

# Production of Nuclear Transfer Horse Embryos by Piezo-Driven Injection of Somatic Cell Nuclei and Activation with Stallion Sperm Cytosolic Extract<sup>1</sup>

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## ABSTRACT

We investigated the use of direct nuclear injection using the Piezo drill and activation by injection of stallion sperm cytosolic extract for production of cloned equine embryos. When metaphase II horse oocytes were injected with either of two dosages of sperm extract and cultured 20 h, similar activation rates (88% vs. 90%) and cleavage rates (49% vs. 46%) were obtained. The successful reconstruction rate of horse oocytes with horse somatic cell donor nuclei after direct injection using the Piezo drill was 82%. Four dosages of sperm extract (containing 59, 176, 293, or 1375 µg/ml protein) and two activation times (1.5–2 vs. 8–10 h after nuclear transfer) were examined. Cleavage and activation (pseudopronucleus formation) rates of oocytes injected with sperm extract containing 59 µg/ml protein were significantly ( $P < 0.05$ ) lower than any other dosage. The percentage of embryos cleaving with normal nuclei in oocytes injected with the 1375 µg/ml preparation 1.5–2 h after donor injection was significantly ( $P < 0.05$ ) higher than that of the 293 µg/ml preparation 8–10 h after donor injection (22 vs. 6%). Embryos developed to a maximum of 10 nuclei. Interspecies nuclear transfer was performed by direct injection of horse nuclei into enucleated bovine oocytes, followed by chemical activation. This resulted in 81% reconstruction (successful injection of the donor cell), 88% cleavage, and 73% cleavage with normal nuclei. These results indicate that direct nuclear injection using the Piezo drill is an efficient method for nuclear transfer in horse and cattle oocytes and that sperm extract can efficiently activate horse oocytes both parthenogenetically and after nuclear transfer

*assisted reproductive technology, early development, embryo, gamete biology, oocyte development*

## INTRODUCTION

Somatic cell nuclear transfer has been successfully performed in several species, including sheep [1], mice [2], cattle [3, 4], goats [5, 6], and pigs [7, 8]. To reconstruct recipient oocytes with somatic cell nuclei, inactivated Sendai virus, electrofusion, and intracytoplasmic direct nuclear injection have been used, with the majority of work being done by electrofusion. In cattle, the rate of reconstruction after electrofusion of host cytoplasts with somatic cells has ranged from 36% to 89% [3, 9–13]. These rates are lower

than those achieved when cloning with embryo-derived blastomeres in the same species (86%–99%) [10, 14]. The reported percentages of reconstruction with somatic cells by electrofusion in the pig are 59%–81% [15, 16] and in sheep are 63%–85% [1, 17]. When inactivated Sendai virus is used for fusion, relatively lower fusion rates were reported (43%–69%) [18, 19]. Cytoplasmic direct injection using the Piezo drill, which was introduced by Wakayama et al. [2], has produced a high reconstruction rate in mouse oocytes (79%–95%) [2, 20, 21].

Little information is available on nuclear transfer in the horse. There are no reports of nuclear transfer with embryonic blastomeres in this species. Electrofusion of horse oocytes with adult somatic donor cells has been reported in brief communications, with fusion rates from 20% to 67% [22–24]. In a recent presentation, a fusion rate of up to 82% was reported in equine oocytes fused with fetal or adult fibroblasts using electrofusion in combination with Sendai virus [25]. Direct injection of nuclei into the cytoplasm of equine oocytes, using a standard micropipette, has also been reported, with 13% to 30% reprogramming (criteria undefined) after activation [26]. In these studies, activation of reconstructed oocytes was achieved by treatment with a calcium ionophore followed by incubation with cycloheximide or 6-DMAP or by treatment with a calcium ionophore in combination with ethanol. Cleavage rates after nuclear transfer of adult fibroblasts to horse oocytes were low (0%–37%) [22–26].

Recently, our laboratory has reported a high rate of normal fertilization and cleavage (>70%) after intracytoplasmic sperm injection (ICSI) of horse oocytes using the Piezo drill [27]. This high rate of cleavage is in sharp contrast with disappointing cleavage rates achieved in reconstructed embryos using chemical methods of activation, as described above, or in metaphase II (MII) oocytes after chemical activation [28]. Because sperm appear to be efficient activators of horse oocytes, we examined the activation of reconstructed horse and bovine oocytes with stallion sperm that had been treated with Hoechst 33342 and exposed to UV light to inhibit sperm involvement in the reconstructed embryo (unpublished data). The nuclear decondensation rates of horse and bovine oocytes reconstructed with horse somatic cell nuclei and injected with horse sperm were 37% (13/35) and 77% (24/31), respectively. This suggested that factor(s) from sperm have the ability to stimulate activation of bovine or horse reconstructed oocytes. However, after injection, some oocytes possessed a decondensed sperm head, or two or three pronuclei. An alternative to use of an intact sperm for activation is the use of cytosolic sperm extract. Sperm extract has been used to parthenogenetically activate MII mammalian oocytes [29–31]. In one study, sperm extract was used to activate bovine oocytes after nu-

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clear transfer, with lower efficiency than that achieved with chemical activation [32]. There are no previous reports on the use of sperm extract for activation of horse oocytes.

We report here the results of reconstruction of horse oocytes using direct nuclear injection with a Piezo-driven pipette. Further, we produced a crude sperm cytosolic extract from stallion sperm and investigated the dose effect of this sperm extract on parthenogenetic activation of horse oocytes and on activation of horse oocytes reconstructed with horse somatic cell nuclei. Because of poor development achieved after nuclear transfer using horse oocytes, we validated the Piezo-driven direct injection technique by performing transfer of horse somatic cell nuclei into bovine host cytoplasts and monitoring their development. Bovine cytoplasts have previously been shown to develop to the 8- to 16-cell stage after electrofusion with horse somatic cell nuclei [22].

