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Time course of pronuclear formation and fertilisation after insemination *in vitro* and intracytoplasmic sperm injection of *in vitro* matured sheep oocytes

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Summary

The time course of sperm decondensation, oocyte activation, pronuclear formation and the possible causes of abnormalities after intracytoplasmic sperm injection (ICSI) and *in vitro* fertilisation (IVF) were examined. Frozen-thawed and pooled fresh semen from three different rams were washed and capacitated for ICSI or IVF. *In vitro* matured oocytes were cultured after sperm injection for 0.5, 0.75, 1, 2, 3, 4, 5, 6, 8, 18, 21 and 23 h, and oocytes were cultured after *in vitro* insemination for the same times other than 18 and 23 h. All oocytes were cultured in bicarbonate-buffered synthetic oviduct fluid medium (BSOF) supplemented with 2% oestrous sheep serum. A total of 746 metaphase II oocytes were injected with a single spermatozoon and 986 oocytes were inseminated for IVF. The earliest oocyte activation after ICSI was observed at 0.5 h, when 14.8% of oocytes were in anaphase II; this was earlier than after IVF, when only 6.4% of the oocytes exhibited anaphase II 1 h after insemination. Decondensing spermatozoa were first observed 1 h after ICSI and 3 h after insemination for IVF. The earliest female and male pronuclei after ICSI were observed at 2 and 3 h respectively, while the female and male pronuclei after IVF were observed at 4 h after insemination. The overall fertilisation rate was lower after ICSI (28.6%) than IVF (70.4%) but the percentage of abnormal fertilisation was not different between ICSI (8.7%) and IVF (15.2%). It was concluded that the fertilisation events were more advanced for ICSI than IVF, using injection and insemination time as reference points. The formation of male and female pronuclei were asynchronous after ICSI, in contrast to IVF when they appeared simultaneously at 4 h. Abnormalities found in fertilisation after ICSI may therefore be induced by the injection technique.

Keywords: ICSI, IVF, Oocyte activation, Sheep, Sperm decondensation

Introduction

During fertilisation, the sequence of events after the spermatozoon has been incorporated into the egg cytoplasm *in vivo* or *in vitro* is the breakdown of the spermatozoon nuclear envelope, contact of the chromatin with the egg cytoplasm, spermatozoal nuclear decondensation and male pronuclear formation. Simultaneously, egg activation is induced by penetration of the spermatozoon, resulting in completion of metaphase II, extrusion of the second polar body and formation of the female pronucleus. The timing of these fertilisation events may be altered during intracytoplasmic sperm injection (ICSI), resulting in abnormal fertilisation. After ICSI in humans the events of fertilisation occur earlier than after *in vitro* fertilisation (IVF) (Nagy *et al.*, 1994; Dozortsev *et al.*, 1995) and the difference in time has been estimated as 2–4 h. ICSI has also been associated with asynchrony of the appearance of pronuclei (Nagy *et al.*, 1994), imbalance between the cell cycle stage of the oocyte and the spermatozoon (Edwards, 1995), abnormal activation (Cohen *et al.*, 1995; Edwards, 1995) and abnormal fertilisation (Staessen & Van Steirteghem, 1997). These results suggest that the pattern of oocyte activation after ICSI

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might be different from the activation pattern after normal fertilisation.

Although the birth of normal lambs has been obtained after ICSI (Catt *et al.*, 1996; Gómez *et al.*, 1998), the fertilisation rate (30.1%; Gómez *et al.*, 1977) and rate of development to blastocyst (8%; Gómez *et al.*, 1988) of sheep oocytes are still low after ICSI. A variety of factors could be responsible for the low rate of fertilisation and blastocyst development after ICSI, but it is known that the principal cause of fertilisation failure after ICSI is inadequate oocyte activation (Gómez *et al.*, 1997) possibly combined with abnormal fertilisation. In the present study, the time course of sperm decondensation, oocyte activation, pronuclear formation and the possible causes of abnormalities after ICSI and IVF have been examined.

