

Developmental competence *in vivo* and *in vitro* of *in vitro*-matured equine oocytes fertilized by intracytoplasmic sperm injection with fresh or frozen–thawed spermatozoa

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This study was undertaken to evaluate the development of equine oocytes *in vitro* and *in vivo* after intracytoplasmic sperm injection (ICSI) with either fresh or frozen–thawed spermatozoa, without the use of additional activation treatments. Oocytes were collected from ovaries obtained from an abattoir and oocytes classified as having expanded cumulus cells were matured in M199 with 10% fetal bovine serum and 5 μ U FSH ml⁻¹. After 24–26 h of *in vitro* maturation, oocytes with a first polar body were selected for manipulation. Fresh ejaculated stallion spermatozoa were used for the experiment after swim-up for 20 min in sperm–Tyrode’s albumen lactate pyruvate. Frozen–thawed spermatozoa from the same stallion were treated in a similar way. Spermatozoa were immobilized and injected into the oocytes using a Piezo drill. Presumptive zygotes were cultured in G1.2 medium for 20 or 96 h after the injection was administered, or were transferred to the oviducts of recipient mares and recovered 96 h later. In addition, bovine oocytes with first polar bodies were injected with the two types of stallion spermatozoa and fixed 20 h after injection to examine pronuclear formation. Fertilization rate (pronucleus formation and

cleavage) at 20 h after injection of spermatozoa was not significantly different between fresh and frozen–thawed sperm groups in either equine or bovine oocytes. Pronucleus formation after injection of spermatozoa into bovine oocytes was significantly higher than that for equine oocytes ($P < 0.05$). There were no significant differences in cleavage rate or average number of nuclei at 96 h between equine oocytes injected with fresh or frozen–thawed spermatozoa. However, embryos developed *in vivo* for 96 h had a significantly higher number of nuclei in both sperm treatments compared with those cultured *in vitro*. These results indicate that good activation rates may be obtained after injection of either fresh or frozen–thawed equine spermatozoa without additional activation treatment. Injection of frozen–thawed equine spermatozoa results in similar embryo development to that obtained with fresh equine spermatozoa. *In vitro* culture of equine zygotes in G1.2 medium results in a similar cleavage rate but reduced number of cells compared with *in vivo* culture within the oviduct. Bovine oocytes may be useful as models for assessing sperm function in horses.

Introduction

Over the last decade, only low rates of fertilization have been achieved after conventional IVF in horses (Bezard *et al.*, 1989; Dell’Aquila *et al.*, 1999). This low rate of fertilization appears to be related to the inability of spermatozoa to penetrate the equine zona pellucida *in vitro*, as fertilization rates are greatly improved by partial removal or by drilling of the zona pellucida (Choi *et al.*, 1994; Li *et al.*, 1995). Only two foals have been produced by conventional IVF, both resulting from insemination of an oocyte matured *in vivo* (Palmer *et al.*, 1991; Bezard *et al.*, 1992). Intra-

cytoplasmic sperm injection (ICSI) has been used in horses (Squires *et al.*, 1996; Grondahl *et al.*, 1997; Dell’Aquila *et al.*, 1997a,b, 1999; Cochran *et al.*, 1998; Li *et al.*, 2000) because it bypasses critical fertilization events, such as the acrosome reaction, zona pellucida binding and penetration, and sperm–egg membrane fusion.

The percentage of male pronucleus formation in oocytes after ICSI in horses varies from 21 to 68% (Dell’Aquila *et al.*, 1997a; Grondahl *et al.*, 1997; Guignot *et al.*, 1998; Li *et al.*, 2000). Cleavage rates after ICSI of equine oocytes have typically been reported without fixation or staining, and thus the actual proportion of embryos with normal development is not clear. Reported cleavage rates range from 20 to 65% (Dell’Aquila *et al.*, 1997b; Grondahl *et al.*, 1997; Kato *et al.*, 1997; Cochran *et al.*, 1998; Guignot *et al.*, 1998; McKinnon *et al.*, 2000; Schmid *et al.*, 2000; Li *et al.*, 2001).

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Three different preparations of equine spermatozoa have been used for ICSI: fresh (Kato *et al.*, 1997; Schmid *et al.*, 2000), cooled (Cochran *et al.*, 1998; Li *et al.*, 2000) and frozen–thawed (Grondahl *et al.*, 1997; Dell’Aquila *et al.*, 1997a,b, 1999; Li *et al.*, 2001). There appears to be controversy over the necessity for activation treatment after ICSI in horses. Researchers working with fresh semen achieved minimal pronucleus formation unless oocytes were chemically activated after ICSI (Kato *et al.*, 1997; Guignot *et al.*, 1998; Schmid *et al.*, 2000). Use of cooled semen either required (Li *et al.*, 2000) or did not require (Cochran *et al.*, 1998) chemical activation for pronucleus formation. Groups using frozen–thawed spermatozoa have reported good rates of pronucleus formation (about 50%) without activation treatment after ICSI (Grondahl *et al.*, 1997; Dell’Aquila *et al.*, 1997a,b, 1999; McKinnon *et al.*, 2000), although some workers have combined the use of frozen–thawed spermatozoa with activation treatment (Li *et al.*, 2001).

Spermatozoa injected into the oocyte by ICSI are still enveloped by their plasma membrane, but must be capable of releasing the factors involved in oocyte activation. In conventional ICSI, spermatozoa are treated by pressing the tail of the spermatozoa against the bottom of a dish to immobilize it and damage the sperm plasma membrane to facilitate release of sperm-borne oocyte-activation factors (Dozortsev *et al.*, 1995). It is possible that the process of freezing spermatozoa results in changes to the sperm membrane that allow more rapid or more complete diffusion of sperm factors into the cytoplasm of the oocyte. Therefore, the differences in reported requirements for chemical activation after ICSI in horses may be related to the type of spermatozoa used.