## MATERIALS AND METHODS

### *Oocyte Collection*

Ovaries were transported from slaughterhouses to the laboratory at room temperature (3- to 4-h transport time). Adnexa were trimmed from the ovaries with scissors and the ovaries were cleaned with sterilized gauze. All visible follicles were opened with a scalpel blade and the granulosa layer of each follicle was scraped using a 0.5-cm bone curette. The contents of the curette were washed into individual Petri dishes with Hepes-buffered TCM199 with Hanks salts (H-TCM199; Gibco Life Technologies, Inc., Grand Island, NY) plus ticarcillin (0.1 mg/ml; SmithKline Beecham Pharmaceuticals, Philadelphia, PA). The contents of the Petri dishes were examined using a dissection microscope at 10–20 $\times$ . Oocyte-cumulus complexes were classified as compact, expanded, or degenerating depending on the expansion of both mural granulosa and cumulus as described previously [33, 34]. Oocytes with any sign of expansion of either the cumulus or the mural granulosa, from having individual cells visible protruding from the surface to having full expansion with copious matrix visible between cells, led to the classification of expanded (Ex). Oocytes having both compact cumulus and compact mural granulosa were classified as compact (Cp). Ex oocytes were considered to have higher potential meiotic and developmental competence than Cp oocytes [33, 34] and were used for the nuclear transfer studies. Cp oocytes were used for evaluation of parthenogenetic activation.

### *In Vitro Maturation*

Oocytes were washed twice in maturation medium (TCM199 with Earle salts [Gibco], 5  $\mu$ U/ml FSH [Sioux Biochemicals, Sioux Center, IA], 10% fetal bovine serum [Gibco], and 25  $\mu$ g/ml gentamycin [Gibco]). Oocytes were cultured in droplets of maturation medium at a ratio of 10  $\mu$ l medium per oocyte under light white mineral oil (Sigma Chemical Co., St. Louis, MO) at 38.2°C in 5% CO<sub>2</sub> in air for 24–26 h. After maturation, oocytes were denuded of cumulus by pipetting in a solution of 0.05% hyaluronidase in H-TCM199 with 5% FBS. Denuded oocytes were selected for presence of a polar body. Oocytes not having a polar body were fixed in buffered formal saline, mounted on a slide with 6.5  $\mu$ l of 9:1 glycerol:PBS containing 2.5  $\mu$ g/ml Hoechst 33258, and examined using fluorescence microscopy to determine the chromatin configuration.

### *Preparation of Stallion Sperm Cytosolic Extract*

Stallion sperm cytosolic extracts were prepared from sperm from one stallion as described previously for the mouse [30] with modifications. Ejaculated stallion sperm were centrifuged at 900  $\times$  g for 10 min to remove seminal plasma. The pellet was then suspended in Sperm-TALP (Sp-TALP) containing 6 mg/ml bovine serum albumin [35] and centrifuged at 900  $\times$  g for 10 min. The resulting pellet was resuspended to a final concentration of either 5 or 20  $\times$  10<sup>8</sup> sperm/ml in nuclear isolation medium (NIM: 125 mM KCl, 2.6 mM NaCl, 7.8 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.4 mM KH<sub>2</sub>PO<sub>4</sub>, 3.0 mM EDTA disodium salt; pH 7.45) [36] and centrifuged to remove Sp-TALP. The pellet was then resuspended to the same volume with NIM containing 1 mM dithiothreitol, 100  $\mu$ M leupeptin, 100  $\mu$ M antipain, and 100  $\mu$ g/ml soybean trypsin inhibitor. The suspension was subjected to four cycles of freezing (5 min per cycle in liquid N<sub>2</sub>) and thawing (5 min per

cycle at 15°C), then sperm were pelleted at 20 000  $\times$  g for 50 min at 2°C. The resultant supernatant was carefully removed, aliquoted, and kept at –80°C until used. The amount of protein in the sperm extracts was analyzed by Coomassie dye binding.

### *Preparation of Donor Cells*

Fibroblast cells were obtained from an oral mucosa biopsy specimen from a 4-yr-old mare. Pieces of tissue were minced and placed in a flask with DMEM/F-12 (Sigma) supplemented with 10% FBS and 1% antimicrobials (10 000 U/ml penicillin G, 10 mg/ml streptomycin, and 25  $\mu$ g/ml amphotericin B; Sigma). Cells were cultured in 5% CO<sub>2</sub> at 37 to 38.2°C until fibroblast cells became confluent and were passaged by trypsinization. For this experiment, cells at passage 3–7, grown to confluence without serum starvation, were used. Cells were trypsinized before use and held in H-TCM199 plus 2%–10% polyvinylpyrrolidone (PVP; Sigma) for nuclear transfer by direct injection.

### *Statistical Analysis*

Differences among groups were evaluated using chi-square analysis, with Fisher exact test used when the expected value for any parameter was less than five. The numbers of embryonic nuclei at 96 h were compared among treatments by analysis of variance.

### *Parthenogenetic Activation of Cp Oocytes with Sperm Extract*

Matured horse Cp oocytes having a first polar body were selected. Injection of sperm extract was performed using a Piezo drill (Burleigh Instruments Inc., Fishers, NY). For injection, supernatants from the 5 and 20  $\times$  10<sup>8</sup> sperm/ml preparations were diluted 1:1 in NIM plus 20% PVP. Oocytes were held in H-TCM199 with 10% FBS with a holding pipette (120–140- $\mu$ m outer diameter) under an inverted microscope equipped with Narishige manipulators. The diluted sperm extract was injected using a pipette with an inner diameter of approximately 5  $\mu$ m, and the injection volume (2–4  $\mu$ l) was controlled by the movement of mercury within the pipette [31]. Injected oocytes were held for 20 min at room temperature in the same medium to heal the broken membrane slowly. Oocytes were transferred into G1.2 medium (G1.2/G2.2, IVF Science, Denver, CO) at a ratio of 10  $\mu$ l medium/oocyte and were incubated at 38.2°C under 5% CO<sub>2</sub> in air. At 20 h postinjection, oocytes were fixed, stained, and evaluated as described above. Oocytes in anaphase II—metaphase III (metaphase plate with two polar bodies) were considered to be activated but arrested in development (having incomplete activation). Oocytes having one to three pronuclei, syngamy, first mitotic figure, or cleavage with presence of nuclei in each blastomere, with one or two polar bodies, were considered fully activated.

