Special Report

Present status of equine cloning and clinical characterization of embryonic, fetal, and neonatal development of three cloned mules

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The ability to clone mammals via somatic cell nuclear transfer has been recognized as a major scientific milestone because it proved that a fully differentiated somatic cell can be genetically reprogrammed back to the undifferentiated state of a 1-cell zygote (embryo) and initiate and undergo complete embryonic-fetal development, resulting in the birth of an animal that is genetically identical to the original cell donor. Although the specific methods used for cloning via nuclear transfer can differ, it is generally performed by micromanipulating and fusing 2 cells (**Figure 1**). One cell, referred to as the nuclear donor or karyoplast, is derived from the animal to be cloned; typically, donor cells are maintained in tissue culture, from which 1 cell is selected for each nuclear transfer procedure. The other cell, referred to as a cytoplast, is a mature unfertilized oocyte from which the genetic material (polar body and metaphase plate) has been removed. The cytoplast contains numerous cellular factors (eg, mRNA and proteins) that play an important role in the reprogramming of the genetic material (ie, genes) of the donor cell, which enables the cloned embryo to initiate the complex sequence of events leading to embryonic and fetal development. The reconstituted embryo uses the donor cell DNA as the template for subsequent gene expression, which results in a genetic clone of the donor animal.

The ability to clone animals via somatic cell nuclear transfer offers tremendous potential for application in the areas of animal agriculture (eg, genetic improvement[1,2] and disease resistance[3]), conservation biology through the preservation of endangered species,[4,5] and medical biotechnology (eg, production of transgenic animals for biopharming[6,7] and xenotransplantation of organs[3,8]). For the equine

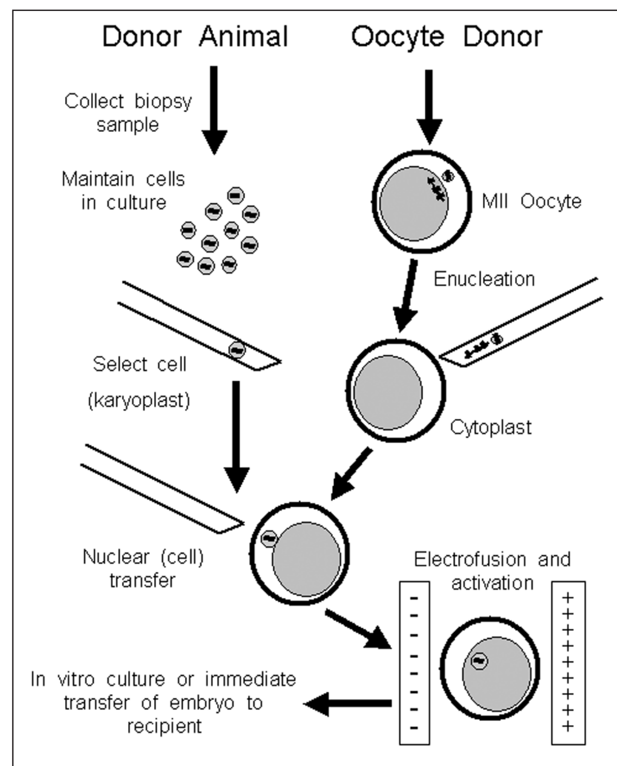

Figure 1—Schematic representation of the nuclear transfer cloning procedure. A tissue biopsy specimen (generally skin) is collected from the donor animal, from which a population of viable cells is isolated. Donor cells can be maintained in culture and actively used for cloning or preserved and stored indefinitely in liquid nitrogen for future use. A mature (metaphase II [MII]) unfertilized oocyte is obtained and enucleated by removing the first polar body and metaphase plate with micromanipulators. A single donor cell (karyoplast) is then placed under the zona pellucida of the enucleated oocyte (cytoplast) in contact with the cytoplast, and the 2 cells are induced to fuse by use of a pulse of direct electrical current. After fusion, the reconstructed 1-cell cloned embryo is stimulated to initiate embryonic development by a chemical activation process that mimics the events of fertilization.