It has been suggested that the sperm-borne oocyte-activation factor is both soluble (Swan, 1990), and is released after repeated freezing and thawing in liquid nitrogen, and insoluble (Perry *et al.*, 1999), that is, present in the membrane. The sperm-borne oocyte-activation factor is not species-specific: pig cytosolic sperm factor can activate mouse and bovine oocytes (Fissore *et al.*, 1998; Perry *et al.*, 1999). In addition, spermatozoa from species, such as hamsters, rats, rabbits, humans or sea urchins can activate mouse oocytes (Rybouchikin *et al.*, 1995; Wakayama *et al.*, 1997; Yazawa *et al.*, 2000). In other reports, interspecies fertilization after ICSI has been reported in conjunction with additional activation treatments. Injection of bovine, mouse or human spermatozoa, combined with electrical stimulus, resulted in activation of pig oocytes (Kim *et al.*, 1999), and injected killed ovine, bovine or minke whale spermatozoa activated bovine oocytes when combined with ethanol treatment (Wei and Fukui, 2000). However, there are no reports available on the capacity of spermatozoa of different species to activate bovine oocytes without additional activation stimulation. Bovine oocytes have been used as a model for zona-free penetration assay using equine spermatozoa (Choi *et al.*, 1999a; Landim-Alvarenga, 2001). Bovine oocytes have also

been used as host cytoplasm for nuclei from other species (Dominko *et al.*, 1999), including horses (Hinrichs *et al.*, 2000).

In vitro culture systems have not been developed to support equine embryonic development, and few studies have been carried out in this area. This lack of research is due, in part, to the small number of equine embryos available for investigation, as equine oocytes are difficult to recover in large numbers (Hinrichs, 1998). *In vitro* culture of ICSI-derived embryos for only 26–28 h reduced the pregnancy rate after oviductal transfer compared with those embryos transferred earlier (4–8 h) after ICSI (McKinnon *et al.*, 2000). *In vivo*-derived one- to two-cell equine embryos did not produce blastocysts unless co-cultured with uterine tubal epithelial cells (Ball *et al.*, 1993). In co-culture, 35% of embryos developed to blastocysts. Because of inadequate *in vitro* embryo culture systems, few reports are available on the development of ICSI embryos *in vitro*. Three laboratories have produced blastocysts from ICSI-embryos in culture *in vitro*, but in small to moderate proportions (1 of 79, Dell’Aquila *et al.*, 1997b; 1 of 76, Guignot *et al.*, 1998; 10 of 33, Li *et al.*, 2001). Pregnancies and foals have resulted from the transfer of ICSI-produced zygotes and embryos to the oviducts of recipient mares, but again, in small proportions (Squires *et al.*, 1996; Cochran *et al.*, 1998; McKinnon *et al.*, 2000). Transfer of ICSI blastocysts produced *in vitro* to the uterus with the subsequent birth of live foals, has been reported by Li *et al.* (2001). There is no information available comparing development of ICSI embryos in the oviduct *in vivo* to that achieved *in vitro*.

In this study, the activation rate of oocytes fertilized by ICSI was investigated using fresh or frozen–thawed equine spermatozoa, and their subsequent development both *in vivo*, after transfer to the oviducts of recipient mares, and *in vitro* was evaluated. In addition, the activation rate of the two types of spermatozoa was evaluated when injected into bovine oocytes to determine whether bovine oocytes are a suitable model for equine oocytes.

Materials and Methods

Oocyte collection

Ovaries were transported from the abattoir to the laboratory at room temperature (3–4 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 TCM-199 with Hank’s salts (Gibco Life Technologies, Inc, Grand Island, NY) plus ticarcillin (0.1 mg ml⁻¹, SmithKline Beecham Pharmaceuticals, Philadelphia, PA). The contents of the Petri dishes were examined under a dissection microscope at ×10–20 magnification. Cumulus–oocyte complexes were classified as compact, expanded or degenerating depending

on the expansion of both mural granulosa and cumulus as described previously (Hinrichs and Williams, 1997; Hinrichs and Schmidt, 2000). 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) were classified as expanded. Oocytes with both compact cumulus cells and compact mural granulosa were classified as compact. Only expanded oocytes were used in this study.

In vitro maturation

Selected oocytes were washed twice in maturation medium (TCM-199 with Earle's salts (Gibco), 5 μ U FSH ml⁻¹ (Sioux Biochemical Inc, Sioux Center, IA), 10% fetal bovine serum (FBS) and 25 μ g gentamycin ml⁻¹). Oocytes were cultured in droplets at a ratio of 10 μ l medium per oocyte, under light white mineral oil (Sigma Chemical Co., St Louis, MO) at 38.2°C in 5% CO₂ in air for 24–26 h. After maturation, oocytes were denuded of cumulus cells by pipetting in a solution of 0.5% (w/v) hyaluronidase in TCM-199 with 5% FBS. Denuded oocytes were selected for the presence of a polar body. Oocytes without a polar body were fixed in buffered formal saline, mounted on a slide with 6.5 μ l of 9:1 glycerol:PBS containing 2.5 μ g Hoechst 33258 ml⁻¹, and examined using fluorescence microscopy to determine the chromatin configuration.

