Effects of different activation treatments on fertilization of horse oocytes by intracytoplasmic sperm injection

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The effects of four reagents on the activation and subsequent fertilization of equine oocytes, and the development of these after intracytoplasmic sperm injection, were investigated. Cumulus–oocyte complexes collected from equine ovaries obtained from an abattoir were matured in vitro for 40–44 h in TCM199 medium before being injected, when in metaphase II, with an immobilized stallion spermatozoon. The cumulus–oocyte complexes were then subjected to one of five activation treatments: (a) 10 μmol ionomycin l⁻¹ for 10 min; (b) 7% (v/v) ethanol for 10 min; (c) 100 μmol thimerosal l⁻¹ for 10 min; (d) 250 μmol inositol 1,4,5-triphosphate l⁻¹ injection; and (e) no treatment (control). After 18–20 h further culture, the cumulus–oocyte complexes were assessed for activation by observing whether they had progressed through second anaphase–telophase and had formed a female pronucleus. The proportions of oocytes activated after each treatment were: 16/27 (59%) for ionomycin; 14/25 (56%) for ethanol; 22/28 (79%) for thimerosal; 15/27 (56%) for inositol 1,4,5-triphosphate; and 0/20 (0%) for the untreated controls. Thus, significantly more oocytes (P < 0.05) were activated by treatment with thimerosal than by the other four treatments. The proportions of oocytes that cleaved to the two-cell stage at 24–30 h after sperm injection in the groups treated with ionomycin, ethanol and thimerosal were 7/20 (35%), 5/19 (26%) and 11/23 (48%), respectively. No cleavage was observed in any of the control oocytes or those treated with inositol 1,4,5-triphosphate. Furthermore, evidence of normal fertilization was observed in 2/7 (29%), 2/5 (40%) and 7/11 (64%) of the oocytes treated with ionomycin, ethanol and thimerosal, respectively. These results demonstrated that: (a) it is possible to activate equine oocytes with the chemical stimulants, ionomycin, ethanol, thimerosal and inositol 1,4,5-triphosphate; (b) thimerosal is more effective than the other three reagents in facilitating both meiotic activation and normal fertilization of equine oocytes; and (c) chemical activation may also stimulate parthenogenetic cleavage of oocytes without concurrent changes in the head of the spermatozoon.

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decondensation of the sperm head in the ooplasm. In vitro fertilization (IVF) has been less successful in equids than in other large domestic species, either using conventional incubation of oocytes with spermatozoa, or ICSI (Zhang et al., 1989, 1990; Grondahl, 1998). The calcium ionophore A23187, ionomycin and ethanol have all been used to stimulate activation of equine oocytes for ICSI but these treatments have given cleavage rates of only 20–40% (Guignot et al., 1996; Dell Aquila et al., 1997; Kato et al., 1997) and only three live foals have been produced to date using ICSI (Squires et al., 1996; McKinnon et al., 1998).

The pattern of the increase in intracellular Ca²⁺ in oocytes is reported to differ among the various chemical treatments used to activate the oocytes and facilitate normal fertilization (Miyazaki et al., 1992; Fissore and Robl, 1993, 1994; Nakada and Mizuno, 1998). For example, reagents like the calcium ionophore A23187, ionomycin and ethanol induce only one increase in intracellular Ca²⁺ during a single treatment, which differs from the repeated spike-like increases in intracellular Ca²⁺ that occur characteristically during normal fertilization. However, thimerosal induces a pattern of spikes in intracellular Ca²⁺ concentrations that resembles normal fertilization by a spermatozoon (Swan, 1991). Inositol 1,4,5-triphosphate (InsP₃) acts as a second messenger for the fertilization by a spermatozoon (Swan, 1991). Inositol 1,4,5-triphosphate (InsP₃) acts as a second messenger for the release of intracellular Ca²⁺ and has proved to be an effective oocyte activator in mammalian ICSI (Fissore and Robl, 1993; Nakada and Mizuno, 1998).

In the present study, four chemicals were compared for their ability to activate equine oocytes after ICSI. The criteria for successful activation included decondensation of the sperm head and formation of the female pronucleus, followed by the development of normal two-cell embryos.