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industry, nuclear transfer is one of several new assisted reproductive techniques (eg, oocyte transfer and intracytoplasmic sperm injection) being developed for clinical use. Potential uses of equine cloning include preservation of genetic material from individual animals that would otherwise not be able to reproduce, such as geldings; preservation of genetic material of endangered or exotic species such as the Mongolian Wild Horse (Przewalski's horse); and, because of the companion animal role that horses fill for some individuals, emotional fulfillment of the owner. Of these, cloning geldings to produce sexually intact males for breeding purposes will likely be the first direct application of equine cloning. Although some breed associations (eg, The Jockey Club and American Quarter Horse Association) do not presently allow the registration of cloned animals, for some equine sporting activities (dressage and show jumping), breed registry status is irrelevant, which eliminates that regulatory impediment to the use of cloning technology.

Veterinarians can help their clientele prepare for cloning by banking tissue from animals to be cloned. Presently, several commercial companies[a-e] will isolate and store cells from tissue collected from animals. These companies typically provide the veterinarian with a tissue collection and transportation kit; the procedure involves aseptically collecting a small skin biopsy specimen that is placed in tissue culture medium and returned to the company where cells are grown in tissue culture. After the cells have grown in culture, they can be used immediately for cloning or harvested and stored frozen in liquid nitrogen for use in the future. Ideally, tissue should be collected from a live animal; however, in an emergency, it may be possible to collect a suitable sample after death. To maximize the likelihood of recovering viable cells, tissue from the dead animal should be kept refrigerated (3° to 8°C [37° to 47°F]); the chance of recovering viable cells diminishes when the tissue has been frozen or stored at > 10°C (50°F).[a]

Status of Equine Cloning

The year 2003 was notable for equine cloning. We reported the live birth of 3 mule foals cloned from a fetal fibroblast cell line,[9] and Galli et al[10] reported the live birth of a horse foal cloned from an adult fibroblast cell line. We have also produced 7 cloned horse pregnancies by use of adult cumulus cells, although in each instance, embryonic loss occurred spontaneously prior to day 80 of gestation.[11,f] Prior to 2003, limited in vitro development of cloned equine embryos was reported.[12-16] As in other species, the efficiency of equine cloning is low; < 3% of cloned embryos produced with our present cloning system[9] resulted in the birth of live offspring. On the basis of experience with other species, we believe that inefficiency of cloning is primarily because of cloned offspring syndrome,[17] which is characterized by a high incidence of embryonic, fetal, or placental developmental abnormalities that result in extremely high rates of embryonic loss, abortion, and stillbirths throughout gestation and compromised neonatal health after birth.[18] Cloned offspring syndrome has been especially problematic in cattle and sheep in which problems occur throughout gestation and the neonatal period, whereas in goats and swine, problems have generally been limited to early gestation.[18,19] Neonatal health complications that have been observed in cloned calves include lung dysmaturity, pulmonary hypertension, respiratory distress, hypoxia, hypothermia, hypoglycemia, metabolic acidosis, enlarged umbilical veins and arteries, and development of sepsis in umbilical structures or lungs.[20] Similar problems have been observed in cloned sheep.[21] The embryonic, fetal, and placental developmental abnormalities that result in abortion, stillbirth, or compromised neonatal health are thought to reflect incomplete or abnormal genetic reprogramming of the donor nucleus, specifically related to critically important imprinted genes.[22-24] Because the nuclear transfer procedure involves a series of complex procedures that generally includes culture of donor cells, in vitro maturation of oocytes, oocyte enucleation, cell or nucleus injection, fusion, activation, in vitro culture of reconstructed embryos, and embryo transfer, the production of cloned offspring may be adversely affected if results of any of these procedures are suboptimal.[24] Our recent experience with the live birth of 3 cloned mules has provided an opportunity to develop an initial baseline of information on the characteristics of pregnancy, parturition, and neonatal health of equine clones, which can serve as an important benchmark for comparison with noncloned equine pregnancies. By making these comparisons, we can begin to address whether cloned offspring syndrome will be a problem in equine clones and, if it is, how it is manifested.

Embryonic, Fetal, and Placental Development of Viable Cloned Mules

Three light-horse mares (A [8 years old], B [10 years old], and C [12 years old]) of unknown prior reproductive history that were pregnant with cloned mule embryos were monitored throughout gestation. The cloned mule embryos were produced from a fetal fibroblast cell line via nuclear transfer procedures, as described.[9] Immediately after completion of the in vitro nuclear transfer procedures, four 1-cell cloned embryos were transferred to each mare by surgically placing them into the oviduct ipsilateral to ovulation, which had occurred during the preceding 24 hours. An initial pregnancy examination was performed via transrectal ultrasonography from days 13 to 16 of gestation (day 0 = day of transfer). Subsequent examinations were performed at 7- to 10-day intervals until day 60 and then approximately every 2 to 4 weeks throughout gestation. Parameters evaluated at each examination during early gestation (< 60 days) were size and location of the embryonic vesicles, presence of an embryo within the vesicle, and presence of an embryonic heartbeat. The primary parameter evaluated ultrasonographically, manually, or both at each examination during midgestation (60 to 270 days) was fetal movement. Parameters evaluated during late gestation (> 270 days) were fetal movement and transrectal ultrasonographic assessment of the **combined thickness of the uterus and placenta (CTUP)**, as described.[25]

