Evaluation of foal production following intracytoplasmic sperm injection and blastocyst culture of oocytes from ovaries collected immediately before euthanasia or after death of mares under field conditions

Katrin Hinrichs, DVM, PhD, DACT; Young-Ho Choi, DVM, PhD; Jody D. Norris, BS; Linda B. Love, MS; Sylvia J. Bedford-Guaus, DVM, PhD, DACT; David L. Hartman, DVM; Isabel C. Velez, DVM, MS

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Objective—To evaluate the efficiency of foal production following intracytoplasmic sperm injection (ICSI) and blastocyst culture of oocytes from mares that died or were euthanized under field conditions.

Design—Prospective case series.

Animals—16 mares (age, 3 to 19 years) that died or were euthanized for various causes.

Procedures—Ovaries were collected immediately before euthanasia (n = 10) or after death (6). Ovaries were transported to the laboratory for oocyte recovery (15 mares), or oocytes were recovered at a remote location and shipped to the laboratory (1). Oocytes underwent ICSI, and presumptive zygotes were cultured for 7 to 10 days. Blastocysts were shipped to embryo transfer facilities for transcervical transfer to recipient mares.

Results—Ovaries were processed 30 minutes to 12 hours (mean ± SD, 4.6 ± 3.3 hours) after mares' deaths. A mean of 14.1 ± 8.6 oocytes/mare were cultured, and 110 of 225 (49%) matured. Twenty-one blastocysts developed after ICSI and were transferred to recipient mares. Thirteen pregnancies were established; 10 healthy foals were produced from 6 donor mares. The number of blastocysts produced per mare and number of live foals produced per mare were significantly correlated with the number of oocytes recovered.

Conclusions and Clinical Relevance—Foals were produced from mares after death or euthanasia under field conditions. Proportions of foals born overall (10 foals/16 mares) and mares from which ≥ 1 foal was produced (6/16) were greater than those reported following recovery and oviductal transfer of oocytes to inseminated recipients after death of donor mares under field conditions. (*J Am Vet Med Assoc* 2012;241:1070–1074)

The current success of ICSI and in vitro embryo production[1,2] leads to the possibility of use of these techniques to produce embryos and foals from oocytes collected from valuable mares after death. Much research on in vitro equine embryo production has been conducted with oocytes recovered from mares after death in slaughterhouses[1,3–5]; however, work with clinical patients involves additional factors, including the cause of death or reasons for euthanasia, duration of illness, medications received, method of euthanasia, conditions of ovary transport, and interval between death of the mare and receipt of ovaries by the laboratory. One report[6] is available, which incorporates data from an earlier report,[7] on production of foals from clinical patients via in vitro maturation and transfer (surgical transfer to the oviduct of inseminated recipient mares) of oocytes collected after natural death or euthanasia of the donor mare. In that report,[6] oocyte transfer resulted in 8 foals or advanced pregnancies (> 70 days at the time of the report) from 25 donor mares; at least 1 foal or advanced pregnancy was produced for 6 of 25 (24%) mares.

Abbreviations

ICSI	Intracytoplasmic sperm injection
M199	Medium 199

Transfer of oocytes to the oviducts of recipient mares may provide an optimal environment for embryo development, but use of this method is problematic when multiple oocytes are available for transfer, as in the situation when oocytes are recovered after death. A mean recovery of 11 oocytes/mare after death has been reported.[6] The expense, effort, and animal-use issues involved in performing the required surgeries often result in transfer of multiple oocytes to the oviducts of individual recipient mares. In a study by Carnevale et al,[6] up to 14 oocytes were transferred to the oviduct of each recipient mare, with up to 8 conceptuses detected ultrasonographically in individual recipients and presence of multiple conceptuses resulting in embryo loss.

From the Department of Veterinary Physiology and Pharmacology, College of Veterinary Medicine and Biomedical Sciences, Texas A&M University, College Station, TX 77843 (Hinrichs, Choi, Norris, Love, Velez); the Department of Clinical Sciences, College of Veterinary Medicine, Cornell University, Ithaca, NY 14853 (Bedford-Guaus); and Hartman Equine Reproduction Center, 15225 US Hwy 377, Whitesboro, TX 76273 (Hartman).