Materials and methods

Oocyte collection and preparation

Ovaries from adult sheep were obtained from an abattoir and transported (at 25 °C) to the laboratory within 2 h of slaughter. The ovaries were washed three times in phosphate-buffered saline (PBS) solution containing 250 IU/ml penicillin (Sigma, St Louis, MO), 0.09 mg/ml streptomycin (Sigma) and 50 µg/ml neomycin (Sigma) and then washed a further three times in PBS medium at 30 °C. Oocytes were aspirated from follicles (2–8 mm in diameter) using a 1 ml syringe and 21 G needle and collected into tissue culture medium 199 (M-199 with Earle's salts, l-glutamine, without sodium bicarbonate; Gibco-BRL, Grand Island, NY) supplemented with 10.5 mM Hepes (free acid; Sigma), 10.5 mM sodium Hepes (Sigma), 4 mM sodium bicarbonate (Sigma), 0.01 mg/ml heparin sodium salt (Grade 1A, from porcine intestinal mucus; Sigma), 125 IU/ml penicillin (Sigma), 0.05 mg/ml streptomycin (Sigma), 0.08 mg/ml kanamycin (Sigma) and 10% fetal bovine serum (FBS; Multiser, Cytosystems P/L, Castle Hill, NSW, Australia; H199+). After rinsing three times in this medium the cumulus–oocyte complexes were matured in multiwell dishes (Nunclon; Inter Med, Roskilde, Denmark), with each well containing 40–50 oocytes and 500 µl of maturation medium under paraffin oil (Ajax Chemicals, Auburn, NSW, Australia), for 18–20 h (ICSI) or 22 h (IVF) at 39 °C in a humidified atmosphere of 5% CO_2 in air. The maturation medium was M-199 (with Earle's salts, L-glutamine, 25 mM sodium bicarbonate, 25 mM Hepes; Gibco) supplemented with 10% FBS (v/v), 10 µg/ml FSH (Folltropin-V; Vetrepharm, Essendon, Victoria, Australia), 10 µg/ml LH (Lutropin-V; Vetrepharm) and 0.3 mM pyruvate (Sigma).

After *in vitro* maturation the oocytes for ICSO or IVF were denuded of their cumulus by gentle pipetting and washed twice in H199+. The cumulus-free oocytes were transferred to multiwell dishes (Nunclon) for IVF (n = 50 per dish) or 1 ml plastic tubes for ICSI (n = 10 per tube) in 500 µl of bicarbonate-buffered synthetic oviduct fluid medium (BSOF) supplemented with 2% oestrous sheep serum (v/v; collected and processed from a ewe on day 3 of the oestrous cycle), 1 mM pyruvate and 1 mM L-glutamine. The oocytes were then cultured for 2–4 h (ICSI) or 2 h (IVF) at 39 °C in 5% CO_2 in air prior to microinjection or insemination.

Preparation of spermatozoa

Pooled fresh or frozen-thawed semen from three different rams was diluted (1:20, semen:medium) in Hepes-buffered modified SOF (HSOF) supplemented with 3 mg/ml (0.3%) bovine serum albumin (BSA fraction V, Sigma), 4.0 mM sodium bicarbonate, 2.4 mM d-glucose (Analar, BDH Chemicals, Victoria, Australia), 0.33 mM pyruvate, 6.0 mM L-glutamine, 75 µg/ml kanamycin, 125 IU/ml penicillin and 50 µg/ml streptomycin and held for 1 h at room temperature. Two-layer Percoll gradients (90%–45%) were prepared in 1 ml Eppendorf tubes: 500 µl of 90% Percoll was placed at the bottom of the tube, and 500 µl of 45% Percoll was carefully layered on the top, avoiding mixing of the two layers; 100 µl of the diluted semen was placed at the top of the Percoll gradient. The samples were centrifuged at 5000 *g* for 2 min. The top layer of Percoll was discarded and the pellet (containing spermatozoa) was resuspended in 1 ml of HSOF and centrifuged again at 5000 *g* for 1 min. The supernatant was discarded and the spermatozoa purified by the 'swim-up' technique.

Spermatozoa for ICSI were incubated for 1–4 h depending on the time required to complete injection of oocytes. For ICSI, 10 µl of medium with spermatozoa was aspirated from the sperm tube and diluted with 100 µl of 10% (v/v) polyvinylpyrrolidone (PVP, MW 360 000). This reduced the velocity of motility of spermatozoa and facilitated the capture of a spermatozoon. A small drop (about 2 µl) of this suspension of spermatozoa was kept under paraffin oil in a plastic Petri dish before injection into oocytes.