Mares A and B were treated prophylactically with a

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long-acting progesterone formulation[g] (3 mg/kg [1.4 mg/lb], IM); treatment was initiated on days 3 (mare B) and 22 (mare A) and was repeated every 7 days until day 100. All 3 mares received routine veterinary care throughout gestation, which included vaccination against equine herpes virus-1 and influenza virus[h] at 5, 7, and 9 months of gestation and vaccination against West Nile virus[h] (2-shot series 1 month apart) between days 47 and 176 of gestation (differed among mares). Approximately 6 weeks before the estimated date of parturition, each mare was vaccinated against tetanus and eastern and western encephalomyelitis.[h] Mares were dewormed approximately every 3 months during gestation with a variety of anthelmintics. Because the mares were carrying mule conceptuses, which increases the risk of neonatal isoerythrolysis,[26,27] blood samples were collected within 1 month of their estimated parturition dates for detection of anti-RBC hemolysin or agglutinin antibodies.[i]

Of the 12 cloned mule embryos transferred to these mares, 4 developed into ultrasonographically detectable conceptuses. Twins that subsequently became fixed in opposite uterine horns were detected in mare C. The growth profile of each cloned conceptus was compared with a published[28] growth profile of noncloned horse conceptuses that had normal development from days 11 to 40 (**Figure 2**). At each examination, all 4 cloned conceptuses were within the normal size range for noncloned conceptuses. An embryo was identified within the vesicle of all 4 cloned conceptuses between days 21 and 29; however, a heartbeat was not detected in one of the twin conceptuses in mare C. The conceptus without a heartbeat was spontaneously eliminated between days 29 and 36. The 3 remaining conceptuses continued to have normal development and remained viable throughout gestation. During the 10th and 11th months of gestation, CTUP measurements of the cloned pregnancies were compared with CTUP measurements obtained from commercial broodmares with noncloned horse pregnancies.[29] At each examination at which it was measured, the CTUPs of the cloned pregnancies were within the reference range for noncloned pregnancies (**Table 1**).

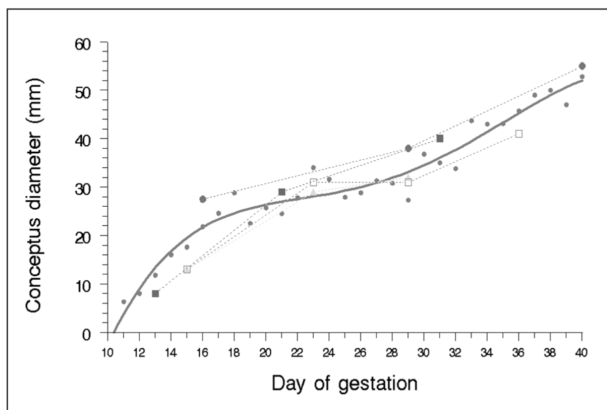

Figure 2—Growth profiles of 4 cloned mule conceptuses (dashed lines; each conceptus is indicated by a unique symbol) and 147 noncloned clinically normal horse conceptuses (solid line depicts regression analysis of the daily mean values [small solid circles]) between days 11 and 40 of gestation. Adapted from Vanderwall et al[28] with permission.

