Theriogenology 79 (2013) 791–796

Contents lists available at SciVerse ScienceDirect

Theriogenology

journal homepage: www.theriojournal.com

# A viable foal obtained by equine somatic cell nuclear transfer using oocytes recovered from immature follicles of live mares

Young-Ho Choi [a], Jody D. Norris [a], Isabel C. Velez [a], Candace C. Jacobson [a,1], David L. Hartman [b], Katrin Hinrichs [a,*]

[a] College of Veterinary Medicine & Biomedical Sciences, Texas A&M University, College Station, Texas, USA
[b] Hartman Equine Reproduction Center, Whitesboro, Texas, USA

ARTICLE INFO

*Article history:*
Received 27 May 2012
Received in revised form 12 December 2012
Accepted 12 December 2012

*Keywords:*
Oocyte
Embryo
Donor cell
Nuclear transfer
Horse

ABSTRACT

The presence of heterogenous mitochondria from the host ooplast affects the acceptance of offspring obtained by somatic cell nuclear transfer. This might be avoided by obtaining oocytes from selected females, but is then complicated by low numbers of available oocytes. We examined the efficiency of equine somatic cell nuclear transfer using oocytes recovered by transvaginal aspiration of immature follicles from 11 mares. Use of metaphase I oocytes as cytoplasts and of scriptaid (a histone deacetylase inhibitor) treatment during oocyte activation were evaluated to determine if these approaches would increase blastocyst production. In experiment 1, blastocyst development was 0/14 for metaphase I oocytes and 4/103 (4%) for metaphase II oocytes. Three blastocysts were transferred to recipient mares, resulting in two pregnancies and one live foal, which died shortly after birth. In experiment 2, blastocyst development was 2/47 (4%) for control oocytes and 1/83 (1%) for scriptaid-treated oocytes. No foals were born from two blastocysts transferred in the control group. The blastocyst from the scriptaid treatment resulted in birth of a live foal. In conclusion, this is apparently the first report of production of a viable cloned foal from oocytes collected from immature follicles of live mares, supporting the possibility of cloning using oocytes from selected mares.



## 1. Introduction

Cloned foals have been successfully produced using adult somatic cells [1–6]. Because of the value of individual horses (up to millions of dollars) cloning presents a unique clinically applicable tool for preservation of valuable genetics in this species. However, the value of a horse might be dependent on its registration by a breed association, and few associations currently allow registration of foals produced by nuclear transfer (NT), or of their progeny. One of the main arguments for exclusion of cloned foals from breed registries is the presence of heterogenous mitochondrial DNA in the foal; that is, mitochondrial DNA from the ooplast used for cloning. This heterogeneity might be avoided by use of oocytes from the same maternal line as the donor animal. The ability to select the source of oocytes used for cloning also allows research regarding the effect of mitochondrial genotype on the phenotype of the cloned offspring. Cloned calves have been produced using oocytes recovered from live cows of selected mitochondrial genotype [7].

Collection of useful numbers of oocytes from selected mares would require the use of transvaginal ultrasound-guided follicle aspiration (TVA), which was first reported in the mare in 1992 [8]. Unfortunately, the tight attachment of the oocyte to the follicle wall [9] makes TVA of equine immature follicles relatively inefficient—published recovery rates are often less than 30% (reviewed in [10]). Additionally, recovery of oocytes from mares must be performed at intervals of no less than 10 days, to avoid reduction in the

* Corresponding author. Tel.: +1 979 862 1338; fax: +1 979 845 6544.
*E-mail address:* khinrichs@cvm.tamu.edu (K. Hinrichs).
[1] Present address: Department of Veterinary Clinical Sciences, Oklahoma State University, Stillwater, Oklahoma, USA.

0093-691X/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.theriogenology.2012.12.005

number of follicles over time [11]. These factors result in a low number of oocytes available when TVA of immature follicles is performed in the mare. In a recent study, TVA yielded an average of three mature oocytes per mare every 2 weeks [10].

The overall efficiency of equine cloning is still low, largely because of the low rate of blastocyst formation after NT (typically less than 10% [2,3,5]). If a limited number of oocytes is available, methods to increase blastocyst development are essential. One straightforward method would be to use both metaphase I (MI) and metaphase II (MII) oocytes as host cytoplasts, thus using those oocytes that fail to reach MII by the end of the maturation period. Although synchrony between oocyte meiotic stage and donor cell cycle stage is important for successful NT [12,13], we previously obtained a pregnancy and live foal after equine NT using a MI ooplast generated from abattoir-derived tissue, and a donor cell synchronized using roscovitine, presumably in G0 or G1 of the cell cycle [5]. Other than this, the relative efficiency of use of MI or MII cytoplasts with roscovitine-treated donor cells is unknown.