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Address correspondence to Dr. Hinrichs (khinrichs@cvm.tamu.edu).

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The use of ICSI, followed by in vitro culture to the blastocyst stage (when the embryo can be transferred transcervically to a recipient mare), is an alternative approach to the production of foals after death of a mare. Use of in vitro embryo production would eliminate the need for surgical transfer and permit transcervical transfer of each blastocyst to a separate recipient mare, thus providing the potential to produce a foal from every competent oocyte. However, this method may not support as high a pregnancy rate as would surgical oocyte transfer to the oviduct. For example, oocytes recovered from preovulatory follicles after gonadotropin stimulation yield a high rate of pregnancy (75% to 83%) after transfer to the oviduct[8–10] but have a much lower rate of blastocyst development (29% to 41%) following ICSI and in vitro culture.[11,12] In addition, not all of the blastocysts transferred to the uteri of recipient mares will establish pregnancy, potentially lowering pregnancy rates still further when this method is used. Thus, it is not clear whether this is an efficient method for producing foals after death of a mare. To the best of our knowledge, there are currently no reports regarding the use of this method to produce foals after the death of mares in clinical cases. The purpose of the study reported here was evaluate the efficiency of foal production following ICSI and in vitro culture to the blastocyst stage of oocytes from mares that died or were euthanized under field conditions.

Materials and Methods

Animals—Sixteen privately owned mares of various breeds (mean ± SD age, 13 ± 4.9 years [range, 3 to 19 years]) that died or were euthanized in the field between April 13, 2006, and October 15, 2009, were donors for oocytes in the study, at the owners' request. Reasons for euthanasia included colic (n = 3), laminitis (4), fractured limb (2), tendon injury and infection (1), joint infection and colitis (1), mandibular fracture with subsequent cecal rupture (1), postpartum uterine tear (1), and necrotizing bronchitis (1). Another 2 mares that had colic died during anesthesia for surgery or during postoperative recovery.

Procedures—Ovaries were excised under general anesthesia immediately prior to euthanasia in 10 mares. Nine of these were anesthetized via IV administration of a combination of ketamine and xylazine; ovaries of the remaining mare were removed during colic surgery under general inhalation anesthesia. Specific information regarding anesthetic doses and method of euthanasia after ovary collection was not available for these horses. Ovaries were collected after death in the remaining 6 mares; these included the 2 horses that died during or after colic surgery, 3 that were euthanized via barbiturate administration, and 1 for which the method of euthanasia was not recorded.

Ovaries collected from 15 of the 16 mares were packaged and shipped to the Equine Embryo Laboratory at Texas A&M University. Ovaries that were expected to arrive at the laboratory ≤ 2 hours after collection were placed in a plastic bag with or without sterile saline (0.9% NaCl) solution and packaged in a container (closed-cell extruded polystyrene foam box, insulated cooler, or passive heat-exchange device[a]) with ballast (1 to 3 L of fluid [water in a bottle or bag or bags of fluid manufactured for IV use]) at approximately 37°C; those expected to arrive > 2 hours after collection were packaged similarly but at less than or equal to room temperature (18° to 23°C). Oocytes were collected from the ovaries of 1 mare by the attending veterinarian after excision, and the isolated oocytes were placed in a modified M199 tissue culture medium (40% M199 with Hanks salts and HEPES, 40% M199 with Earle salts, and 20% fetal bovine serum)[13] at room temperature and shipped to the laboratory overnight.

