cells 2009; 11:377–386.
20. Pedersen HG, Schmidt M, Sangild PT, Strobech L, Vajta G, Callesen H, Greve T. Clinical experience with embryos produced by handmade cloning: work in progress. Mol Cell Endocrinol 2005; 234:137–143.
21. Choi YH, Harding HD, Hartman DL, Obermiller AD, Kurosaka S, McLaughlin KJ, Hinrichs K. The uterine environment modulates trophectodermal OCT-4 levels in equine blastocysts. Reproduction 2009; 138:589–599.
22. Bavister B, Yanagimachi R. The effects of sperm extracts and energy sources on the motility and acrosome reaction of hamster spermatozoa in vitro. Biol Reprod 1977; 16:228–237.
23. Tervit HR, Whittingham DG, Rowson LE. Successful culture in vitro of sheep and cattle ova. J Reprod Fertil 1972; 30:493–497.
24. Holm P, Booth PJ, Schmidt MH, Greve T, Callesen H. High bovine blastocyst development in a static in vitro production system using SOFaa medium supplemented with sodium citrate and myo-inositol with or without serum-proteins. Theriogenology 1999; 52:683–700.
25. Vajta G, Peura TT, Holm P, Paldi A, Greve T, Trouson AO, Callesen H. New method for culture of zona-included or zona-free embryos: the Well of the Well (WOW) system. Mol Reprod Dev 2000; 55:256–264.
26. McKinnon AO, Squires EL. Morphologic assessment of the equine embryo. J Am Vet Med Assoc 1988; 192:401–406.
27. Lagutina I, Lazzari G, Galli C. Birth of cloned pigs from zona-free nuclear transfer blastocysts developed in vitro before transfer. Cloning Stem Cells 2006; 8:283–293.
28. Eckardt S, McLaughlin KJ. Interpretation of reprogramming to predict the success of somatic cell cloning. Anim Reprod Sci 2004; 82:97–108.
29. Yan ZH, Zhou YY, Fu J, Jiao F, Zhao LW, Guan PF, Huang SZ, Zeng YT, Zeng F. Donor-host mitochondrial compatibility improves efficiency of bovine somatic cell nuclear transfer. BMC Dev Biol 2010; 19:10–31.
30. Wilding M, Dale B, Marino M, di Matteo L, Alviggi C, Pisaturo ML, Lombardi L, De Placido G. Mitochondrial aggregation patterns and activity in human oocytes and preimplantation embryos. Hum Reprod 2001; 16:909–917.
31. Westhusin ME, Collas P, Marek D, Sullivan E, Stepp P, Pryor J, Barnes F. Reducing the amount of cytoplasm available for early embryonic development decreases the quality but not the quantity of embryos produced by in vitro fertilization and nuclear transplantation. Theriogenology 1996; 46:243–252.
32. Peura TT, Lewis IM, Trounson AO. The effect of recipient oocyte volume on nuclear transfer in cattle. Mol Reprod Dev 1998; 50:185–191.
33. Tagawa M, Matoba S, Narita M, Saito N, Nagai T, Imai K. Production of monozygotic twin calves using the blastomere separation technique and Well of the Well culture system. Theriogenology 2008; 69:574–582.
34. Chrenek P, Makarevich AV. Production of rabbit chimeric embryos by aggregation of zona-free nuclear transfer blastomeres. Zygote 2005; 13: 39–44.
35. Kurosaka S, Eckardt S, Ealy AD, McLaughlin KJ. Regulation of blastocyst stage gene expression and outgrowth interferon tau activity of somatic cell clone aggregates. Cloning Stem Cells 2007; 9:630–641.
36. Lee SG, Park CH, Choi DH, Kim HS, Ka HH, Lee CK. In vitro development and cell allocation of porcine blastocysts derived by aggregation of in vitro fertilized embryos. Mol Reprod Dev 2007; 74: 1436–1445.
37. Hinrichs K, Schmidt AL, Memon MA, Selgrath JP, Ebert KM. Culture of 5-day horse embryos in microdroplets for 10 to 20 days. Theriogenology 1990; 34:643–653.