To determine whether injection with the sperm extract was compatible with further development, we subsequently activated a small number of Cp oocytes parthenogenetically by injection of supernatant from the 20  $\times$  10<sup>8</sup> sperm/ml preparation, diluted 1:1 as described above, followed by incubation in TCM199 with 10% FBS containing 5  $\mu$ g/ml cytochalasin B (Sigma) for 3 h, and cultured the injected oocytes in G1.2 medium as described above but for 96 h.

### *Nuclear Transfer by Direct Injection and Activation with Sperm Extract*

Matured Ex oocytes were selected for presence of a first polar body and were incubated for 10 min in TCM199 with 10% FBS containing 5  $\mu$ g/ml Hoechst 33342 (Sigma) and 5  $\mu$ g/ml cytochalasin B. Oocytes were then held under an inverted microscope as described above. The zona pellucida of the oocyte was drilled using an enucleation pipette (10- to 13- $\mu$ m outer diameter) attached to a Piezo drill (Prime Tech Ltd., Ibaraki, Japan), and the polar body and metaphase plate were aspirated into the enucleation pipette. After enucleation, the resulting cytoplasts were held in TCM199 plus 10% FBS. The injection of fibroblast cells into the enucleated horse oocytes was modified from the method described by Kimura and Yanagimachi [37], using the Piezo drill. The outside diameter of the injection pipette was 8–9  $\mu$ m. Immediately before injection, a somatic cell held in H-TCM199 plus 2% PVP was gently aspirated in and out of the injection pipette until the cell membrane was broken. Donor cell injection was carried out in a 100- $\mu$ l drop of H-TCM199 containing 0.1% polyvinylalcohol. Reconstructed oocytes were held at 38.2°C in TCM199 plus 10% FBS in 5% CO<sub>2</sub> in air before activation.

TABLE 1. Parthenogenetic activation of horse oocytes injected with sperm extract and cultured in G1.2 medium for 20 h.

Sperm extract protein concentration ( $\mu\text{g/ml}$ )	No. of oocytes examined	No. of oocytes (%) activated with				
		MIII <sup>a</sup>	1-3 PN	First mitosis	Cleaved	Total
293	41	3	9	4	20 (49%)	36 (88%)
1375	39	4	4	9	18 (46%)	35 (90%)

<sup>a</sup> MIII, Metaphase plate with two polar bodies; PN, pronucleus.

Supernatants from the  $5 \times 10^8$  and  $20 \times 10^8$  sperm/ml aliquots were used to prepare sperm extract for oocyte injection. The  $5 \times 10^8$  preparation was diluted 1:9, 1:2.3, or 1:1 with NIM plus 11%–20% PVP to make solutions equivalent to 0.5, 1.5, and  $2.5 \times 10^8$  sperm/ml, all with a final concentration of 10% PVP. The  $20 \times 10^8$  preparation was diluted 1:1 with 20% PVP to make a solution equivalent to  $10 \times 10^8$  sperm/ml. For activation, reconstructed horse oocytes were subjected to intracytoplasmic injection with 2–4  $\mu\text{l}$  of sperm extract solution. Oocytes were injected with sperm extract either 1.5–2 h after donor cell injection or 8–10 h after donor cell injection. The injection of sperm extract was conducted as described above for parthenogenetic activation.

Reconstructed, activated oocytes were cultured in a droplet of 10  $\mu\text{l}$  G1.2 medium per oocyte in 5%  $\text{CO}_2$  in air at 38.2°C for 96 h without a change of medium. Development of embryos was evaluated daily using a dissection microscope at 40–60 $\times$  magnification on a heated stage. At 48 h postactivation, noncleaved embryos were removed, fixed, and stained to examine their activation status. After 96 h of culture, embryos were fixed and stained as described above to examine the number and status of nuclei. Only nuclei that appeared to be normal were included in the nucleus number; nuclei showing signs of degeneration (vacuolization, condensation, or fragmentation) were disregarded.

To evaluate the possibility that injection of buffer alone was capable of causing activation of reconstructed oocytes, an additional study was conducted. After 24 h in vitro maturation, Ex oocytes were selected for presence of a polar body and were reconstructed with donor cells as described above. Reconstructed oocytes were randomly assigned to be injected with NIM buffer or with sperm extract ( $10 \times 10^8/\text{ml}$ ) 1.5–2 h after donor cell injection. Oocytes were cultured as above for 96 h after injection.

### Nuclear Transfer Using Bovine Host Cytoplasts

Bovine oocytes were purchased from Ovagenics (San Angelo, TX) and were cultured overnight in a portable incubator maintained at 39°C. On arrival at the laboratory, the glass tube containing the oocytes was uncapped and placed in an incubator in 5%  $\text{CO}_2$  in air until 22 h of IVM. Oocytes were then denuded by gentle pipetting in 0.05% hyaluronidase (Sigma) in H-TCM199 plus 5% FBS. Oocytes with a first polar body were selected and used for enucleation followed by direct nuclear injection. Direct injection with equine somatic cells was performed as described above for horse oocytes, using the Piezo drill, with the exception that H-TCM199 plus 10% PVP was used for donor cell holding and H-TCM199 plus 10% FBS for intracytoplasmic direct injection of the donor cell.

Activation of reconstructed bovine oocytes was performed by treatment with 10  $\mu\text{M}$  calcium ionophore A23187 (Sigma) in H-TCM199 without serum for 5 min on a heated stage. Oocytes were then washed in H-TCM199 plus 20% FBS and incubated for 8–10 h in 10  $\mu\text{g/ml}$  cycloheximide in TCM199 plus 10% FBS. Subsequently, the oocytes/embryos were cultured for 3–5.5 days in G1.2/2.2 media and were fixed and stained to examine numbers of nuclei as described above.

## RESULTS

Five hundred eighty horse ovaries were processed, and 3977 follicles were scraped, for an average of 6.9 follicles per ovary. We recovered 2092 oocytes, of which 628 were Cp, 1305 were Ex, and 159 were degenerating. Oocytes not used in this study were used in a separate project.