Parturition, Postpartum Placental Evaluation, and Neonatal Health of Viable Cloned Mules

Serum from blood of mares A and C yielded strong positive results for anti-donkey RBC agglutinin antibodies; therefore, prior to parturition, an alternate source of equine colostrum was obtained and screened for anti-donkey RBC agglutinin activity. Colostrum from donor mares was examined for agglutinin activity against whole blood from 1 female donkey by use of an agglutination test.[30] Only colostrum that had negligible agglutinin activity was subsequently administered to the foals of those 2 mares. When the estimated date of parturition approached, the mares were monitored for clinical signs indicative of preparation for parturition, including udder development, relaxation of the sacrosciatic ligaments and vulva, and cervical relaxation or dilatation. For each of these parameters, all 3 mares had the typical temporal pattern of change prior to parturition. When parturition appeared imminent (within 96 hours), the mares were monitored with routine procedures that consisted of direct visual observation during the day when they were turned out on pasture and closed-circuit video observation during the night when they were confined to a box stall. The 3 mares spontaneously initiated and completed parturition without assistance on days 340 (B) and 346 (A and C). All 3 foals were active and healthy at birth, and all 3 mares spontaneously expelled the placenta within 90 minutes of parturition. Physical characteristics of each placenta were evaluated and compared with previously published data[31] for Thoroughbred horses (**Table 2**); of note was the finding that the nonpregnant horn was longer than the pregnant horn in all 3 cloned placentas, whereas the pregnant horn is typically longer than the nonpregnant horn in equine placentas. The clinical importance, if any, of this finding was unknown. In addition, the placenta of mare A had gross and microscopic evidence of mild, focal microcotyledonary necrosis in 3 areas (4.0 × 5.0, 3.0 × 1.5, and 2.0 × 2.0 cm) that were associated with minimal inflammatory changes (lymphocytic placentitis) and yielded growth of moderate numbers of *Aeromonas* and *Klebsiella* organisms. There was no evidence that these areas of focal placentitis compromised fetal or neonatal health of this mare's foal. Although the inciting cause of the placentitis was unknown, it is unlikely that it was related to the cloning procedure.

Table 1—Combined thickness of the uterus and placenta (CTUP) measurements (mean ± SD) during the 10th and 11th months of gestation in 3 mares with cloned mule pregnancies.

	CTUP measurement (mm)	
Month of gestation (d)	**Cloned mule pregnancies (No. of observations)**	**Upper reference limit for noncloned horse pregnancies***
10 (271–300)	6.4 ± 0.9 (5)	8
11 (301–330)	7.8 ± 1.5 (4)	10

*Adapted from Troedsson et al[29] with permission.

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Table 2—Birth weights and placental characteristics of 3 cloned mule foals.

	Mare			
Variable	A	B	C	Reference range*
Foal weight (kg)	36	40	49	36.8–63.2
Placenta weight (kg)†	2.0‡	3.3	4.2	4.0–8.4
Cord length (cm)	58	60	49	32–90
Pregnant horn (cm)				
Length	60	50	61	54–85
Width (middle)	10	18	19	14–34
Nonpregnant horn (cm)				
Length	65	57	65	46–80
Width (middle)	10	18	16	12–29
Body (cm)				
Length	108	100	102	110–152
Width (cranial)	35	~25	32	30–55

*Values for Thoroughbred foals adapted from Whitwell and Jeffcott[31] with permission. †All 3 placentas were stored at 4°C for > 48 hours prior to analysis, which likely resulted in some fluid loss prior to analysis. ‡Amnion not recovered.

Table 3—Hematology profiles of 3 cloned mule foals. Samples were collected between 24 and 36 hours after birth.

	Foal			
Variable	A	B	C	Reference range*
WBC (cells/µL)	3,100	10,400	8,900	4,900–11,700
Band neutrophils (%)	6	0	0	NA
Neutrophils (%)	45	86	83	NA
Lymphocytes (%)	39	12	15	NA
Monocytes (%)	10	0	2	NA
Eosinophils (%)	0	0	0	NA
Basophils (%)	0	2	0	NA
RBC morphology	Slight anisocytosis	Normal	Normal	NA
RBC ($\times 10^6$ cells/µL)	10.07	9.98	9.78	8.2–11.0
Hemoglobin (g/dL)	14.1	13.7	13.4	12.0–16.6
PCV (%)	45	44	42	32–46
Plasma protein† (g/dL)	5.0	6.1	4.8	5.2–8.0
Fibrinogen (mg/dL)	100	200	200	100–400
MCV (fL)	45	44	43	36–46
MCH (pg)	14	14	14	NA
MCHC (%)	31	31	32	32–40
RDW (%)	26	26	26	NA
Platelets (µL)	127,000	41,000	61,000	129,000–409,000
MPV (fL)	4.4	5.7	4.4	NA

*Adapted from Harvey JW. Normal hematologic values. In: Koterba AM, Drummond WH, Kosch PC, eds. *Equine clinical neonatology*. Philadelphia: Lea & Febiger, 1990;561–570. Reprinted with permission. †Determined using a refractometer.