Materials and Methods

Collection and in vitro maturation of oocytes

Horse ovaries were obtained from an abattoir and transported to the laboratory at a temperature of 8–15°C during 22–24 h. Cumulus–oocyte complexes (COCs) were recovered by scraping the walls of follicles, and groups of 20–30 COCs were matured in vitro for 40–44 h at 38°C in 5% CO₂ in air in 500 μl aliquots of 25 mmol Hepes-buffered TCM199 (Gibco, catalogue number 22340-012, Grand Island, NY) supplemented with 20% (v/v) heat inactivated fetal bovine serum (FBS, Gibco), 10 μg FSH ml⁻¹ (Sigma Chemicals, St Louis, MO), 5 μg LH ml⁻¹ (Sigma) and 1 μg oestradiol ml⁻¹ (Sigma) under mineral oil (Sigma) in four-well culture plates.

Preparation of spermatozoa for ICSI

The influence of the acrosome status of stallion spermatozoa has been examined in equine ICSI by Li et al. (2000) who reported that the acrosome reaction is not essential for oocyte activation and formation of the male pronucleus, but the motility of the spermatozoon and its pre-decondensation in vitro before injection will improve the fertilization rate. Therefore, only motile spermatozoa were selected for ICSI in the present study. Semen was collected by artificial vagina from either of two identical twin Pony stallions of proven high fertility. Sperm motility was assessed under a microscope immediately before the ejaculate was diluted with an equal volume of the skimmed milk-based extender described by Kenney et al. (1975). The extended semen was then allowed to cool slowly to 4°C in an insulated container (Equitainer; Animal Reproduction Systems, Chino, CA) before a 1.5 ml aliquot was layered carefully onto 2 ml of a discontinuous 90:45% Percoll (Sigma) density gradient in a 15 ml conical centrifuge tube. The semen was then centrifuged through the Percoll layers, initially for 5 min at 200 × g and then for 15 min at 800 × g. The supernatant was gently aspirated to leave a 100 μl pellet of highly concentrated spermatozoa which was resuspended in 2–3 ml Earle’s balanced salt solution (EBSS, Gibco) containing 0.3% BSA. Finally, the mixture was adjusted to a concentration of 5–7 × 10⁷ spermatozoa ml⁻¹ and maintained at 38°C until used for ICSI. It took 45–60 min to process each semen sample in this manner.

ICSI and activation treatment of oocytes

The ICSI procedure was performed using an Eppendorf micromanipulator (Transferman, Hamburg) attached to an inverted microscope (Olympus IMT-2, Tokyo). All manipulations were performed on a heated stage (CO 102; Linkam, Tadworth), which provided a working temperature of 30°C. ICSI was carried out in 20 μl drops of 20 mmol Hapes-buffered EBSS containing 20% FBS, using spermatozoa that had been held in a separate 10 μl drop of the ICSI medium containing 30% (v/v) polyvinylpyrrolidone (PVP, FW 40000, Sigma).

After 40–44 h of in vitro maturation (IVM), the COCs were placed in EBSS containing hyaluronidase (200 iu ml⁻¹; Sigma) for 3–5 min, after which the cumulus cells were removed by gentle pipetting. Second metaphase oocytes (MII) that had extruded their first polar body were selected from these denuded oocytes and injected, in groups of 5–10, into the drop of ICSI medium. The injection pipette (ICSI Pipette Kit 2, Hunter Scientific Ltd, Saffron Walden) was directed into the drop containing the motile spermatozoa, one of which was immobilized by swiping the injection pipette across its tail before aspirating it into the pipette in a minimal volume of medium. The pipette was guided back into the ICSI drop containing the oocytes and one oocyte was held stationary on the end of the holding pipette by suction (ICSI Pipette Kit 3, Hunter Scientific Ltd). The injection pipette containing the spermatozoon was then pushed through the zona pellucida and the plasma membrane so that the spermatozoon could be injected into the cytoplasm. This procedure was repeated for each selected MII oocyte and all of the injected oocytes were returned to the IVM medium for incubation until the activation treatment was initiated.

Within 30–60 min after ICSI, the oocytes were re-allocated into one of the five following treatment groups: (a) EBSS medium containing 10 μmol ionomycin l⁻¹ (Sigma) for 10 min; (b) EBSS medium containing 7% (v/v) ethanol (BDH, Poole)
for 10 min; (c) EBSS medium containing 100 μmol thimerosal l⁻¹ (Sigma) for 10 min; (d) injection of 10–20 nA of 250 μmol InsP₃ l⁻¹ (Sigma) into the cytoplasm; and (e) no activation treatment (control). After these activation treatments, the oocytes were washed twice in normal culture medium (G1.2, IVF Science, Gothenburg) and incubated in fresh medium at 38°C in an atmosphere of 5% CO₂ in air.