On analysis, the protein contents of the  $5 \times 10^8$  and  $20 \times 10^8$  sperm/ml preparations were found to be 585 and 2750  $\mu\text{g/ml}$ , respectively. Therefore, the protein contents of the diluted solutions used for injection (representing 0.5, 1.5, 2.5, and  $10 \times 10^8$  sperm/ml) were 59, 176, 293, and 1375  $\mu\text{g/ml}$ , respectively.

### Parthenogenetic Activation of Cp Oocytes with Sperm Extract

Four hundred seven Cp oocytes were placed into maturation culture for parthenogenetic activation with stallion sperm extract. Of these, 107 (26%) had a first polar body after a 42-h culture. One hundred five Cp oocytes were intact after injection of sperm extract and two lysed after injection. The activation rates of oocytes treated with the 293 or 1375  $\mu\text{g/ml}$  dosages of sperm extract were 88% and 90%, respectively (Table 1); these were not significantly different. The rates of cleavage with normal nuclei were 49% and 46%, respectively; these were also not significantly different. Photomicrographs of parthenogenetically activated oocytes in first mitosis and cleavage are presented in Figure 1, A and B.

Twenty-five additional MII Cp oocytes were parthenogenetically activated and then evaluated after the 96-h culture. Of these, 23 (92%) cleaved and 20 (80%) had normal nuclei. The average nucleus number (mean  $\pm$  SEM) was  $5.1 \pm 0.7$ . A parthenogenetic embryo with 13 nuclei is presented in Figure 1C.

### Nuclear Transfer by Direct Injection and Activation with Sperm Extract

Of 823 Ex oocytes examined after maturation for 24–26 h, 11 were broken during denuding and 812 were evaluated for presence of a polar body. Of these, 489 (60%) had a polar body and 488 were used for nuclear transfer. Of oocytes without polar bodies, 50 (6% of the cultured oocytes) were found to be in MI on fixation and staining and the remainder were degenerating.

The 488 mature Ex horse oocytes were subjected to enucleation and nuclear transfer with direct injection using the Piezo-driven pipette. Of these, 436 (89%) were successfully enucleated and 399 (82%) survived injection of the donor cell nucleus. Twelve oocytes lysed after injection of sperm extract for activation. The in vitro development of 293 reconstructed horse oocytes activated with different sperm extract concentrations is shown in Table 2. The remaining 94 reconstructed oocytes were used on separate projects. Cleavage and activation (pronucleus-like formation) rates of oocytes injected with the 59  $\mu\text{g/ml}$  preparation were significantly ( $P < 0.05$ ) lower than any other dosage. The percentage of embryos cleaving with normal nuclei in oocytes injected with the 1375  $\mu\text{g/ml}$  preparation 1.5–2 h after donor injection was significantly ( $P < 0.05$ ) higher than that of the 293  $\mu\text{g/ml}$  preparation 8–10 h after donor injection (22% vs. 6%). Embryos developed to a maximum of 10 nuclei (Fig. 2). There was no significant difference in average number of nuclei in embryos from the different sperm extract treatments.

Sixty-five additional reconstructed Ex oocytes were used to evaluate the effect of injection of buffer alone. Thirty-three were injected with NIM buffer and, of these, two (6%) were morphologically cleaved but without normal nuclei

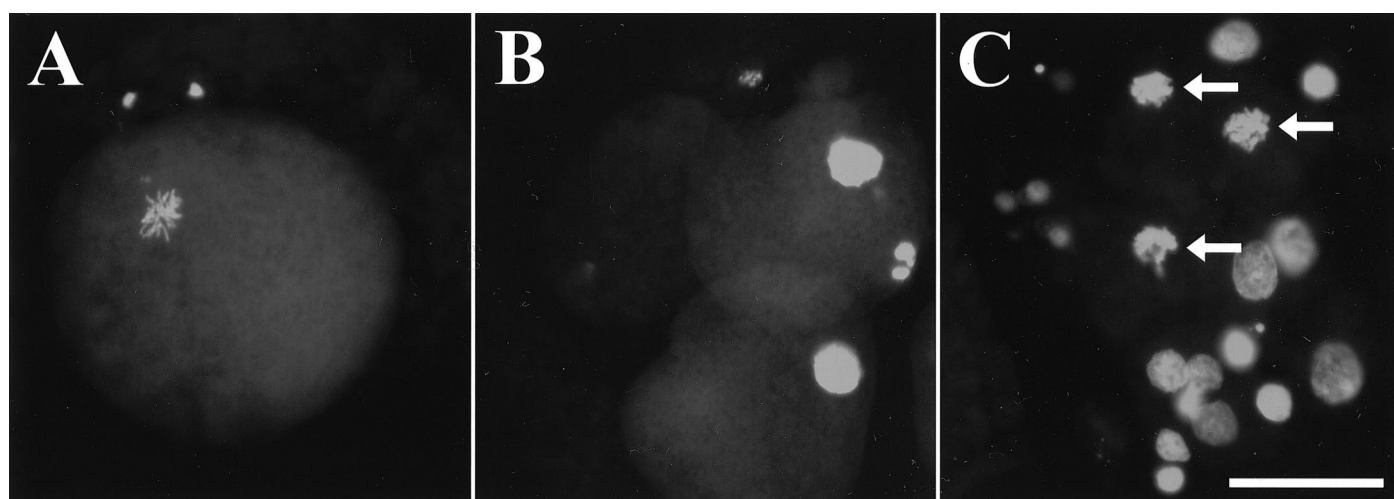


FIG. 1. Horse Cp oocytes parthenogenetically activated by injection of sperm extract, stained with Hoechst 33258. **A**) Mitotic figure of the first division, with two polar bodies 20 h after activation. **B**) Two-cell embryo with two nuclei 20 h postactivation. **C**) Cleaved embryo with 13 nuclei and 3 mitotic figures (arrow) after a 4-day culture in vitro. Bar = 50  $\mu$ m.

when examined at 96 h. Thirty-two reconstructed oocytes were activated with the 1375  $\mu$ g/ml sperm preparation and, of these, 19 (59%) cleaved and 4 (13%) cleaved with normal nuclei, with an average of  $2.8 \pm 0.3$  nuclei per embryo. Extract-injected oocytes had significantly higher rates of both morphological cleavage ( $P < 0.001$ ) and normal cleavage ( $P = 0.053$ ) than did oocytes injected with buffer.