Sperm preparation

Fresh semen was collected from one stallion using a Missouri model artificial vagina and was then diluted to 80 $\times 10^6$ spermatozoa ml⁻¹ with a commercial skimmed-milk glucose extender (Animal Reproduction Systems, Chino, CA) before further processing. Semen from the same stallion was frozen using a skimmed milk–egg yolk-based extender (Blanchard *et al.*, 1998). Semen straws were thawed at 50°C for 42 s. Two hundred μ l of semen (fresh extended or frozen–thawed) was placed in the bottom of a 5 ml tube containing 1 ml of modified Tyrode's albumin lactate pyruvate (TALP) (sp-TALP, Parrish *et al.*, 1988) and incubated in an atmosphere of 5% CO₂ in air for swim-up. After 20 min, 0.6 ml of medium was collected from the top of the tube and centrifuged at 327 *g* for 3 min in a 1.7 ml polypropylene tube. The sperm pellet was resuspended and washed once with the same medium, and the supernatant was removed and the pellet was used for ICSI.

Intracytoplasmic sperm injection

The ICSI technique was modified from the method described by Kimura and Yanagimachi (1995), using the Piezo injection system. The outside diameter of the sperm injection pipette was 7–8 μ m. A 120–140 μ m (outside diameter) pipette was used to hold the oocytes. Immediately before injection, 1 μ l sperm suspension was placed in 3 μ l sp-TALP containing 10% (w/v) polyvinylpyrrolidone (Sigma)

under oil. The injection of spermatozoa was carried out in a separate 50 μ l drop of Hepes-buffered TCM-199 containing 10% FBS. Each spermatozoon was immobilized by applying a few pulses with the Piezo drill (Burleigh Instruments Inc, Fishers, NY) to the sperm tail immediately before injection. All manipulations were performed at room temperature. Injected oocytes were held for 20 min at room temperature in the same medium to slowly heal the broken membrane, and then oocytes to be cultured *in vitro* were transferred into G1.2 medium (G1.2/G2.2, IVF Science, Denver, CO) and incubated at 38.2°C under 5% CO₂ in air. These oocytes were assessed at either 20 or 96 h after ICSI. Oocytes to be transferred to a recipient mare were placed in 50 μ l G1.2 medium and incubated at 38.2°C under 5% CO₂ in air until transfer (< 2 h; see below).

Preparation of bovine oocytes and ICSI

Bovine oocytes were purchased from Ovagenix (San Angelo, TX) and were cultured overnight in a portable incubator maintained at 39°C. Upon arrival at the laboratory, the glass tube containing the oocytes was uncapped and placed in an incubator at 5% CO₂ in air, until 24 h of IVM. Oocytes were then denuded by gentle pipetting in 0.05% (w/v) hyaluronidase (Sigma) in TCM-199 plus 5% FBS. Oocytes with a first polar body were selected and used for manipulation. Sperm preparation and ICSI were performed as described above. After insemination, bovine oocytes were cultured for 20 h in G1.2 medium.

Assessment of oocyte fertilization and activation

At 20 h after ICSI, assigned equine oocytes and all bovine oocytes were fixed, stained and evaluated as described above. Oocytes in metaphase II, with either no spermatozoa visible or a condensed sperm head present, were considered to be non-activated. Oocytes in anaphase II to metaphase III (metaphase plate with two polar bodies) with individualized sperm chromosomes or a decondensing sperm head were considered to be activated but arrested in development (having incomplete activation). Oocytes with two or three pronuclei, syngamy or cleavage with the presence of nuclei in each blastomere, with one or two polar bodies, were considered to be fertilized. Three pronuclei were considered to be formed when activation of both halves of the metaphase II chromatin and decondensation of the sperm head occurred.

In vitro culture

Oocytes assigned to the 96 h group were cultured in their original droplet of G1.2 medium, without a change of medium. Development of embryos was evaluated each day under a dissection microscope at $\times 40$ –60 magnification, on a heated stage. At 48 h after ICSI, non-cleaved embryos were removed, fixed and stained to examine their fertilization status. After 96 h of culture, embryos were fixed and stained as described above to examine the number and

Table 1. Pronucleus formation of equine oocytes fixed 20 h after intracytoplasmic sperm injection (ICSI) with two different types of equine spermatozoa

Spermatozoa	Number of oocytes examined (number of replicates)	Number of unfertilized oocytes ^a (%)	Number of incompletely activated oocytes with			Number of activated oocytes with				
			Sperm chromosome ^b	Decondensed sperm head ^c	Total (%)	2 PN	3 PN	First mitosis	Two-cell	Total (%)
Fresh	41 (3)	4 (10)	4	4	8 (20)	24	1	1	3	29 (71)
Frozen–thawed	40 (3)	3 (8)	7	6	13 (33)	17	2	4	1	24 (60)

^aUnfertilized oocytes include oocytes with an intact sperm head or no spermatozoa present.

^bSperm chromosomes with oocyte in metaphase III.

^cDecondensed sperm head with oocytes in pronucleus, decondensed chromosome or telophase stages.

2PN: two pronuclei with one or two polar bodies.

3PN: three pronuclei present.

status of nuclei. Only nuclei that appeared to be normal were included in the number of nuclei recorded; nuclei with signs of degeneration (vacuolization, condensation, or fragmentation) were disregarded.