Assessment of oocyte activation

At 18–20 h after sperm injection, half the cultured oocytes in each group were mounted on a glass microscope slide as described by Iwasaki and Li (1994), fixed overnight in a solution of ethanol:acetic acid (3:1, v/v), stained for 1 h with 1% (w/v) aceto-orcein and examined under a microscope for signs of activation. These signs included: (a) progression of the oocyte nucleus from the MII to the anaphase–telophase (AII–TII) stage; (b) formation of the female pronucleus (FPN); and (c) progression through the first cleavage division. Examination of the injected spermatozoon using a phase-contrast microscope enabled it to be classified into one of three categories: (a) condensed sperm head (CS); (b) decondensed sperm head (DS); and (c) male pronucleus formed (MPN; Fig. 1).

Assessment of normal fertilization from parthenogenetic cleavage

The other half of the injected oocytes in each group were cultured for 24–30 h and those that had cleaved to form two-cell stage embryos were stained with Hoechst 33342 (Sigma) in 5 μg G1.2 medium ml⁻¹ for 10 min at 30–35°C before being examined under ultraviolet light (excitation wavelength 330–380 nm) using a fluorescent microscope (Fig. 2). Embryos...
at the two-cell stage of development that had been fertilized normally could be expected to show a single nucleus in each of the two blastomeres. Parthenogenetic cleavage was distinguished from fertilization by identifying a condensed sperm head in one of the two blastomeres.

A total of 219 oocytes was used in the experiment and each treatment was repeated 3–5 times. The results were evaluated by chi-squared analysis.

**Results**

**Oocyte activation**

The results of the five oocyte activation treatments are shown (Table 1). At 18–20 h after injection of the spermatozoon, the proportions of oocytes that had undergone successful activation (AII–TII, FPN and progression throughout the first cleavage division) were 16/27 (59%), 14/25 (56%), 22/28 (79%), 15/27 (56%) and 0/20 (0%) after treatment with, respectively, ionomycin, ethanol, thimerosal, InsP$_3$ and no treatment (control group). Treatment with thimerosal significantly ($P < 0.05$) increased the proportion of oocytes that were activated compared with the other four treatments. The proportions of oocytes that exhibited a condensed sperm head and appeared to have been unaffected by the activation treatment were 13/27 (48%), 18/25 (72%), 8/28 (29%), 6/27 (22%) and 18/20 (90%) for the five treatment groups, respectively. The untreated (control) and the ethanol-treated groups both showed a significantly ($P < 0.05$) higher proportion of oocytes containing non-decondensed sperm heads than did the other three treatment groups. Treatment with thimerosal resulted in the highest proportion of oocytes (8/28, 29%) that had formed a male pronucleus ($P < 0.01$) compared with treatment with the other reagents. After sperm injection alone, in the absence of any chemical activation treatment, 16/20 control oocytes remained at MII and 18/20 control oocytes showed sperm heads that had not begun to decondense.

![Fig. 2. Fertilization status of two-cell stage horse embryos derived from intracytoplasmic sperm injection and cultured in vitro for 36–40 h (5 μg Hoechst stain ml$^{-1}$ for 10 min). Fertilized two-cell embryo before (a) and after (b) Hoechst staining. Parthenogenetic two-cell embryo before (c) and after (d) Hoechst staining, showing a condensed sperm head (CS) in a blastomere. Scale bar represents 20 μm.](image_url)
Normal fertilization versus parthenogenetic cleavage

Fertilization rates achieved in the four different oocyte-activation treatment groups are compared (Table 2). The proportion of oocytes that cleaved to the two-cell stage was significantly higher in the thimerosal treatment group (11/23; \(P < 0.05\)) than in the other three treatment groups. Neither fertilization nor parthenogenetic cleavage had occurred in the InsP3-treated or the untreated (control) groups of oocytes. Normal fertilization had occurred in 2/7 oocytes activated with ionomycin, 2/5 activated with ethanol and 7/11 activated with thimerosal. Thus, thimerosal activation resulted in a significantly higher proportion of oocytes being fertilized normally by ICSI compared with the other activation treatments (\(P < 0.05\)). Parthenogenetic cleavage occurred in 5/7 oocytes treated with ionomycin, 2/5 treated with ethanol and 3/11 treated with thimerosal.