Oocytes were recovered from ovaries by opening the ovarian follicles and scraping the granulosa layer with a bone curette, as described elsewhere.[14] Cells were washed from the curette into Petri dishes with a solution of M199 with Hanks salts and HEPES.[b] Recovered oocytes were evaluated with a dissecting microscope at 10 to 60X magnification, and degenerating oocytes (ie, cells that had misshapen or shrunken cytoplasm or lacked an ooplasmic membrane) were discarded. Nondegenerating oocytes were cultured for maturation in vitro for 27 to 30 hours in M199 with Earle salts[b] with 10% fetal bovine serum[b] and 5 mU/mL of follicle-stimulating hormone[c] at 38.2°C in 5% CO_2 in air as described elsewhere.[1]

After culture, oocytes were denuded of cumulus, and those with visible polar bodies underwent ICSI with fresh-cooled or previously frozen and thawed sperm collected from the stallion of the client's choice. Sperm injection was performed as described, with a Piezo drill.[13] The resulting presumptive zygotes were cultured in Dulbecco modified Eagle medium/nutrient F-12 Ham[d] with 10% fetal bovine serum at 38.2°C in an atmosphere of 5% CO_2, 5% O_2, and 90% N_2. Presumptive embryos were evaluated on days 7 to 10 after ICSI for blastocyst development. Embryos with organization of an outer presumptive trophoblast layer with decreasing density of inner cells were considered to be blastocysts.

Blastocysts produced were placed in 1.1 mL of equilibrated Dulbecco modified Eagle medium/nutrient F-12 Ham supplemented with 10% fetal bovine serum at 38.2°C in a nominal 1-mL glass vial. Vials were wrapped in a 120-mL ballast bag at 38.2°C and placed in a passive heat-exchange device[a] in which the container coolant cans had been warmed to 38.2°C. The package was shipped by air or driven by car to the embryo transfer program of the client's choice for transcervical transfer of embryos to recipient mares (1 embryo/recipient). Follow-up information regarding detection of pregnancy and abortion in recipient mares and delivery and health status of live foals was obtained from owners and attending veterinarians via telephone or email.

Statistical analysis—Descriptive data (proportions, mean ± SD, and range) are reported. Differences in blastocyst production and foal production between categories of age, underlying condition (acute vs chronic), method of ovary removal, and time to receipt of ovaries were evaluated via Fisher exact tests with a statistical computer program.[e] Correlations between the number of oocytes recovered and number of blastocysts or foals produced from each donor mare were determined via linear regression analysis after normality was confirmed via a Shapiro-Wilk test with a statistical software package.[f] Values of $P < 0.05$ were accepted as significant.

Results

Blastocyst production and foal production for donor mares were summarized **(Table 1)**. The interval between death of the mare and ovary processing for oocyte recovery ranged from 30 minutes to 12 hours (mean, 4.6 ± 3.3 hours). One of 16 mares had no oocytes recovered. In total, 282 oocytes were recovered (mean, 17.6 ± 9 oocytes/mare; range, 0 to 35 oocytes/mare). Of these, 225 oocytes (mean, 14.1 ± 8.6 oo-

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Table 1—Summary of blastocyst and foal production for 16 mares from which ovaries (n = 15) or oocytes (1) were collected during general anesthesia before euthanasia (10) or after death (6) and transferred to the Equine Embryo Laboratory at Texas A&M University for ICSI and subsequent blastocyst culture.

	No. of mares			
	Total	Blastocysts not produced (n = 6)	Blastocysts produced (n = 10)	
Variable			Foal not produced (n = 4)	Foal produced (n = 6)
Age (y)				
3–11	**4**	1	1	2
12–15	**8**	4	2	2
16–19	**4**	1	1	2
Underlying condition*				
Acute (≤ 2 wk)	**9**	2	3	4
Chronic (> 2 wk)	**7**	4	1	2
Ovary removal				
Before euthanasia	**10**	3	3	4
After euthanasia	**4**	2	1	1
After death, no euthanasia	**2**	1	0	1
Time from mare death to receipt of tissues (h)†				
Ovaries				
≤ 2	**4**	2	0	2
3–4	**4**	3	1	0
5–7	**5**	0	3	2
9–12	**2**	1	0	1
Oocytes				
20	**1**	0	0	1
No. of viable oocytes recovered†				
0	**1**	1	0	0
4–9	**4**	2	1	1
11–16	**7**	3	3	1
20–32	**4**	0	0	4

Cultured oocytes underwent ICSI, and presumptive zygotes were cultured for 7 to 10 days. Blastocysts were shipped to embryo transfer facilities for transfer to recipient mares.