#### Nuclear Transfer Using Bovine Host Cytoplasts

Two hundred eleven bovine oocytes were shipped, and 25 oocytes were lost during denuding. After denuding, 159 of 186 oocytes (85%) were selected for nuclear transfer. Ninety-three percent of bovine oocytes survived enucleation using the Piezo drill. The rate of reconstruction (successful injection of the horse donor cell) was 81%. After culture for 3–5.5 days, 72/82 (88%) of reconstructed oocytes cleaved morphologically (Fig. 3A) and 60 (73%) showed normal cleavage with nuclei present within the blastomeres. Thirty-four embryos (41% of reconstructed oocytes) had more than eight nuclei when assessed after Hoechst staining. An interspecies cloned embryo with 13 nuclei is shown in Figure 3B.

#### DISCUSSION

In this study, Piezo-actuated microinjection resulted in a higher rate of reconstruction of horse oocytes with horse somatic cell nuclei than we have previously found using

electrofusion (82% in this study vs. 20%–48% [22, 24]). This reconstruction rate is also higher than those previously reported in the horse by other laboratories, with the exception of use of electrofusion in combination with Sendai virus [25]. The reconstruction rate when bovine oocytes were injected with horse somatic cell nuclei in this study was higher than that reported by Dominko et al. [38] for most interspecies nuclear transfers. In mouse oocytes, Piezo-driven microinjection also produced higher reconstruction rates than did electric- or virus-mediated fusion [2, 18–20].

There have been only a few reports on methods of activation of horse oocytes either parthenogenetically [28, 39, 40] or after intracytoplasmic sperm injection [41]. In these reports, activation was attempted by exposure to chemicals, including ionomycin, ethanol, thimerosal, inositol 1,4,5-triphosphate, and calcium ionophore A23187, alone or in combination with cycloheximide or 6-DMAP. The highest rate of activation (52% pronucleus formation plus 24% first mitosis) was achieved by treatment with calcium ionophore A23187 followed by culture in cycloheximide [40]. In the present study, injection of sperm extract into MII horse Cp oocytes resulted in 88%–90% total parthenogenetic activation, including 46%–49% normal cleavage, after a 20-h culture.

When calcium ionophore A23187 and cycloheximide were used to activate reconstructed nuclear transfer (NT) horse Ex oocytes, only 29% activation and 9% cleavage were obtained [24]. In the present study, injection of sperm

TABLE 2. In vitro development of horse oocytes reconstructed with somatic cell donor nuclei and cultured for 96 h after activation with stallion sperm cytosolic extract.

Time of oocyte activation	Sperm extract protein concentration ( $\mu$ g/ml)	No. of oocytes cultured	No. (%) of oocytes cleaved	No. (%) of cleaved oocytes with normal nuclei	Average nuclei number (mean $\pm$ SEM)	No. (%) of activated oocytes
1.5–2 h after injection	59	45	5 (11) <sup>a</sup>	4 (9)	2.0 $\pm$ 0.4	11 (24) <sup>a</sup>
	176	49	19 (39) <sup>b</sup>	6 (12)	2.7 $\pm$ 0.3	26 (53) <sup>b</sup>
	293	55	26 (47) <sup>b</sup>	7 (13)	2.7 $\pm$ 0.6	39 (71) <sup>b</sup>
	1375	50	23 (46) <sup>b</sup>	11 (22) <sup>c</sup>	3.5 $\pm$ 0.7	28 (56) <sup>b</sup>
8–10 h after injection	293	47	20 (43) <sup>b</sup>	3 (6) <sup>d</sup>	3.3 $\pm$ 0.9	34 (72) <sup>b</sup>
	1375	47	24 (51) <sup>b</sup>	5 (11)	4.4 $\pm$ 1.4	27 (57) <sup>b</sup>

<sup>a,b</sup> a vs. b:  $P < 0.01$ .

<sup>c,d</sup> c vs. d:  $P < 0.05$ .

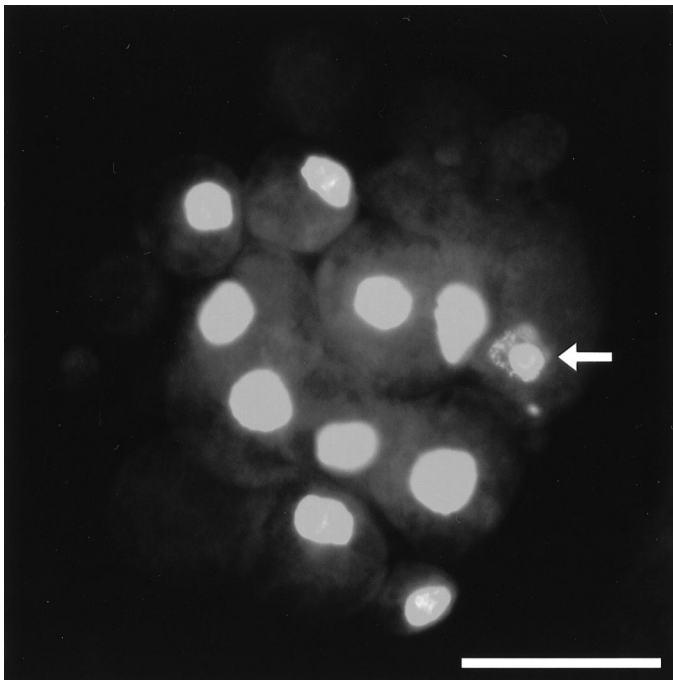


FIG. 2. Horse NT embryo after in vitro culture for 96 h in G1.2 medium, stained with Hoechst 33258. Ten normal nuclei and one abnormal nucleus (arrow) are present. Bar = 50  $\mu$ m.

extract was effective in activating reconstructed NT horse Ex oocytes, as a 72% activation rate (combined nuclear decondensation and cleavage) and up to 51% cleavage rate was achieved. These activation and cleavage rates are high in comparison with those previously reported after transfer of adult somatic cell nuclei in the horse. However, the percentage of reconstructed embryos with normal nuclei after 4 days of culture was low (6%–22%) and the average nucleus number was only two to four. Ex oocytes, selected and matured in the same manner as in this study but fertilized by ICSI, develop to an average of 8.2 nuclei at 96 h [27]. The cleavage rate and development of Cp oocytes parthenogenetically activated with sperm extract indicates that sperm extract is capable of triggering normal embryonic development. There is one previous brief report on the use of sperm extract for activation of NT oocytes in other species (bovine cytoplasts reconstructed with adult fibroblast nuclei; [32]). In that study, in vitro development of oocytes injected with sperm extract after reconstruction was lower than that of chemically activated or in vitro fertilized oocytes.