Discussion

During fertilization of mammalian oocytes, the spermatozoon binds to the zona pellucida and then penetrates through it to fuse with the plasma membrane. This fusion results in oocyte activation. One component of activation is the resumption of meiosis, which results in pronucleus formation, the initiation of DNA synthesis and the first cleavage division (Cuthbertson and Cobbold, 1985; Swan, 1991; Ben-Yosef and Shalgi, 1998). Once the spermatozoon has been incorporated into, and has activated, the ooplasm, it undergoes decondensation and the initial stages of sperm–oocyte interaction are then complete. The use of chemical reagents to activate oocytes during the ICSI procedure provides a means of bypassing the requirement for the spermatozoon to perform this function.

The calcium ionophore A23187, thimerosal, ionomycin, ethanol and electrophoresis have all been used widely in nuclear transfer and ICSI experiments to activate oocytes after injection of either a nucleus or a spermatozoon (Nagai, 1987; Onodera and Tsunoda, 1989; Hagen et al., 1991; Greenblatt et al., 1995; Kono et al., 1996; Catt et al., 1996; Keskintepe et al., 1997; Hamano et al., 1999). These activation treatments induce a transient increase in the intracellular concentration of Ca\(^{2+}\) in the oocytes. For nuclear transfer, the object of the activation treatment is to induce fusion of the injected nucleus with the host cytoplasm and thereby initiate oocyte division. For the ICSI procedure, activation of the oocyte is required to induce the resumption of meiosis, decondensation of the sperm head and subsequent formation of the male pronucleus. In normal fertilization (both \textit{in vivo} and \textit{in vitro}), almost all of the penetrated spermatozoa become decondensed within an activated cytoplasm. In the present study, treatment of horse oocytes with thimerosal resulted in the highest rate of oocyte activation (79%). A smaller percentage (57%) of the thimerosal-treated oocytes injected with a spermatozoon showed sperm head changes (decondensing head, MPN and the first cleavage division), but only 33% of the oocytes treated with ionomycin and 16% of those treated with ethanol showed similar sperm head changes indicative of fertilization. These findings indicate that oocyte activation and sperm changes are not necessarily stimulated by chemicals used to induce oocyte activation.

The pattern of intracellular Ca\(^{2+}\) changes in the oocyte during fertilization has been investigated in various mammals. The initial Ca\(^{2+}\) release is relatively prolonged and...
is followed over a 1–3 h period by intermittent Ca\(^{2+}\) spikes at 5–20 min intervals (Kline and Kline, 1992; Miyazaki et al., 1992; Nakada et al., 1995; Nakada and Mizuno, 1998). It has been found that thimerosal induces multiple intracellular calcium transients in unfertilized mammalian oocytes by sensitizing InsP\(_3\)-induced intracellular calcium release (hamster: Swann, 1991; Miyazaki et al., 1992; rabbit: Fissore and Robl, 1993; mouse: Kline and Kline, 1994; cow: Nakada and Mizuno, 1998; pig: Machaty et al., 1997; Wang et al., 1999), in contrast to other artificial activators (for example, electrical pulse, ethanol, calcium ionophore A23187, and ionomycin) which induce only one calcium transient. Treatment with ionomycin or ethanol induces a single increase in intracellular Ca\(^{2+}\) after 1–5 min (Nakada and Mizuno, 1998), whereas Nakada and Mizuno (1998) showed that treatment of bovine oocytes with thimerosal induces repeated spike-like increases in intracellular Ca\(^{2+}\) concentration, more reminiscent of normal fertilization. The higher proportion of activated oocytes and sperm head changes observed after thimerosal treatment compared with ionomycin or ethanol in the present study implies that repeated increases in intracellular Ca\(^{2+}\) are necessary to induce activation and fertilization of equine oocytes subjected to ICSI. In cattle, the duration of the first intracellular calcium transient induced by thimerosal was shorter than that during fertilization, and the subsequent oscillatory intracellular calcium responses had short-lived increases and shorter intervals between peak values than the responses of bovine oocytes during fertilization. In the present study, activation of horse oocytes was induced more effectively by treatment with thimerosal than by the other treatments, which all resulted in lower activation rates and increased proportions of parthenogenetic embryos. Therefore, it may be assumed that the mechanism of oocyte activation during fertilization is different from that of the sensitizing InsP\(_3\)-induced intracellular calcium release stimulated by artificial treatment. However, the exact pattern of Ca\(^{2+}\) release in horse oocytes induced by these treatments needs to be elucidated further.