MCV = Mean corpuscular volume. MCH = Mean corpuscular hemoglobin. MCHC = Mean corpuscular hemoglobin concentration. RDW = Red cell distribution width. MPV = Mean platelet volume. NA = Not available.

Immediately after birth, all 3 foals were weighed (Table 2) and underwent a complete physical examination. In addition, they received routine postpartum care consisting of disinfection of the umbilical stump with 0.5% chlorhexidine[j] every 6 hours for 3 days; a single enema[k]; and prophylactic treatment with vitamin E and selenium[l] (0.03 mg/kg [0.014 mg/lb], IM, once), gentamicin[m] (5 mg/kg [2.3 mg/lb], IM, q 24 h, for 3 days), and procaine penicillin G[n] (24,000 U/kg [10,909 U/lb], IM, q 12 h, for 3 days). All 3 foals stood unassisted within 30 minutes of birth. To minimize the risk of neonatal isoerythrolysis, the foals of mares A and C were muzzled to prevent them from nursing and they received the described donor colostrum via a nasogastric tube. The mammary secretions of mares A and C were manually expressed, and the colostrum was checked for RBC agglutinin activity against their foal's blood; agglutinin activity was negligible within 6 hours of parturition in both mares, at which time each foal was allowed to nurse its respective dam. In addition to the supplemental equine colostrum administered to the foals of mares A and C, all 3 foals received 150 to 250 mL of a commercial equine IgG formulation[o] via a nasogastric tube within 4 hours of birth. Between 24 and 36 hours after birth, blood samples were collected from each foal for routine hematologic (**Table 3**) and serum biochemical analyses (**Table 4**).[p] All 3 foals had mild to moderate thrombocytopenia

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Table 4—Serum biochemical profiles of 3 cloned mule foals. Samples were collected between 24 and 36 hours after birth.

Variable	Foal A	Foal B	Foal C	Reference range*
SDH (U/L)	6	3	3	0.6–4.6
GGT (U/L)	25	23	29	18–43
AST (U/L)	86	135	136	146–340
ALP (U/L)	1,734	1,358	1,131	861–2,671
CK (U/L)	474	92	217	40–909
BUN (mg/dL)	15	11	9	9–40
Creatinine (mg/dL)	2.0	1.6	1.4	1.2–4.3
Glucose (mg/dL)	130	133	144	121–233
Total protein† (g/dL)	4.0	5.9	4.9	4.3–8.1
Albumin (g/dL)	3.1	3.0	3.2	2.5–3.6
Globulin (g/dL)	0.9	2.9	1.7	1.5–4.6
Calcium (mg/dL)	12.1	11.6	12.0	11.7 ± 2.0
Phosphorus (mg/dL)	3.2	4.5	5.1	5.6 ± 1.8
Sodium (mEq/L)	144	142	139	141 ± 18
Potassium (mEq/L)	4.0	4.2	3.6	4.6 ± 1.0
Chloride (mEq/L)	110	105	102	102 ± 12
CO_2 (mmol/L)	26	28	29	27 ± 6
Anion gap (mEq/L)	12	13	11.7	16 ± 8

*Adapted from Bauer JE. Normal blood chemistry. In: Koterba AM, Drummond WH, Kosch PC, eds. *Equine clinical neonatology*. Philadelphia: Lea & Febiger, 1990;602–614. Reprinted with permission. †Determined by use of Biuret method.

SDH = Sorbitol dehydrogenase. GGT = γ-glutamyl transferase. AST = Aspartate aminotransferase. ALP = Alkaline phosphatase. CK = Creatine kinase.

(Table 3), which resolved spontaneously and was not associated with any clinical signs. The underlying cause of the thrombocytopenia was unknown but may have been the result of absorption from colostrum of alloantibodies against foal platelets.[32] Clinically, the foals remained healthy throughout the neonatal period and continue to be healthy and vigorous at 15 to 18 months of age.