When direct nuclear injection is used, the possibility exists for incomplete breakage of the oocyte or donor cell membrane, resulting in failure of transfer. In addition, media components as well as cell membrane are injected into the oocyte cytoplasm. To determine whether use of the Piezo drill or methods associated with this technique were compromising the ability of the embryo to develop normally, we evaluated the use of the Piezo drill for transfer of equine nuclei to bovine cytoplasts. The reconstruction rate achieved in interspecies nuclear transfer using the Piezo drill (81%) was higher than that we previously achieved using electrofusion (109/159, 69% [22]). Rates of cleavage and embryo development ( $\geq 8$  cells) of Piezo-manipulated interspecies NT embryos in this study (88% and 41%, respectively) compared favorably with those of interspecies embryos obtained using electrofusion (cultured on Vero cell

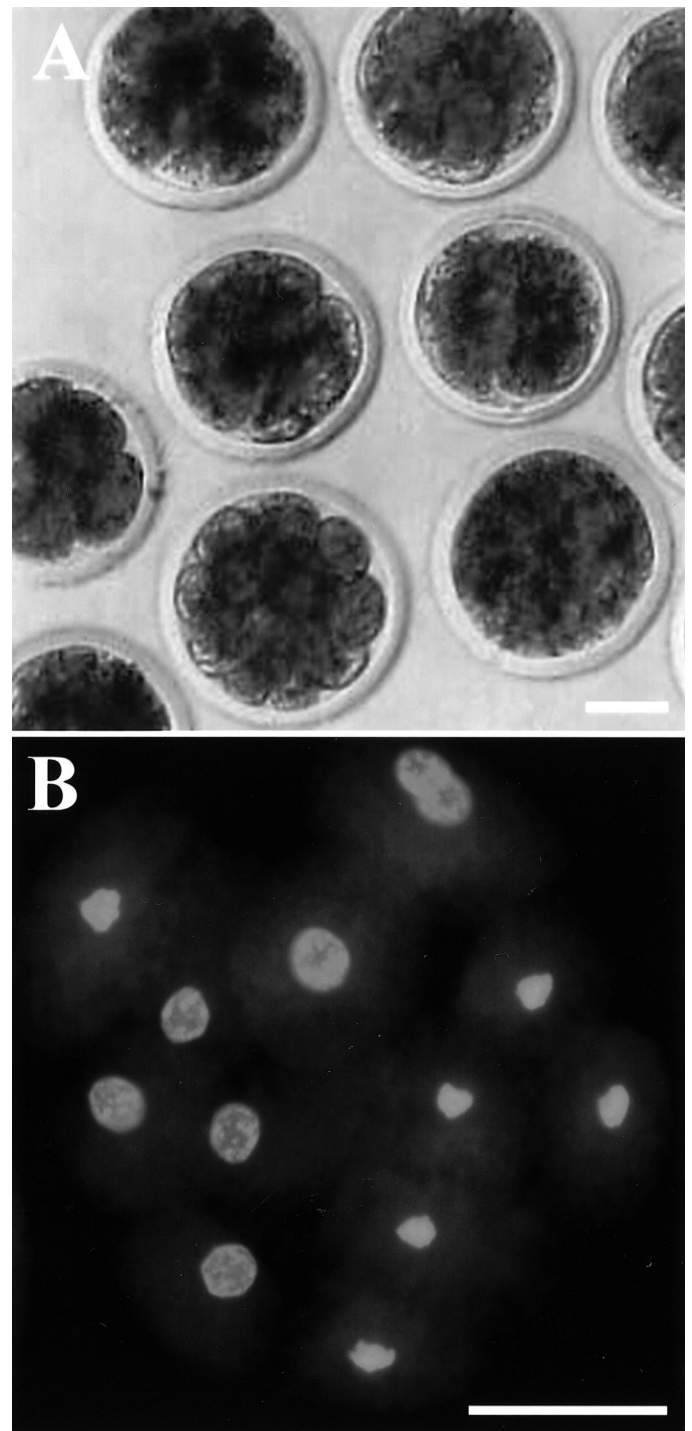


FIG. 3. Interspecies NT embryos (horse somatic cell nuclei into bovine ooplasts). Embryos were cultured in G1.2 medium for 3.5 days. **A**) Bright-field image showing cleavage. **B**) Fluorescence image of a single embryo, showing 13 nuclei. Bars = 50  $\mu$ m.

or cumulus cell monolayers; 53% cleavage and 39%  $\geq 8$  cells; [22]). It is notable that markedly better development was achieved in the interspecies transfers than in the equine-equine transfers. Similar increases in development when bovine cytoplasts rather than equine cytoplasts were reconstructed with equine somatic cell nuclei by electrofusion have been previously reported [23]. We have activated interspecies reconstructed oocytes (horse nuclei in bovine cytoplasm) with stallion sperm extract and have ob-

tained 97% (32/33) cleavage, including 64% (21/33) normal cleavage (normal nuclei present), and average nucleus number of 7.4 after a 96-h culture (unpublished data). These data suggest that it is the horse oocyte rather than the donor cell or the reconstruction and activation methods that is the limiting factor in the development of equine NT embryos.