Oocyte transfer and recovery

Oocytes were surgically transferred to the oviducts of recipient mares within 2 h of ICSI. For oocyte transfer, detomidine (Pfizer Inc., Lees Summit, MO, 3–5 mg, i.v.) and butorphanol (Fort Dodge Animal Health Co., Fort Dodge, IA, 5 mg i.v.) were administered to the mares. Transfer of presumptive zygotes to the oviduct was performed via standing flank laparotomy, as described previously for oocyte transfer (Hinrichs *et al.*, 1998). Oocytes that had been subjected to ICSI with fresh and frozen–thawed spermatozoa were transferred to separate oviducts. At 94 h after transfer, mares were killed and the uterus, ovaries and oviducts were removed. Embryos were recovered within 2 h by cannulating the oviductal papilla and flushing the oviduct with 50 ml Dulbecco's PBS. Recovered embryos were evaluated under a dissection microscope, and then fixed and stained with Hoechst 33258 as described above. The uteri of the first two mares to receive transferred embryos were flushed with Dulbecco's PBS to recover any embryos that may have descended from the oviducts.

Statistical analysis

Differences among groups were evaluated using chi-squared analysis. Fisher's exact test was used when the expected value for any parameter was < 5. The numbers of embryonic nuclei were compared between treatments by the Student's *t* test. The correlation between number of embryonic nuclei and number of oviductal nuclei present in embryos transferred *in vivo* was calculated by regression analysis.

Results

A total of 236 ovaries were processed, from which 1644 follicles were scraped, an average of seven follicles per

ovary. Nine hundred and eighteen oocytes were recovered, of which 254 were compact, 595 were expanded and 69 were degenerating. Five hundred and forty-four expanded oocytes were used for this study, and the remainder were used on a separate project.

When the oocytes were examined after 24–26 h of maturation, seven were damaged during denuding and 537 were evaluated for presence of a polar body. Of these, 312 (58%) had a polar body and 310 were used for ICSI. After ICSI, 301 oocytes (97%) remained intact. Two oocytes that underwent ICSI were lost during transfer to oviducts. Of oocytes without polar bodies, 40 (18%) were found to be in metaphase I when fixed and stained and the remainder was degenerating.

In equine presumptive zygotes fixed after 20 h of culture, there were no significant differences in total activation rate (complete plus incomplete) between the fresh or frozen–thawed sperm groups (90 and 93%, respectively, Table 1). The rate of fertilization (pronucleus formation and cleavage) was 71 and 60% for oocytes injected with fresh and frozen–thawed spermatozoa, respectively. These values were not significantly different. On evaluation at 20 h of culture, three oocytes injected with fresh spermatozoa and one oocyte injected with frozen–thawed spermatozoa had developed to two-cell embryos. An equine oocyte with two pronuclei and two polar bodies injected with frozen–thawed spermatozoa is shown (Fig. 1a).

One hundred and sixty-two bovine oocytes were obtained, and 150 oocytes (93%) contained a polar body after IVM and were used for ICSI. Of these, 140 oocytes (93%) survived manipulation (ICSI or sham injection; Table 2). The activation rates of bovine oocytes injected with fresh or frozen–thawed equine spermatozoa were not significantly different (81 and 80%, respectively). One oocyte for each treatment group had developed to a two-cell embryo at 20 h. The activation rate (pronucleus formation and cleavage) for bovine oocytes injected with frozen–thawed equine spermatozoa was significantly higher than that for equine oocytes injected with frozen–thawed equine spermatozoa ($P < 0.05$), and the overall activation rate for

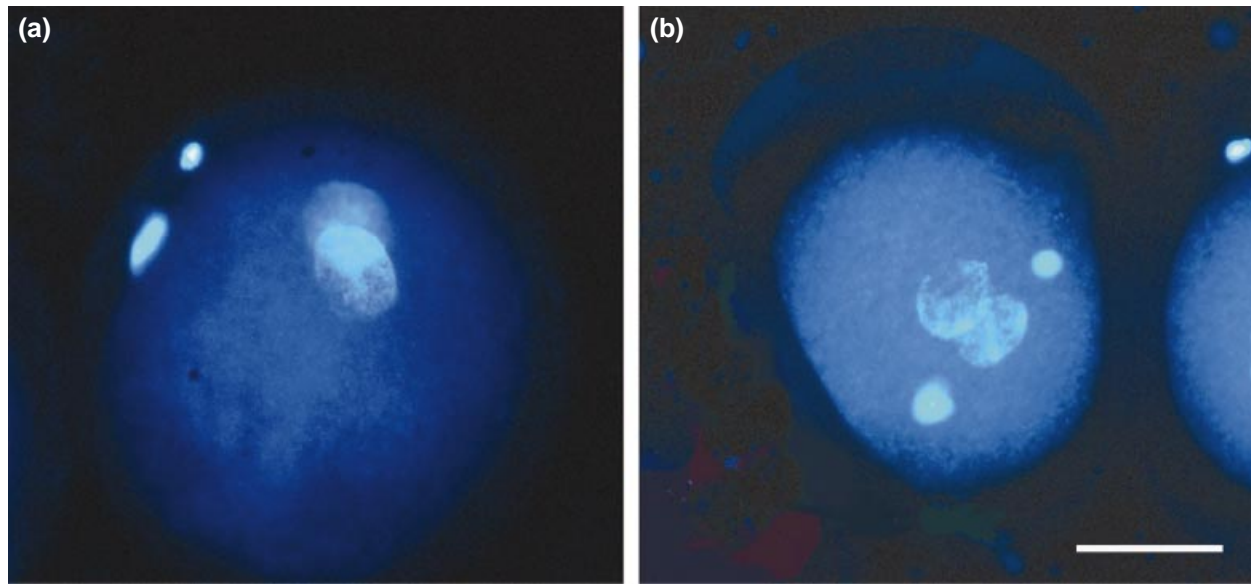


Fig. 1. Pronucleus formation after intracytoplasmic sperm injection (ICSI) in (a) equine and (b) bovine oocytes, with two pronuclei and two polar bodies visible after Hoechst 33258 staining after culture *in vitro* for 20 h. Scale bar represents 50 μ m.