Discussion

Three of the 4 cloned mule conceptuses that established ultrasonographically detectable pregnancies remained viable throughout gestation and had prototypical patterns of embryonic, fetal, and placental development; parturition; and neonatal health. The twin conceptus in 1 mare (C) that was spontaneously lost between days 29 and 36 was 1 of 18 cloned mule conceptuses in a larger study[9] that were spontaneously lost prior to day 60; therefore, cloned mule conceptuses had a high rate of early pregnancy loss (18/21 [86%]), which is similar to that seen with cloned conceptuses in other species.[18] However, the normality of fetal and placental development, parturition, and neonatal health in the 3 mule conceptuses that remained viable after day 60 was markedly different from that typically observed in clones of many other species, most notably cattle and sheep.[18] The fact that all 3 cloned foals were carried to term and were healthy is remarkable when compared with the extremely high incidence of mid- to late-term abortion, stillbirth, and neonatal problems in cattle and sheep. For example, most cloned bovine embryos that are carried to term are born with health complications.[20] Certain aspects of our cloning system may aid in understanding problems seen in cattle and sheep clones. Although it is likely that reprogramming errors in the donor mule cells contributed to the high incidence of early embryonic loss (86%), in contrast to cattle and sheep, these errors were apparently only expressed during early gestation.

It is important to note that when generating the cloned mule embryos, a concerted effort was made to minimize in vitro handling and incubation of oocytes and cloned embryos. Specifically, in vivo matured oocytes were used as host cytoplasts and reconstructed embryos were immediately transferred to recipient mares after completion of the activation procedure. We chose to minimize the amount of time oocytes and embryos were maintained in vitro because optimal culture systems for maturing equine oocytes and supporting the development of equine embryos in vitro have not been developed. This has direct implications for cloning because in sheep, the use of oocytes matured in vivo results in higher pregnancy rates with cloned embryos, compared with oocytes matured in vitro.[33] This may be attributable to decreased ability of oocytes matured in vitro to fully reprogram the donor nucleus.[22] In addition to possible effects on the oocyte, in vitro incubation of embryos in medium containing serum contributes to large offspring syndrome in ruminants, which, like incomplete nuclear reprogramming, is thought to perturb imprinted genes,[22] leading to an up to 2-fold increase in birth weight and multiple organ defects.[34]

There is anecdotal evidence that maturation of oocytes in vitro and embryo culture increase mid- to late-term gestational abnormalities of equine clones. Galli et al[10] used oocytes matured in vitro and incubated cloned embryos to the blastocyst stage before transfer to recipient mares; of 17 blastocysts transferred to recipients, 4 resulted in ultrasonographically detectable pregnancies at day 21. Of those 4 conceptuses, 2 were lost soon after detection, 1 was aborted at day 187 (cause unknown), and 1 resulted in the birth of a healthy foal. Scientists at Texas A&M University also produced a cloned equine pregnancy

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using an oocyte matured in vitro and incubation of the cloned embryo to the blastocyst stage before transfer, but the fetus was aborted during late gestation.[q]

Further work will be necessary to determine whether minimizing handling and culturing is important for preventing the types of abnormalities seen with cloned cattle and sheep or whether there are fundamental species differences that account for the lack of problems observed in cloned equine offspring. As further advancements are made, we anticipate that the clinical use of equine cloning will increase and that the practitioner's role will progress from simply providing tissue collection services for cell banking to the clinical management of mares with cloned pregnancies and the neonatal care of cloned foals.

[a]Cyagra Inc, Worcester, Mass.
[b]Geneticas, Los Angeles, Calif.
[c]Genetic Savings & Clone, Sausalito, Calif.
[d]Lazaron BioTechnologies LLC, Baton Rouge, La.
[e]PerPETuate Inc, Farmington, Conn.
[f]Vanderwall DK, Woods GL, Aston KI, et al. Cloned horse pregnancies produced using adult cumulus cells (abstr). *Reprod Fertil Dev* 2004;16:160.
[g]BioRelease P4 LA 150, Betpharm, Lexington, Ky.
[h]Fort Dodge Animal Health, Fort Dodge, Iowa.
[i]Hematology Laboratory, Veterinary Medical Teaching Hospital, University of California, Davis, Calif.
[j]Nolvasan solution, Fort Dodge Laboratories Inc, Fort Dodge, Iowa.
[k]Unico Inc, Lake Worth, Fla.
[l]E-SE Injection, Schering-Plough Animal Health, Union, NJ.
[m]GentaVed 100, VEDCO Inc, St Joseph, Mo.
[n]US Vet, G.C. Hanford Mfg Co, Syracuse, NY.
[o]Seramune, Sera Inc, Shawnee, Kan.
[p]Clinical Pathology Laboratory, College of Veterinary Medicine, Washington State University, Pullman, Wash.
[q]Hinrichs K, Texas A&M University, College Station, Tex: Personal communication, 2003.

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