The time of oocyte activation after donor cell injection is an important factor in the success of nuclear transfer. Time from reconstruction to activation in bovine NT studies has varied from essentially immediately [12] to 3–5 h [11], 4–6 h [4], and ~10 h [42]. In the mouse, embryo development of oocytes activated 1–6 h after donor injection was significantly higher than that of simultaneously activated oocytes [2]. These authors reported that chromosome condensation of donor nuclei occurred within 1 h of injection. The high MPF level within the recipient cytoplasm is thought to trigger nuclear membrane breakdown and condensation of the chromatin into individual chromosomes. It is felt that exposure to the oocyte cytoplasm is important in reprogramming of the chromatin. The exposure time needed may be short, however. In a recent report, Wakayama and Yanagimachi [43] found that immediate activation of oocytes after reconstruction was compatible with embryo development and birth of live young if activated oocytes were not treated with cytochalasin B. In contrast, activation of oocytes before donor cell injection resulted in nuclear degeneration. No information was previously available on the effect of time from reconstruction to activation in horse nuclear transfer oocytes. In the present study, there was no difference in embryo development in oocytes activated 1.5–2 h vs. 8–10 h after reconstruction when the same dosage of sperm extract was used.

In conclusion, injection of stallion sperm cytosolic extract parthenogenetically activated MII horse oocytes with high efficiency and resulted in normal cleavage. Injection of stallion sperm extract also activated reconstructed horse oocytes in a dose-dependent fashion, but rates of normal cleavage were low. Piezo-actuated microinjection allowed high reconstruction rates in both horse and cattle oocytes, and good cleavage rates and embryonic development were obtained in interspecies cloned embryos. Further research on factors influencing the in vitro development of equine nuclear transfer embryos is needed to improve the efficiency of this procedure in the horse.

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## REFERENCES

1. Wilmut I, Schnieke AE, McWhir J, Kind AJ, Campbell KHS. Viable offspring derived from fetal and adult mammalian cells. *Nature* 1997; 385:810–813.
2. Wakayama T, Perry ACF, Zuccotti M, Johnson KR, Yanagimachi R. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. *Nature* 1998; 394:369–374.
3. Kato Y, Tani T, Sotomaru Y, Kurokawa K, Kato J, Doguchi H, Yasue H, Tsunoda Y. Eight calves cloned from somatic cells of a single adult. *Science* 1998; 282:2095–2098.
4. Wells DN, Misica PM, Tervit HR. Production of cloned calves following nuclear transfer with cultured adult mural granulosa cells. *Biol Reprod* 1999; 60:996–1005.
5. Baguisi A, Behboodi E, Melican DT, Pollock JS, Destrempes MM, Cammuso C, Williams JL, Nims SD, Porter CA, Midura P, Palacios MJ, Ayres SL, Denniston RS, Hayes ML, Ziomek CA, Meade HM, Godke RA, Gavin WG, Overstrom EW, Echelard Y. Production of goats by somatic cell nuclear transfer. *Nat Biotechnol* 1999; 17:456–461.
6. Keefer CL, Baldassarre H, Keyston R, Wang B, Bhatia B, Bilodeau AS, Zhou JF, Leduc M, Downey BR, Lazaris A, Karatzas CN. Generation of dwarf goat (*Capra hircus*) clones following nuclear transfer with transfected and nontransfected fetal fibroblasts and in vitro-matured oocytes. *Biol Reprod* 2001; 64:849–856.
7. Onishi A, Iwamoto M, Akita T, Mikawa S, Takeda K, Awata T, Hanada H, Perry ACF. Pig cloning by microinjection of fetal fibroblast nuclei. *Science* 2000; 289:1188–1190.
8. Polejaeva IA, Chen SH, Vaught TD, Page RL, Mullins J, Ball S, Dai Y, Boone J, Walker S, Ayares DL, Colman A, Campbell KHS. Cloned pigs produced by nuclear transfer from adult somatic cells. *Nature* 2000; 407:86–90.
9. Kubota C, Yamakuchi H, Todoroki J, Mizoshita K, Tabara N, Barber M, Yang X. Six cloned calves produced from adult fibroblast cells after long-term culture. *Proc Natl Acad Sci U S A* 2000; 97:990–995.
10. Zakhartchenko V, Durcova-Hills G, Stojkovic M, Scherthaner W, Prella K, Steinborn R, Muller M, Brem G, Wolf E. Effects of serum starvation and re-cloning on the efficiency of nuclear transfer using bovine fetal fibroblasts. *J Reprod Fertil* 1999; 115:325–331.
11. Hill JR, Winger QA, Long CR, Looney CR, Thompson JA, Westhusin ME. Development rates of male bovine nuclear transfer embryos derived from adult and fetal cells. *Biol Reprod* 2000; 62:1135–1140.
12. Kato Y, Tani T, Tsunoda Y. Cloning of calves from various somatic cell types of male and female adult, newborn and fetal cows. *J Reprod Fertil* 2000; 120:231–237.
13. Dinnyes A, Dai Y, Jiang S, Yang X. High developmental rates of vitrified bovine oocytes following parthenogenetic activation, in vitro fertilization, and somatic cell nuclear transfer. *Biol Reprod* 2000; 63:513–518.
14. Peura TT, Lane MW, Lewis IM, Trounson AO. Development of bovine embryo-derived clones after increasing rounds of nuclear recycling. *Mol Reprod Dev* 2001; 58:384–389.
15. Verma PJ, Du ZT, Crocker L, Faast R, Grupen CG, McIlpatrick SM, Ashman RJ, Lyons IG, Nottle MB. In vitro development of porcine nuclear transfer embryos constructed using fetal fibroblasts. *Mol Reprod Dev* 2000; 57:262–269.
16. Park KW, Lai L, Cheong HT, Im GS, Sun QY, Wu G, Day BN, Prather RS. Developmental potential of porcine nuclear transfer embryos derived from transgenic fetal fibroblasts infected with the gene for the green fluorescent protein: comparison of different fusion/activation conditions. *Biol Reprod* 2001; 65:1681–1685.
17. Wells DN, Misica PM, Day AM, Peterson AJ, Tervit HR. Cloning sheep from cultured embryonic cells. *Reprod Fertil Dev* 1998; 10:615–626.
18. Kato Y, Yabuuchi A, Motosugi N, Kato J, Tsunoda Y. Developmental potential of mouse follicular epithelial cells and cumulus cells after nuclear transfer. *Biol Reprod* 1999; 61:1110–1114.
19. Ono Y, Shimozawa N, Ito M, Kono T. Cloned mice from fetal fibroblast cells arrested at metaphase by a serial nuclear transfer. *Biol Reprod* 2001; 64:44–50.
20. Ogura A, Inoue K, Takano K, Wakayama T, Yanagimachi R. Birth of mice after nuclear transfer by electrofusion using tail tip cells. *Mol Reprod Dev* 2000; 57:55–59.
21. Wakayama T, Yanagimachi R. Mouse cloning with nucleus donor cells of different age and type. *Mol Reprod Dev* 2001; 58:376–383.
22. Hinrichs K, Shin T, Love CC, Varner DD, Westhusin ME. Comparison of bovine and equine oocytes as host cytoplasts for equine nuclear transfer. In: *Proceedings of the 5th International Symposium on Equine Embryo Transfer*; 2001; Saari, Finland: 43–44.
23. Reggio BC, Sansinena M, Cochran RA, Guitreau A, Carter JA, Denniston RS, Godke RA. Nuclear transfer embryos in the horse. In: *Proceedings of the 5th International Symposium on Equine Embryo Transfer*, 2001; Saari, Finland: 45–46.
24. Choi YH, Shin T, Love CC, Burghardt RC, Varner DD, Hinrichs K. Effect of initial cumulus morphology and addition of cytochalasin B on fusion, activation and cleavage of horse oocytes undergoing nuclear transfer. *Theriogenology* 2001; 55:261 (abstract).
25. Li X, Morris LHA, Allen WR. In vitro development of horse oocytes reconstructed with the nuclei of fetal and adult cells. In: *From epididymis to embryo: Havemeyer Foundation Workshop*; 2001; New Orleans, LA: 29.
26. Li X, Morris LHA, Allen WR. Chromatin reprogramming in enucleated horse oocytes injected with cumulus cell nuclei. *J Reprod Fertil Abstr Ser* 2000; 25:77 (abstract 211).