38. Hinrichs K. In vitro production of equine embryos: state of the art. Reprod Domest Anim 2010; 45(suppl 2):3–8.
39. Tremoleda JL, Stout TAE, Lagutina I, Lazzari G, Bevers MM. Effects of In vitro production on horse embryo morphology, cytoskeletal characteristics, and blastocyst capsule formation. Biol Reprod 2003; 69:1895–1906.
40. Stout TA, Meadows S, Allen WR. Stage-specific formation of the equine blastocyst capsule is instrumental to hatching and to embryonic survival in vivo. Anim Reprod Sci 2005; 87:269–281.
41. Guest DJ, Allen WR. Expression of cell-surface antigens and embryonic stem cell pluripotency genes in equine blastocysts. Stem Cells Dev 2007; 16:789–795.
42. Hill JR, Burghardt RC, Jones K, Long CR, Looney CR, Shin T, Spencer TE, Thompson JA, Winger QA, Westhusin, ME. Evidence for placental abnormality as the major cause of mortality in first-trimester somatic cell cloned bovine fetuses. Biol Reprod 2000; 63:1787–1794.
43. Chavatte-Palmer P, Remy D, Cordonnier N, Richard C, Issenham H, Laigre P, Heyman Y, Mialot J-P. Health status of cloned cattle at different ages. Cloning Stem Cells 2004; 6:94–100.
44. McDowell KJ, Sharp DC, Grubaugh W, Thatcher WW, Wilcox CJ. Restricted conceptus mobility results in failure of pregnancy maintenance in mares. Biol Reprod 1988; 39:340–348.

45. Ginther OJ. Intrauterine movement of the early conceptus in barren and post partum mares. Theriogenology 1984; 21:633–644.
46. Ginther OJ. Equine pregnancy: physical interactions between the uterus and conceptus. Am Assoc Equine Pract Proc 1998; 44:73–104.
47. Ginther OJ, Douglass RH. The outcome of twin pregnancies in mares. Theriogenology 1982; 18:237–244.
48. Tecirlioglu RT, Cooney MA, Lewis IM, Korfiatis NA, Hodgson R, Ruddock NT, Vajta G, Downie S, Trounson AO, Holland MK, French AJ. Comparison of two approaches to nuclear transfer in the bovine: hand-made cloning with modifications and the conventional nuclear transfer technique. Reprod Fertil Dev 2005; 17:573–585.
49. Miglino MA, Pereira FT, Visintin JA, Garcia JM, Meirelles FV, Rumpf R, Ambrósio CE, Papa PC, Santos TC, Carvalho AF, Leiser R, Carter AM. Placentation in cloned cattle: structure and microvascular architecture. Theriogenology 2007; 68:604–617.
50. Wei Y, Zhu J, Huan Y, Liu Z, Yang C, Zhang X, Mu Y, Xia P, Liu Z. Aberrant expression and methylation status of putatively imprinted genes in placenta of cloned piglets. Cell Reprogram 2010; 12(2):213–222.
51. Guillomot M, Taghouti G, Constant F, Degrelle S, Hue I, Chavatte-Palmer P, Jammes H. Abnormal expression of the imprinted gene Phlda2 in cloned bovine placenta. Placenta 2010; 31:482–490.
52. Bang JI, Bae DW, Lee HS, Deb GK, Kim MO, Sohn SH, Han CH, Kong IK. Proteomic analysis of placentas from cloned cat embryos identifies a set of differentially expressed proteins related to oxidative damage, senescence and apoptosis. Proteomics 2011; 11:4454–4467.
53. Vanderwall DK, Woods GL, Sellon DC, Tester DF, Schlafer DH, White KL. Present status of equine cloning and clinical characterization of embryonic, fetal, and neonatal development of three cloned mules. J Am Vet Med Assoc 2004; 225:1694–1699.
54. Johnson AK, Clark-Price SC, Choi YH, Hartman DL, Hinrichs K. Physical and clinicopathologic findings in foals derived by use of somatic cell nuclear transfer: 14 cases (2004–2008). J Am Vet Med Assoc 2010; 236:983–990.