Microinjection procedure

The method of microinjection has been described previously (Gómez *et al.*, 1997). Briefly, the ICSI procedure was carried out on the heated stage (39 °C) of an inverted microscope (Diaphot; Nikon, Tokyo, Japan) at x2000 magnification using Normarski optics (Nikon). A 9 cm Petri dish modified with four holes that were covered with a glass coverslip to ensure compatibility with the Nomarski optics was used as an injection dish. The injection dish contained two droplets covered with

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paraffin oil. One droplet contained sperm–PVP suspension (2 µl) and the other droplet 10 µl of HSOF containing 5 oocytes. For the injection, a single spermatozoon was immobilised by striking the tail with the pipette and aspirated tail first into the tip of the injection pipette together with a small amount of cytoplasm (to ensure plasmalemma breakage). The oocyte was held with the holding pipette while the injection pipette was introduced into the cytoplasm. The sperm was then deposited within the oocyte cytoplasm with a minimum amount of PVP medium (< 5 pl). After the injection the oocyte was moved to the edge of the droplet and released from the holding pipette to avoid repeating the manipulation. Injected oocytes were washed twice in BSOF medium, and incubated in groups of 10–15.

Oocyte fertilisation and culture

For ICSI each treatment was replicated 6 times. After ICSI, the oocytes were cultured in groups of 10–15 in multiwell dishes in 500 µl of BSOF medium supplemented with 2% sheep serum, covered by paraffin oil, for 0.5, 0.75, 1, 2, 3, 4, 5, 6, 8, 18, 21 and 23 h in 5% CO_2 in humidified air at 39 °C.

For IVF each treatment was replicated 2 times. The oocytes were co-incubated with 6×10^6 motile sperm / ml in groups of 30 in multiwell dishes in 500 µl of BSOF medium supplemented with 2% sheep serum, covered by paraffin oil, in 5% CO_2 in humidified air at 39 °C for the same times as for ICSI other than 18 and 23 h.

Assessment of survival, fertilisation and activation of oocytes

Injected and IVF oocytes were examined for evidence of degenerative changes after their respective culture treatments. Those oocytes that survived after ICSI and IVF (no signs of degeneration) were fixed (acetic acid and ethanol, 1:3) for about 24 h and then stained with aceto-orcein (0.25% in 45% acetic acid). The oocytes were examined using phase contrast microscopy for oocyte activation, sperm decondensation and pronuclear formation. Activated oocytes were classified as follows: early oocyte activation (those oocytes that were activated and were at anaphase II or telophase II and contained an undecondensed spermatozoon), parthenogenetic activation (those oocytes with one or two polar bodies, one or two pronuclei and an undecondensed spermatozoon. Fertilisation was classified as: normal fertilisation (those oocytes containing two polar bodies, two pronuclei and a sperm tail within their cytoplasm), abnormal fertilisation (those oocytes containing one to three pronuclei, one polar body and a sperm tail within their cytoplasm) and polyspermy (those IVF oocytes that contained more than three pronuclei and more than two sperm tails). Unactivated oocytes consisted of those in metaphase II with one polar body and an undecondensed spermatozoon (intact head), and those with a decondensed spermatozoon (swollen head) and sperm tail.

Decondensation of spermatozoa was classified as follows: intact head, swollen head and pronucleus formed. For IVF, an intact head indicated that the spermatozoon had penetrated the oocyte but its head had not decondensed.

Statistical analysis

The data on normal and abnormal fertilisation, unactivated oocytes, activation and parthenogenetic activation were analysed by chi-squared test. The chi-squared test with Yates' correction was also used to test the differences between treatments. The relationship between early oocyte activation and sperm decondensation was determined by regression analysis. All analyses were performed by the Minitab (version 8.2) computer program.

Results

A total of 746 metaphase II oocytes were injected with a single spermatozoon (ICSI) and 986 oocytes were inseminated (IVF). One hundred and fifty-nine oocytes (21.3%) were degenerated after ICSI, which was a higher percentage than the degeneration obtained after IVF (21 oocytes; 2.1%, $p < 0.001$).

Oocyte activation and pronuclear formation

Of 587 oocytes that survived after ICSI, 225 (38.3%) were activated, which was a significantly lower proportion than was activated after IVF (413 / 800, 51.6%; $p < 0.001$). In the IVF treatments 165 oocytes fixed at 0.5 and 0.75 h after insemination were not included in the analysis because no activation was found at that stage.