*Acute conditions included fractured limbs (n = 2), colic (5), postpartum uterine tear (1), and laminitis (1). Chronic conditions included joint infection and colitis (n = 1), laminitis (3), tendon injury with infection (1), necrotizing bronchitis (1), and mandibular fracture with a subsequent cecal rupture (1). †No mares had values between the indicated categories.

cytes/mare; range, 0 to 32 oocytes/mare) appeared viable and were cultured for maturation. The proportion of degenerating oocytes ranged from 0 of 11 (in a mare with joint infection and colitis) to 12 of 16 (in a mare with necrotizing bronchitis). One hundred ten of 225 (49%) oocytes matured to metaphase II.

After ICSI and culture, 21 of 110 (19.1%) injected oocytes yielded blastocysts (mean, 1.3 ± 1.3 blastocysts/mare; range, 0 to 4 blastocysts/mare). These 21 blastocysts originated from 10 donor mares. After transfer to recipient mares, 13 of 21 (62%) blastocysts established pregnancy. Two pregnancies were aborted before 3 months of gestation, and 1 was aborted between 3 and 6 months of gestation. Ten healthy foals were produced from 6 donor mares. The number of foals from each mare was 1 (3 mares), 2 (2 mares), or 3 (1 mare).

The 6 mares from which foals were produced ranged from 6 to 19 years of age. Ovaries from these mares were collected prior to euthanasia for various causes under general inhalation (n = 1) or IV (3) anesthesia, < 15 minutes after death during general inhalation anesthesia for surgery (1), or 3 hours after euthanasia via barbiturate administration (1). Ovaries were shipped to our laboratory for oocyte collection from 5 of these 6 mares; time from death of the mare to processing of the ovaries ranged from 30 minutes to 9 hours. A healthy foal was also produced from the mare that had oocytes recovered by the attending veterinarian; ovaries were recovered 3 hours after euthanasia of the mare, and oocytes were isolated immediately afterward and received at the laboratory > 20 hours after death of this mare.

The mare from which 3 foals were obtained was 6 years old and had been treated for > 1 month for a tendon injury and infection; ovaries were recovered under general (IV) anesthesia and were transported to the laboratory (duration, 1 hour). Thirty-five oocytes were recovered, of which 32 were viable and 20 matured in vitro. Four blastocysts were produced and transferred, resulting in 3 pregnancies and 3 live foals.

An additional 4 mares that had blastocysts produced without live foal production ranged in age from 7 to 16 years. Ovaries from these mares were collected under general (IV) anesthesia (n = 3) or 1 hour after euthanasia via barbiturate administration (1), and time from death to ovary processing ranged from 3.5 to 7 hours.

There were no significant differences in blastocyst or foal production among mares grouped according to age, underlying condition (acute vs chronic), ovary removal categories, or time from death to receipt of the tissues for processing. The number of blastocysts produced per mare was significantly ($P < 0.01$) correlated with the number of oocytes recovered ($r^2 = 0.45$). Similarly, the number of foals produced per mare was significantly ($P < 0.05$) correlated with the number of oocytes recovered ($r^2 = 0.32$). In general, excluding 1 mare from which no oocytes were collected, blastocyst production rates per mare were 0.6 (3 blastocysts/5 mares), 1.5 (9 blastocysts/6 mares), and 2.25 (9 blastocysts/4 mares) for mares that had 8 to 12, 15 to 23, and 26 to 35 total oocytes collected, respectively (no mare had oocyte counts between these categories). Similarly, foal production rates per mare were 0.2

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(1 foal/5 mares), 0.67 (4 foals/6 mares), and 1.25 (5 foals/4 mares) for mares in these 3 categories, respectively.