Table 2. Nuclear decondensation and pronucleus formation of bovine oocytes fixed 20 h after intracytoplasmic sperm injection (ICSI) with two different types of equine spermatozoa

Spermatozoa	Number of oocytes examined (number of replicates)	Number of oocytes surviving (%)	Number of unfertilized oocytes ^a	Number of incompletely activated oocytes with			Number of activated oocytes with				
				Sperm chromosome ^b	Decondensed sperm head ^c	Total (%)	2 PN	3 PN	First mitosis	Two-cell	Total (%)
Fresh	58 (3)	54 (93)	5 (9)	4	1	5 (9)	40	1	2	1	44 (81)
Frozen–thawed	59 (3)	55 (93)	4 (7)	7	0	7 (13)	34	4	5	1	44 (80)
Sham	33 (3)	31 (94)	31 (100)	0	0	0 (0)	0	0	0	0	0 (0)

^aUnfertilized oocytes include oocytes with an intact sperm head or no spermatozoa present.

^bSperm chromosomes with oocyte in metaphase III.

^cDecondensed sperm head with oocytes in pronucleus, decondensed chromosome or telophase stages.

2PN: two pronuclei with one or two polar bodies.

3PN: three pronuclei present.

bovine oocytes (fresh and frozen–thawed spermatozoa combined) was significantly higher than that for equine oocytes ($P < 0.05$). A bovine oocyte with two pronuclei and two polar bodies after injection with frozen–thawed equine spermatozoa is shown (Fig. 1b).

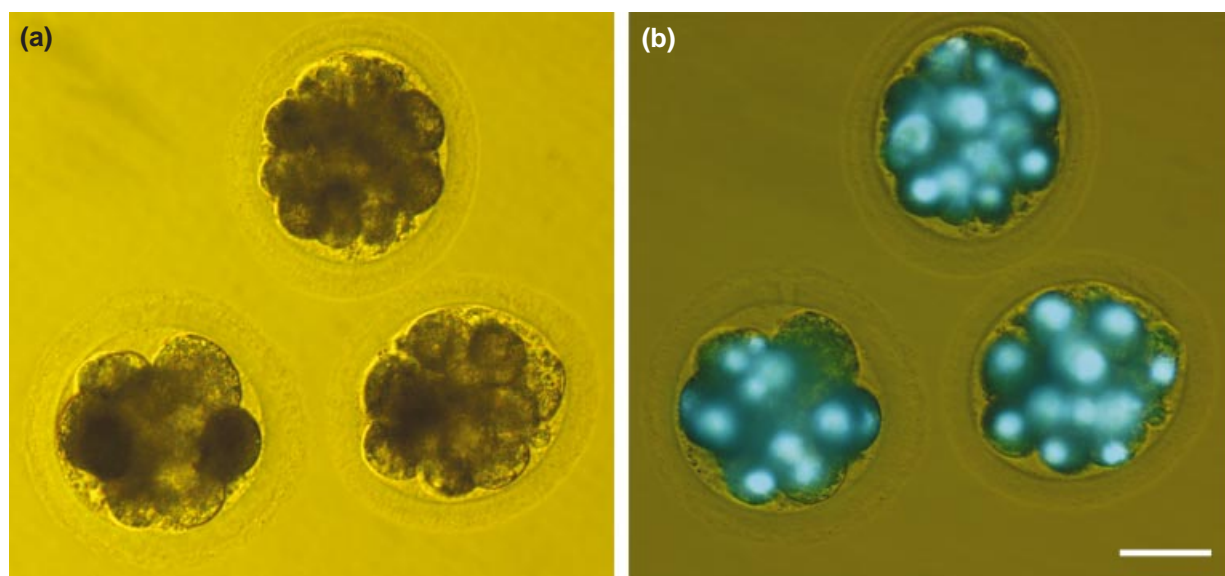
The *in vitro* development of equine oocytes cultured for 96 h after ICSI is shown (Table 3). Cleavage rates assessed morphologically were not significantly different between sperm treatments (84% for fresh versus 76% for frozen–thawed). However, the proportion of oocytes undergoing cleavage with normal nuclei tended to be higher in the oocytes injected with fresh spermatozoa than in oocytes injected with frozen–thawed spermatozoa (42 of 58 (72%) versus 32 of 58 (55%) for oocytes injected with frozen–thawed spermatozoa), although the effect was not significant. There was no significant difference between sperm

groups in the proportion of cleaved oocytes with one to seven nuclei or eight or more nuclei. Only one embryo, in the fresh sperm group, had > 16 nuclei. The average number of nuclei was 8.6 and 7.7 for fresh and frozen–thawed sperm treatments, respectively. Three equine embryos cultured *in vitro* after staining with Hoechst 33258 are shown to demonstrate cell nuclei (Fig. 1b).