The chronology of changes in oocyte activation after ICSI and IVF are summarised in Figs. 1 and 2 respectively.

The earliest oocyte activation after ICSI was observed at 0.5 h after injection, when 4 oocytes (14.8%) were in anaphase II (Fig. 3). This was earlier than IVF, where 6 oocytes (6.4%) exhibited anaphase II 1 h after insemination. The number of oocytes exhibiting anaphase II after ICSI treatment decreased 4 h after injection, whereas after IVF the proportion of oocytes in anaphase II decreased after 6 h. One oocyte in each treatment (ICSI and IVF) remained in anaphase II at 21 h after fixation. Oocytes were observed at telophase II (Fig. 4) as early as 0.75 h after ICSI, but the proportion of oocytes at telophase II decreased at 8 h. After IVF,

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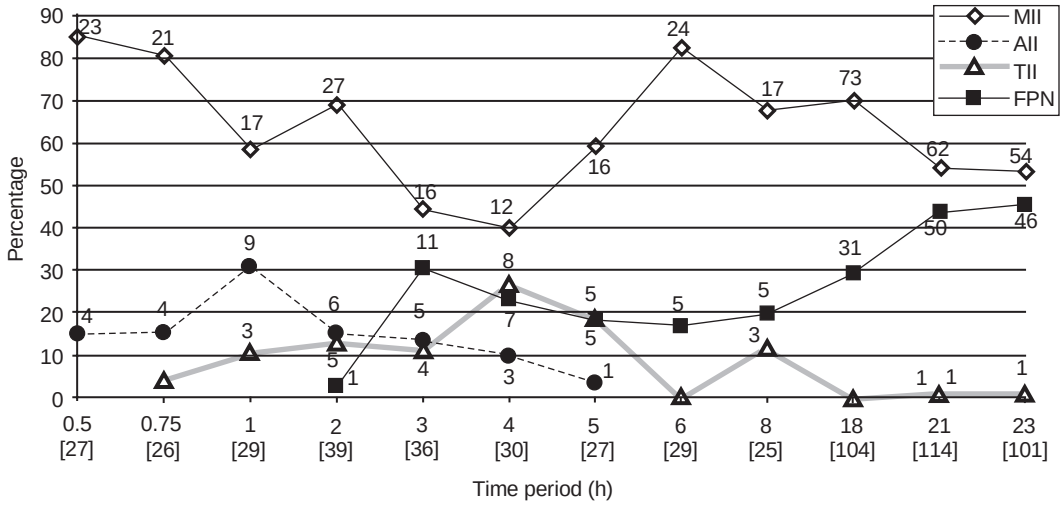

Figure 1 Early oocyte activation observed at different time intervals after intracytoplasmic sperm injection (ICSI). MII, metaphase II; AII, anaphase II; II, telophase II; FPN, female pronucleus. Numbers are total oocytes by period of time.

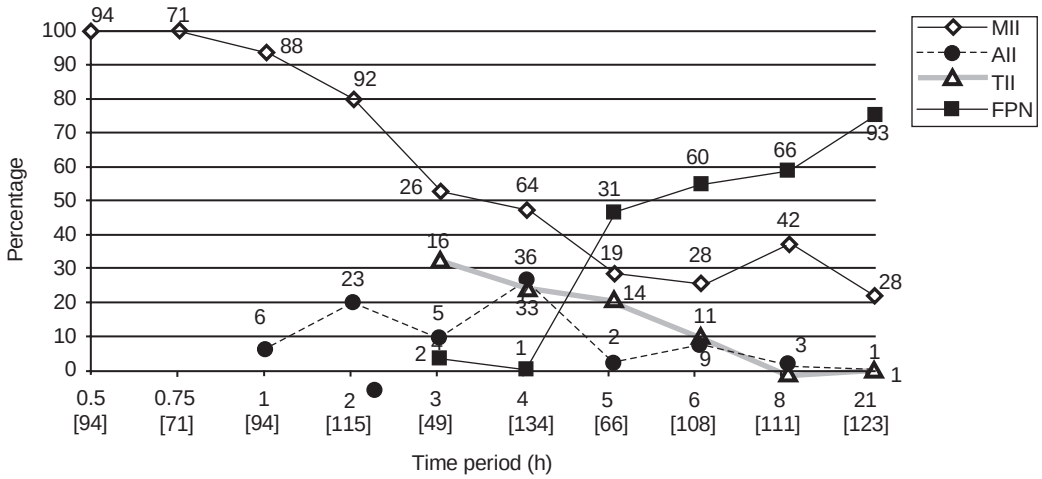

Figure 2 Early oocyte activation observed at different time intervals after *in vitro* fertilisation. Abbreviations as in Fig. 1.