27. Choi YH, Love CC, Love LB, Varner DD, Brinsko B, Hinrichs K. Developmental competence in vivo and in vitro of in vitro-matured equine oocytes fertilized by intracytoplasmic sperm injection with fresh or frozen-thawed sperm. *Reproduction* 2002; 123:455–465.
28. Carneiro G, Lorenzo P, Pimentel C, Pegoraro L, Bertolini M, Ball B, Anderson G, Liu I. Influence of insulin-like growth factor-1 and its interaction with gonadotropins, estradiol, and fetal calf serum on in vitro maturation and parthenogenic development in equine oocytes. *Biol Reprod* 2001; 65:899–905.
29. Swann K. A cytosolic sperm factor stimulates repetitive calcium increases and mimics fertilization in hamster eggs. *Development* 1990; 110:1295–1302.
30. Fissore RA, Gordo AC, Wu H. Activation of development in mammals: is there a role for a sperm cytosolic factor? *Theriogenology* 1998; 49:43–52.
31. Perry ACF, Wakayama T, Yanagimachi R. A novel trans-complementation assay suggests full mammalian oocyte activation is coordinately initiated by multiple, submembrane sperm components. *Biol Reprod* 1999; 60:747–755.
32. Knott JG, Kasinathan P, Wu H, Spell A, Fissore RA, Robl JM. Effect of porcine sperm factor on intracellular calcium and activation of bovine oocytes and development of nuclear transfer embryos. *Theriogenology* 2001; 55:455(abstract).
33. Hinrichs K, Williams KA. Relationships among oocyte-cumulus morphology, follicular atresia, initial chromatin configuration, and oocyte meiotic competence in the horse. *Biol Reprod* 1997; 57:377–384.
34. Hinrichs K, Schmidt AL. Meiotic competence in horse oocytes: interactions among chromatin configuration, follicle size, cumulus morphology, and season. *Biol Reprod* 2000; 62:1402–1408.
35. Parrish JJ, Susko-Parrish JL, Winer MA, First NL. Capacitation of bovine sperm by heparin. *Biol Reprod* 1998; 38:1171–1180.
36. Kuretake S, Kimura Y, Hoshi K, Yanagimachi R. Fertilization and development of mouse oocytes injected with isolated sperm heads. *Biol Reprod* 1996; 55:789–795.
37. Kimura Y, Yanagimachi R. Mouse oocytes injected with testicular spermatozoa or round spermatids can develop into normal offspring. *Development* 1995; 121:2397–2405.
38. Dominko T, Mitalipova M, Haley B, Beyhan Z, Memili E, McKusick B, First NL. Bovine oocyte cytoplasm supports development of embryos produced by nuclear transfer of somatic cell nuclei from various mammalian species. *Biol Reprod* 1999; 60:1496–1502.
39. Hinrichs K, Schmidt AL, Selgrath JP. Activation of horse oocytes. *Biol Reprod Mono* 1995; 1:319–324.
40. Choi YH, Love CC, Varner DD, Thompson JA, Hinrichs K. Activation of cumulus-free equine oocytes: effect of maturation medium, calcium ionophore concentration and duration of cycloheximide exposure. *Reproduction* 2001; 122:177–183.
41. Li X, Morris LHA, Allen WR. Effects of different activation treatments on fertilization of horse oocytes by intracytoplasmic sperm injection. *J Reprod Fertil* 2000; 119:253–260.
42. Kasinathan P, Knott JG, Moreira PN, Burnside AS, Jerry DJ, Robl JM. Effect of fibroblast donor cell age and cell cycle on development of bovine nuclear transfer embryos in vitro. *Biol Reprod* 2001; 64:1487–1493.
43. Wakayama T, Yanagimachi R. Effect of cytokinesis inhibitors, DMSO and the timing of oocyte activation on mouse cloning using cumulus cell nuclei. *Reproduction* 2001; 122:49–60.