Discussion

Results of the study reported here demonstrate that ICSI and culture to the blastocyst stage of oocytes from ovaries of mares that died or were euthanized under field conditions can result in pregnancy and live foal delivery following transcervical embryo transfer to recipient mares. In the present study, 10 healthy foals were produced from 6 of 16 donor mares. The overall proportions of foals born (10 foals/16 mares) and mares from which ≥ 1 foal was produced (6/16) were greater than those reported in an earlier study[6] in which oocytes were recovered after death of mares under field conditions and surgically transferred to the oviducts of inseminated recipients (8 foals or ongoing pregnancies/25 donor mares; ≥ 1 foal produced from 6/25). The higher proportions of foals produced in the present study may have been attributable to the ability to transfer each blastocyst to a separate recipient mare, thus eliminating the potential for multiple conceptuses in 1 recipient mare and associated pregnancy loss.

The number of oocytes recovered was directly correlated with blastocyst and foal production in the present study, indicating that it is important to ensure that every possible follicle is scraped efficiently for optimal oocyte recovery. No blastocysts were produced from 6 of 16 donor mares; however, a mean of 2.1 blastocysts/mare were produced from the remaining 10 mares. Thus, the developmental capacity of oocytes recovered may differ from mare to mare, according to variables associated with the mare's illness and the handling of the ovaries. Within the parameters of the present study, there was no significant relationship between success of the procedure and mare age, underlying condition, ovary removal categories, or time from mare death to receipt of the tissues for processing. Foals were produced from oocytes recovered from ovaries of mares up to 19 years of age and from ovaries received at the laboratory up to 9 hours after removal from the mare. Foals were produced from mares that died during inhalation anesthesia, from mares euthanized via barbiturate overdose, and from mares that had ovaries removed under general (IV or inhalation) anesthesia immediately before euthanasia. For the mare that had oocytes recovered by the attending veterinarian and shipped overnight to the laboratory, 2 blastocysts were produced, resulting in 1 healthy foal.

The number of oocytes recovered from mares in the present study, a mean of almost 18 oocytes/mare, was higher than that obtained in our laboratory when mare ovaries obtained from slaughterhouses were used (typically approx 6 oocytes/pair of ovaries[1,15]). This may be attributable to closer attention to detail when only 1 pair of ovaries is processed; a greater number of oocytes may be collected from small follicles (< 5 mm in diameter) under these conditions. The proportion of degenerating oocytes in the present study (57/282 [20%]) was higher than expected when slaughterhouse tissue is processed (5% to 7%),[16] and the proportion of viable oocytes that matured to metaphase II (110/225 [49%]) was lower than expected; this may also be related to the recovery of oocytes from smaller follicles because these have a higher incidence of atresia and a lower degree of meiotic competence.[17] The higher degeneration and lower maturation rates may also have been related to factors such as the duration and nature of the mare's illness, medications received, method of euthanasia, duration of ovary transport, or temperature at which ovaries were kept during transportation.

Similarly, the proportion of oocytes from which blastocysts were produced following ICSI and culture (21/110 [19%]) was lower than that typically achieved in our laboratory with mare ovaries collected at slaughterhouses (25% to 35%[1,13,16,18]). This result may also have been influenced by mare condition, tissue handling, use of oocytes from small follicles, or use of oocytes that may have been marginal in quality.

One of the major shortcomings of the present study was the inability to standardize ovary collection and transport conditions because the ovaries were collected by attending veterinarians, packaged using available resources, and shipped across different distances by various means before receipt. In addition, we could not control the conditions for transfer of embryos to recipient mares because embryos were shipped for various distances (transport time, up to 10 hours) to the transfer facility of the client's choice. Control of these factors would better enable evaluation of the relationship of mare status and ovary handling methods to the success of postmortem production of embryos and foals via ICSI and embryo culture.