The actions of inositol phosphate acting as the second messenger to induce intracellular Ca\(^{2+}\) release have been reported in several mammalian oocytes. For example, in golden hamsters, addition of anti-inositol phosphate antibodies during IVF effectively blocks the normal oscillations in Ca\(^{2+}\) concentrations (Miyazaki et al., 1992). In the present study, 56% of the oocytes injected with InsP\(_3\) underwent activation (from MII to ALI–TLI, FPN formation and the first cleavage division). Although this rate of activation was lower than the rates achieved in the other treatment groups, the rate of sperm head transformation was higher in the InsP\(_3\)-injected oocytes than in the ionomycin and ethanol treatment groups (56 versus 33 and 16%, respectively, P < 0.05). Nakada and Mizuno (1998) suggested that the single increase in intracellular Ca\(^{2+}\) after InsP\(_3\) injection resembles that induced by the ionomycin and ethanol treatments. However, the present findings indicate that the fertilization process in oocytes activated by inositol phosphate ceased before or during the first cleavage metaphase, and that no further development occurred. Clarification of this point is required before InsP\(_3\) can be considered for activation of horse oocytes after ICSI.

In both sheep and cattle, 50–80% of oocytes subjected to ICSI cleaved to the two-cell stage when stimulated chemically to induce activation. However, the developmental potential of these two-cell embryos was lower, when either cultured in vitro or transferred in vivo, than that of embryos derived from conventional IVF (Li et al., 1993; Catt et al., 1996; Gomez et al., 1998; Hamano et al., 1999). Similar poor developmental capacity has been reported after ICSI of horse oocytes (Dell Aquila et al., 1997; Kato et al., 1997; Grondahl et al., 1998) with the result that only three live foals have been produced to date (Squires et al., 1996; McKinnon et al., 1998). At normal fertilization, oocytes are activated by the penetration of the spermatozoon, and the heads of most spermatozoa in the ooplasm decondense before transforming into MPN stages (Squires et al., 1996; McKinnon et al., 1998). After ICSI in bovine oocytes, the rate of development of two-cell embryos to the eight-cell stage was 38%, which was approximately half the rate achieved after conventional IVF. This finding indicated that many (30–50%) of the two–eight-cell embryos obtained after ICSI were parthenogenetic (Li et al., 1999). When there is a low rate of sperm head decondensation in the cytoplasm, the oocyte activation treatment may still induce parthenogenetic

### Table 2. Ratio of normal fertilization to parthenogenetic development in two-cell embryos obtained after intracytoplasmic sperm injection (ICSI) after different chemical treatments to induce activation of the oocytes

<table>
<thead>
<tr>
<th>Activation treatment</th>
<th>Number of two-cell embryos/number of oocytes (%)</th>
<th>Development pattern/number of oocytes (%)</th>
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<tbody>
<tr>
<td></td>
<td>Fertilization</td>
<td>Parthenogenesis</td>
</tr>
<tr>
<td>10 μmol ionomycin l(^{-1}) for 10 min</td>
<td>7/20 (35%)</td>
<td>2/7 (29%)</td>
</tr>
<tr>
<td>7% ethanol for 10 min</td>
<td>5/19 (26%)</td>
<td>2/5 (40%)</td>
</tr>
<tr>
<td>100 μmol thimerosal l(^{-1}) for 10 min</td>
<td>11/23 (48%)</td>
<td>7/11 (64%)</td>
</tr>
<tr>
<td>10–20 nA of 250 μmol InsP(_3) l(^{-1}) injected into cytoplasm</td>
<td>0/15 (0%)</td>
<td>0/15 (0%)</td>
</tr>
<tr>
<td>No activation treatment (control)</td>
<td>0/15 (0%)</td>
<td>0/15 (0%)</td>
</tr>
</tbody>
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Values in a column with different superscripts are significantly different (P < 0.05).
development of ICSI oocytes. In the present study, between 27 and 71% of the two-cell embryos produced after ICSI were parthenogenetic, which is a higher figure than those reported for other farm animals and has important implications in terms of the potential use of ICSI to produce foals from high class mares remaining in active competition. Further investigation is needed to determine the reason why such a high rate of parthenogenesis should occur after ICSI in this species.

In summary, the present study demonstrated that it is possible to activate equine oocytes with the chemical stimulants, ionomycin, ethanol, thimerosal and inositol trisphosphate, and that thimerosal is more effective than the other three in facilitating both meiotic activation and normal fertilization of equine oocytes during the ICSI procedure. Further research is needed to investigate the relationship between differential maturation of the nucleus and cytoplasm in equine oocytes and its influence on fertilization and the developmental capacity of early embryos after ICSI.

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