One hundred and two oocytes subjected to ICSI were transferred to oviducts and 68 (67%) were recovered from the oviducts at 96 h after ICSI (Table 4). In one case at the time of transfer the oviduct appeared to have a stricture in the ampulla. Cannulation of the oviduct was attempted three times before the presumptive zygotes (oocytes injected with fresh spermatozoa) were expressed from the pipette shallowly in the ampulla. In this case, 21 oocytes were transferred and only five were recovered from the

Table 3. *In vitro* development of equine oocytes fertilized by intracytoplasmic sperm injection (ICSI) and cultured for 96 h in G1.2 medium

Spermatozoa	Number of oocytes examined (number of replicates)	Number of oocytes cleaved (%)	Number of oocytes cleaved with normal nuclei (%)	Number of embryos (%) with			Average number of nuclei (mean \pm SEM)
				1–7 nuclei	8–16 nuclei	> 16 nuclei	
Fresh	58 (4)	49 (84)	42 (72)	16 (38)	25 (60)	1 (2)	8.6 \pm 0.8
Frozen–thawed	58 (4)	44 (76)	32 (55)	16 (50)	16 (50)	0 (0)	7.7 \pm 0.8
Total	116 (4)	93 (80)	74 (64)	32 (43)	41 (55)	1 (1)	8.2 \pm 0.5

**Fig. 2.** Three equine embryos cultured in G1.2 medium for 96 h after intracytoplasmic sperm injection (ICSI) as visualized by (a) bright field and (b) fluorescence and bright field microscopy after Hoechst 33258 staining (14, 16, 23 nuclei; not all nuclei are visible). Scale bar represents 50 μ m.

oviduct at 96 h after transfer. If this replicate is disregarded, of 81 transferred oocytes, 63 (78%) were recovered. No embryos were recovered from the uterine flushings. There were no significant differences in fertilization rate, cleavage rate, proportion of embryos that had 1–7, 8–16 or > 16 nuclei, or average number of nuclei between oocytes injected with fresh or with frozen–thawed spermatozoa (Table 4). Overall (both sperm treatments together), the proportion of oocytes undergoing cleavage with normal nuclei after oviductal transfer was similar to that for oocytes cultured *in vitro* (75 and 64%, respectively). The mean number of nuclei present in the *in vivo*-recovered embryos was significantly higher than that for *in vitro*-cultured embryos (16 versus 8; $P < 0.01$). Two embryos recovered from the oviducts after transfer and stained with Hoechst 33258 are visualized under bright field and fluorescent exposure (Fig. 3). Some embryos recovered from the oviduct appeared to be infiltrated by cells apparently of

oviductal origin (Fig. 4). The numbers of potential oviductal cell nuclei visible after Hoechst 33258 staining ranged from 0 to > 100. There was no apparent relationship between the number of oviductal cells present and the extent of embryonic development (number of embryonic nuclei; $r = 0.042$).

Discussion

The results from the present study show that high fertilization and cleavage rates may be obtained after ICSI of equine oocytes with either fresh or frozen–thawed spermatozoa, without the use of additional activation treatment. The apparent requirement for chemical activation after ICSI as reported previously (Kato *et al.*, 1997; Guignot *et al.*, 1998; Li *et al.*, 2000; Schmid *et al.*, 2000) may be related to a lesser degree of sperm damage induced during conventional ICSI (scoring the sperm tail with the injection pipette or rolling the spermatozoa against the bottom of dish before

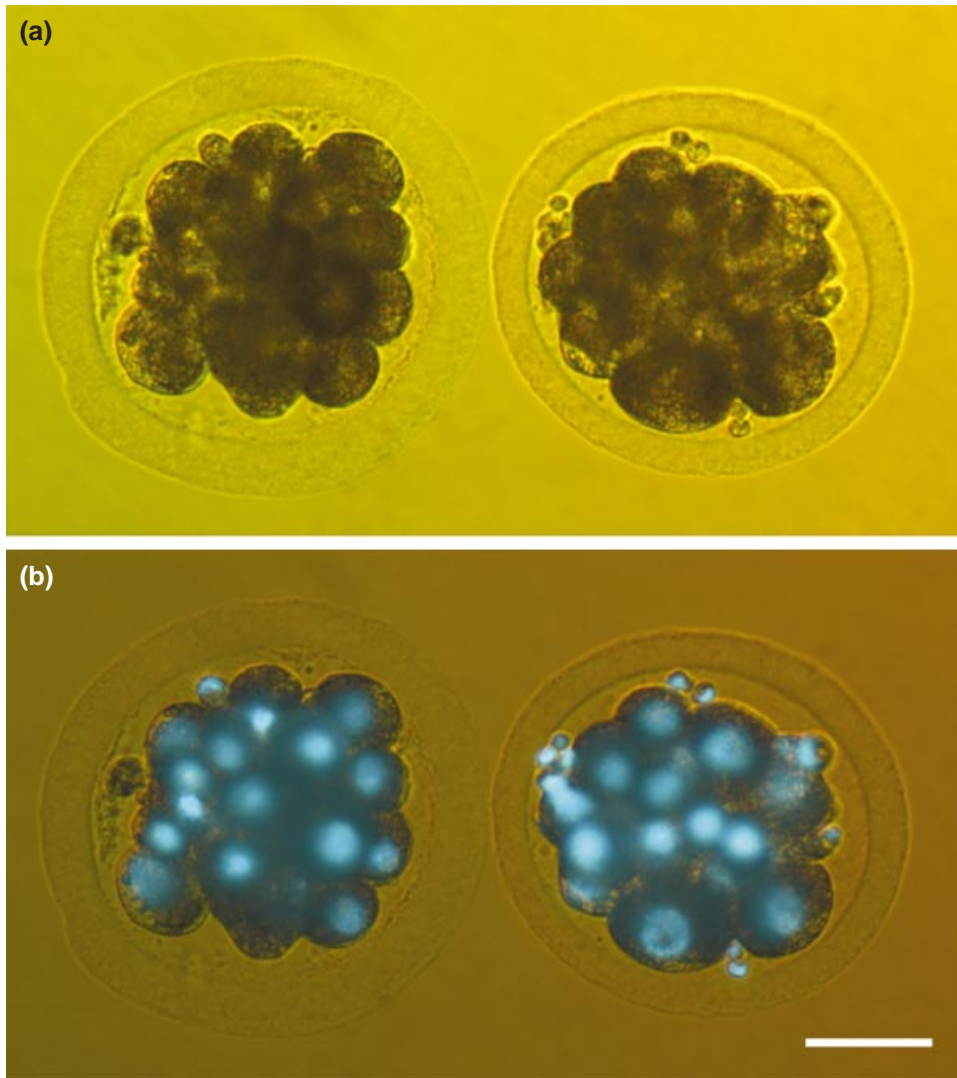


Fig. 3. Equine embryos fertilized by intracytoplasmic sperm injection (ICSI) and transferred to the oviduct for 96 h. Two fertilized embryos both with 22 nuclei as visualized by (a) bright field and (b) fluorescence and bright field microscopy after Hoechst 33258 staining. Some nuclei are not in the plane of focus. Scale bar represents 50 μm .