Use of the histone deacetylase inhibitor, 6-(1,3-dioxo-1*H*,3*H*-benzo[*de*]isoquinolin-2-yl)-hexanoic acid hydroxyamide (scriptaid), during oocyte activation has been associated with increased blastocyst formation rate and proportion of live young after NT in mice and pigs [14–16], and in increased *in vitro* embryo development in cattle [17]. Scriptaid works by maintaining histone acetylation, thus enhancing transcriptional activity [18] and by promoting timely onset of embryonic gene transcription by activation of ribosomal RNA genes and support of nucleolar protein allocation during the early phase of embryonic gene activation [19]. Use of histone deacetylase inhibitors could increase the efficiency of equine NT; however, to the best of our knowledge, there is no information available on their use in this species.

In the present study, we examined the efficiency of equine somatic cell NT using equine oocytes recovered by transvaginal aspiration of immature follicles from a small herd of mares. We evaluated the use of oocytes in MI or MII after *in vitro* maturation, and the effect of treatment with scriptaid during oocyte activation on blastocyst production, pregnancy, and live foal rates.

## 2. Materials and methods

### *2.1. Experiment 1: Use of equine oocytes recovered from immature follicles by TVA, in MI or MII after in vitro maturation*

#### *2.1.1. Follicular aspiration*

For this study, 11 Quarter Horse-type mares aged 5 to 16 years were used. All experimental procedures were performed according to the United States Government Principles for the Utilization and Care of Vertebrate Animals Used in Testing, Research and Training and were approved by the Laboratory Animal Care Committee at Texas A&M University (AUP 2009-226).

Immature oocytes were harvested from all ovarian follicles greater than 8 mm diameter via TVA, as described [10]. Mares were not selected in relation to follicle activity or follicle size and there was no attempt to manipulate or monitor the cycle. A total of 72 mare TVA sessions were conducted for experiment 1; additional aspiration sessions were conducted in the same group of mares for separate experiments. For oocytes used in experiment 1, the interval from the last aspiration session in the same mare was 14 days in 62 sessions, 16 to 44 days in eight sessions, and 6 to 7 days in two sessions. Aspirations were scheduled so that three or four mares underwent aspiration sessions on a given day, and aspirations were performed 1 or 2 days per week. Briefly, mares were tranquilized and held in stocks. The operator positioned a transvaginal ultrasound probe then grasped the ovary via transrectal palpation and manipulated the probe to visualize follicles on the ovary. Follicles were punctured using a 12- or 15-ga double-lumen needle. When possible, each follicle was flushed six times with M199 with Hanks' salts with 25 mmol/L HEPES (Invitrogen, Carlsbad, CA, USA) containing 0.4% fetal bovine serum (FBS; Invitrogen), 8 IU/mL heparin, and 25 μg/mL gentamicin, while the follicle was curetted by rotation of the needle.

#### *2.1.2. In vitro maturation*

The aspirated fluid was filtered through an embryo filter (EmCon filter, Immuno Systems, Inc., Spring Valley, WI, USA) and the collected cellular material rinsed into a Petri dish. Oocytes were identified under a dissection microscope, washed twice, and placed in EH holding medium [20], consisting of 40% M199 with Earle's salts (Invitrogen), 40% M199 with Hanks' salts, and 25 mmol/L HEPES, and 20% FBS (Invitrogen). Oocytes were held in this medium at room temperature (22 °C–26 °C) overnight (14–17 hours). They were then cultured in M199 with Earle's salts supplemented with 10% FBS, 5 mU FSH (Sioux Biochemicals, Sioux Center, IA, USA) and 25 μg/mL gentamicin, for 24 to 30 hours. Oocytes were cultured in droplets of medium at a ratio of 10 μL of medium per oocyte under light white mineral oil (Sigma-Aldrich, St. Louis, MO, USA) at 38.2 °C in a humidified atmosphere of 5% $CO_2$ in air.

#### *2.1.3. Sperm extract preparation*

Stallion sperm cytosolic extract was prepared as previously described [21] but using Chatot-Ziomek-Bavister(CZB) medium modified for use with sperm (Sp-CZB; [22]) for sperm washing [22]. Briefly, the procedures as reported for the mouse [23] were used with modification. Fresh ejaculated stallion sperm were centrifuged at 900× *g* for 10 minutes to remove seminal plasma. The pellet was then resuspended with Sp-CZB containing 5 mg/mL BSA and centrifuged at the same setting. The resulting pellet was resuspended to a final concentration of $20 \times 10^8$ sperm per mL in nuclear isolation medium [24] and centrifuged to remove Sp-CZB. The pellet was then resuspended to the same volume with nuclear isolation medium containing 1 mmol/L dithiothreitol, 100 μmol/L leupeptin, 100 μmol/L antipain, and 100 μg/mL soybean trypsin inhibitor. The suspension was subjected to four cycles of freezing (5 minutes per cycle in liquid $N_2$) and thawing (5 minutes per cycle at 15 °C), then sperm were pelleted at 20,000× *g* for 50 minutes at 2 °C. The resulting supernatant was carefully removed, aliquoted, and kept at −80 °C until use.