Collection of oocytes from ovaries at the mare's location followed by shipment of isolated oocytes to an in vitro fertilization laboratory may allow for production of foals from mares that die in locations far away from the laboratory. Storage of immature oocytes overnight in the described modified tissue culture medium[13] at room temperature, as performed following the death of 1 mare in the present study, has been shown to have no detrimental effect on oocyte maturation or blastocyst production rates after ICSI,[13] whereas storage of ovaries for > 7 hours before oocyte collection was associated with lower oocyte maturation and blastocyst production rates, compared with results from ovaries that were stored 3.5 to 7 hours before oocyte collection.[16] In the present study, oocytes that were recovered at the mare's location and shipped overnight to our laboratory were processed > 20 hours after the mare's death, but blastocyst production was achieved, and 1 live foal was produced following embryo transfer. Methods for oocyte collection have recently been reviewed[19]; oocyte collection could be performed in veterinary practices equipped for this procedure, and oocytes could be sent overnight to laboratories that perform ICSI and embryo culture.

Results of the study reported here indicate that in vitro embryo production can be used to obtain foals from mares when ovaries are collected immediately before euthanasia or after death under field conditions. Further research is needed to determine the effects of individual mare factors and ovary handling variables on oocyte maturation, embryo development, and production of live foals.

a Equitainer, Hamilton Research Inc, South Hamilton, Mass.
b. Invitrogen, Carlsbad, Calif.
c. Sioux Biochemicals, Sioux Center, Iowa.
d. Sigma-Aldrich Corp, St Louis, Mo.
e. Epistat, Tracy Gustafson, Round Rock, Tex.
f. SigmaPlot for Windows, version 11.0, Systat Software Inc, Chicago, Ill.

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Correction: Impact of the Purdue University School of Veterinary Medicine's Boiler Vet Camp on participants' knowledge of veterinary medicine

In the article "Impact of the Purdue University School of Veterinary Medicine's Boiler Vet Camp on participants' knowledge of veterinary medicine" (*J Am Vet Med Assoc* 2011;238:878–882), the McNemar test, rather than the Fisher exact test, should have been used to analyze categorical responses to questionnaires administered before and after camp participation (ie, days 1 and 6).

Reanalysis of the data with the McNemar test yielded minor changes in the results. In the section "Increase understanding of career options in the veterinary medical and veterinary technology professions," there were significant ($P \leq 0.045$) increases between day 1 and day 6 in the percentages of male campers who correctly answered all 4 questions, not just for 3 of the 4 questions as reported. For 3 of the 4 questions, rather than all 4 questions as reported, percentages of Caucasian campers who correctly answered increased significantly ($P < 0.001$) between day 1 and day 6. For all 4 questions, the percentages of underrepresented minority (URM) campers who correctly answered significantly increased between day 1 and day 6, but the *P* value was $P < 0.023$, rather than $P \leq 0.013$ as reported.

In the section "Increase appreciation and understanding of the science of veterinary medicine," for 10 of the 12 questions designed to evaluate specific knowledge of the science of veterinary medicine, there were still significant increases in percentage of correct responses between day 1 and day 6, but the *P* value was $P < 0.006$, rather than $P \leq 0.025$ as reported. The percentage of female campers who correctly answered that an ECG displays electrical activity of the heart significantly increased, but the *P* value was $P < 0.023$, rather than $P \leq 0.045$ as reported. Female campers had a significant ($P \leq 0.027$) increase between day 1 and day 6 in the percentage who correctly listed the two main parts of the chicken egg, and male campers did not have a significant increase between day 1 and day 6 in the percentage who correctly answered the question on how much water a group of 10 cows would drink in a day. Results for Caucasian and URM campers remained the same, with one exception. Finally, the percentage of URM campers who correctly answered the question regarding the name for a female pig significantly ($P \leq 0.041$) increased between day 1 and day 6.

Reanalysis of the data affected a single sentence in the Discussion section. The third sentence in the second paragraph in this section should read: Significant increases between day 1 and day 6 in the percentage of correct answers were detected for 10 of the 12 questions for all campers, 11 of the 12 questions for female campers, 7 of the 12 questions for male campers, 9 of the 12 questions for Caucasian campers, and 6 of the 12 questions for URM campers.

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