injection; Catt and O'Neill, 1995; Dozortsev *et al.*, 1995). Further studies comparing conventional and Piezo-driven ICSI directly are required to confirm this point.

Although the differences observed in development between equine oocytes injected with fresh or frozen-thawed spermatozoa were not significant, it is notable that for every parameter measured, equine oocytes injected with fresh spermatozoa had slightly better development than those injected with frozen-thawed spermatozoa. This non-significant but repeatable superiority of oocytes injected with fresh spermatozoa indicates that further work in this area is warranted. In sheep, fertilization rates were higher after ICSI with fresh rather than with frozen-thawed sper-

matozoa (Gomez *et al.*, 1997). In humans, clinical pregnancy rates after ICSI with fresh epididymal or testicular spermatozoa were significantly higher than (De Croo *et al.*, 1998) or equivalent to (Friedler *et al.*, 1998; Janzen *et al.*, 2000) those for frozen-thawed spermatozoa. It is important to note that the performance of fresh and frozen-thawed spermatozoa in this study may be related to either individual differences in the stallion used for the study, or the method used for freezing the spermatozoa.

Use of the Piezo drill in this study resulted in high pronucleus formation and cleavage rates. Increases in efficiency have been reported in mice, humans and cows when ICSI using the Piezo drill was compared with conventional

Table 4. *In vivo* development of equine oocytes fertilized by intracytoplasmic sperm injection (ICSI) and transferred to the oviduct for 96 h

Spermatozoa	Number of oocytes transferred	Number of oocytes recovered (%)	Number of oocytes cleaved (%)	Number of oocytes cleaved with normal nuclei (%)	Number of embryos (%) with			Average number of nuclei (mean \pm SEM)
					1–7 nuclei	8–16 nuclei	> 16 nuclei	
Fresh	48	22 (46)	21 (95)	19 (86)	3 (16)	7 (37)	9 (47)	18.5 \pm 2.5
Frozen-thawed	54	46 (85)	37 (80)	32 (70)	12 (38)	6 (19)	14 (44)	14.4 \pm 1.8
Total	102	68 (67)	58 (85)	51 (75)	15 (29)	13 (25)	23 (45)	16.0 \pm 1.5

Oocytes were transferred to separate oviducts of the three recipients.

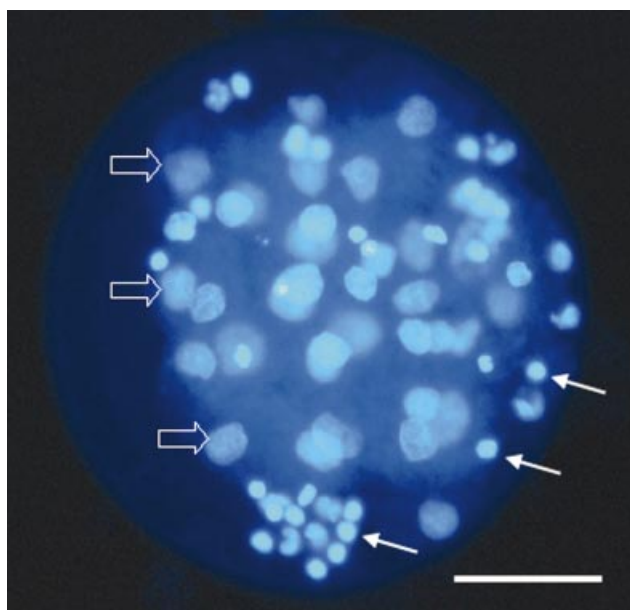


Fig. 4. Hoechst-stained equine embryo recovered from the oviduct 96 h after intracytoplasmic sperm injection (ICSI), showing embryonic nuclei (open arrows) and presumptive oviductal cell nuclei (arrows). Scale bar represents 50 μ m.

ICSI (Kimura and Yanagimachi, 1995; Yanagida *et al.*, 1998; Katayose *et al.*, 1999). Potential reasons for higher efficiency of Piezo microinjection include both increased damage and permeabilization of the sperm membrane, as mentioned above, and more reliable breakage of the oolemma, ensuring that the spermatozoa is deposited in the cytoplasm. Although chemical activation treatment was not required after ICSI, there is a possibility that chemical activation could have a positive effect on the efficiency of ICSI. Some injected equine oocytes (20–33%) had sperm chromosomes at metaphase or an incompletely decondensed sperm head, often with the oocyte in metaphase III. This finding indicates that initial oocyte activation (completion of meiosis) was achieved, a sign that maturation-prompting factor or microtubule-associated protein (MAP) kinase activity was initially reduced, but that the activation stimulus was not maintained long enough to allow

pronucleus formation. Addition of an external activation stimulus may have resulted in full activation of these oocytes.

Although scant data are available on normal development of oviductal embryos in the horse, the development *in vivo* seen in ICSI embryos in the present study compares favourably with that reported for *in vivo*-derived embryos (Betteridge *et al.*, 1982; Peyrot *et al.*, 1987; Bezaud *et al.*, 1989). Recovery of ICSI embryos from the oviducts of mares *in vivo* has been reported by Grohdahl *et al.* (1997) and of five oocytes recovered 48 h after transfer, one had cleaved. Embryonic development (including 24% blastocyst formation) was also reported 7 days after transferring agar-embedded ICSI-derived cleaved equine embryos to the oviducts of sheep (Galli *et al.*, 2000).

The number of nuclei in ICSI presumptive zygotes cultured *in vitro* in the present study was significantly lower than for those transferred to the oviduct. This finding is not surprising in light of previous disappointing results on culture of *in vivo*-recovered equine embryos (Bezaud *et al.*, 1989; Ball *et al.*, 1993). Blastocysts have been produced only sporadically from *in vitro*-derived embryos after co-culture on Vero cell or oviductal monolayers, or trophoblast-conditioned medium (Li *et al.*, 1995; Dell'Aquila *et al.*, 1997b; Guignot *et al.*, 1998; Choi *et al.*, 1999b). Li *et al.* (2001) reported 30% blastocyst development (10 of 33) when two-cell embryos, resulting from oocytes matured in co-culture, were co-cultured with cumulus cells after ICSI. Oocytes not co-cultured during maturation did not develop to blastocysts, indicating the importance of maturation conditions in embryo development after fertilization. Maturation condition (presence of follicular fluid) has also been shown to affect pronucleus formation rate after ICSI of equine oocytes (Dell'Aquila *et al.*, 1997b).

In the present study, G1.2 medium, a low-glucose medium based on synthetic oviductal fluid (SOF; Gardner *et al.*, 1998) was used for embryo culture. G1.2 medium is commonly used for early embryo culture in other species and is typically followed by placement of embryos in G2.2 (higher glucose) medium on day 3 or 4 of culture. Development of human and bovine embryos to the blastocyst stage in G1.2/2.2 medium can be comparable to that achieved *in vivo*; these media were developed after studying the

metabolic requirements of human and bovine oocytes. No such media have been developed for culture of equine embryos on the basis of metabolic studies. The use of G1.2 medium in the present study was based on the work of Azuma *et al.* (1995), in which culture of equine embryos in a low glucose medium for 4 days followed by culture in a higher glucose medium resulted in an increase in the number of nuclei. Cochran *et al.* (1998) also found higher cleavage rates after ICSI (at 48 h) when a low glucose medium (P1) was used, in comparison with M199. Li *et al.* (2000) used G1.2 medium for 30 h of culture after ICSI; however, development of equine embryos after culture for more than 30 h with G1.2 medium has not been reported.

It is difficult to compare the rate of development to the 8–16-cell stage *in vitro* at 96 h in the G1.2 medium as in the present study (53 and 35% of cleaved embryos for fresh and frozen–thawed semen, respectively) with developmental rates reported by other workers. The reported proportion of oocytes developing beyond the two-cell stage is typically on the basis of morphological assessment of blastomeres, rather than on the number of normal nuclei present after staining. Reported rates of development of equine ICSI embryos to 8 or more cells *in vitro* have ranged from approximately 8% (Grondahl *et al.*, 1997), approximately 19% (MacLellan *et al.*, 2000) and 23% (Guignot *et al.*, 1998), to approximately 39% (Dell'Aquila *et al.*, 1997b) and 25–70% (Li *et al.*, 2001).

Oocytes subjected to ICSI have a defect in the zona pellucida of around 8 µm in diameter due to drilling with the Piezo instrument, and it has been hypothesized that after transfer this may allow maternal cellular defences to enter the embryo, resulting in embryo loss. In the present study, numerous cells were observed, apparently from the oviduct, within the perivitelline space of the embryos. With few exceptions, these cells did not appear to be polymorphonuclear. It is possible that these cells represent pyknotic or apoptotic blastomere nuclei; however, their presence in only oviductal-transferred embryos (which have better development than *in vitro*-matured embryos), and their large number, indicates that this is not the case. However, normal development for up to 96 h, as estimated by number and morphology of nuclei, occurred in the presence of these cells, and there was no correlation between number of oviductal cells and extent of the embryonic development.

Bovine oocytes have been used as cytoplasts for transfer of equine nuclei, with development to the 16-cell stage (Hinrichs *et al.*, 2000). Results from the present study indicate that bovine oocytes may be fertilized successfully with equine spermatozoa via ICSI, that is, may achieve both sperm head decondensation and oocyte activation. Thus, bovine oocytes are a possible alternative source of oocytes for research on stallion sperm nuclei transformation mechanisms, as equine oocytes are difficult to obtain in large numbers. It is notable that in many reports in cattle, sperm decondensation rates after ICSI of bovine oocytes have been low (Catt and Rhodes, 1995; Rho *et al.*, 1998; Chung *et al.*, 2000). Use of the Piezo drill appears to overcome the need

for chemical activation in this species (Katayose *et al.*, 1999).

In conclusion, high fertilization and cleavage rates were achieved in the present study, without chemical activation, in equine oocytes fertilized with fresh or frozen–thawed spermatozoa by ICSI using the Piezo drill. Embryo development tended to be more advanced in oocytes inseminated with fresh spermatozoa but no significant differences were noted. Oocytes transferred to the oviduct *in vivo* developed at a rate comparable with that reported for *in vivo*-derived embryos, and had significantly greater embryonic development at 96 h than did embryos cultured *in vitro* in a semi-defined medium (G1.2). Bovine oocytes may serve as a model for equine oocytes in investigation of fertilization in horses.

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