telophase II oocytes were observed from 3 to 6 h after insemination. The duration of anaphase II and telophase II were 4 h 30 min and 4 h 15 min respectively after ICSI, but up to 7 and 3 h respectively after IVF.

The earliest female pronucleus after ICSI was found 2 h after the injection and most activated oocytes had reached this stage between 3 and 23 h after injection (Fig. 1). After IVF the female pronucleus was found 3 h after insemination and most activated oocytes reached this stage between 5 and 21 h after insemination (Fig. 2). The earliest male pronucleus was formed 3 h after ICSI (Fig. 5) and IVF (Fig. 6).

Sperm decondensation

The chronology of sperm decondensation after ICSI and IVF is summarised in Figs. 5 and 6 respectively. After ICSI those oocytes that contained a spermatozoon in a vacuole (Fig. 7) were not included in the analysis. In these cases the sperm did not penetrate the oocyte because the plasmalemma was not broken during the injection procedure.

During IVF, spermatozoa have to undergo capacitation and the acrosome reaction before oocyte penetration, giving a time difference compared with ICSI. Swollen sperm heads were only found if the oocytes were activated, irrespective of insemination method (ICSI or IVF); then, the initial oocyte activation time (anaphase II) was used as a reference point to compare sperm decondensation and male pronuclear formation between ICSI and IVF.

The earliest sperm decondensation time was 1 h after injection and 30 min after the oocyte reached anaphase II. Spermatozoa first penetrated oocytes 0.5 h

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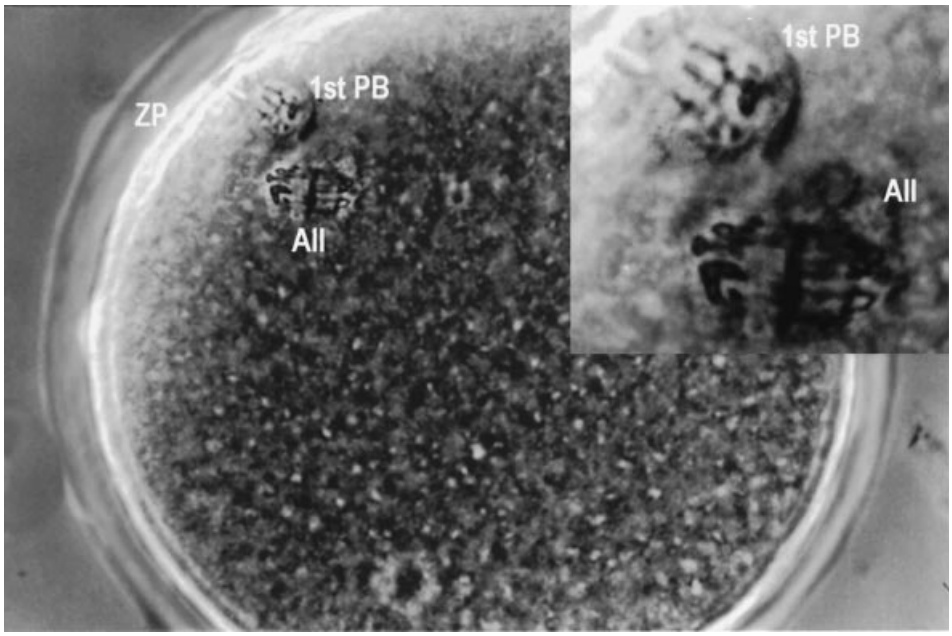

Figure 3 *In vitro* matured sheep oocyte fixed 0.5 h after injection in anaphase II (AII). Homologous chromosomes have started their separation into single chromatids. The first polar body (1st PB) and the zona pellucida (ZP) are visible. × 400.

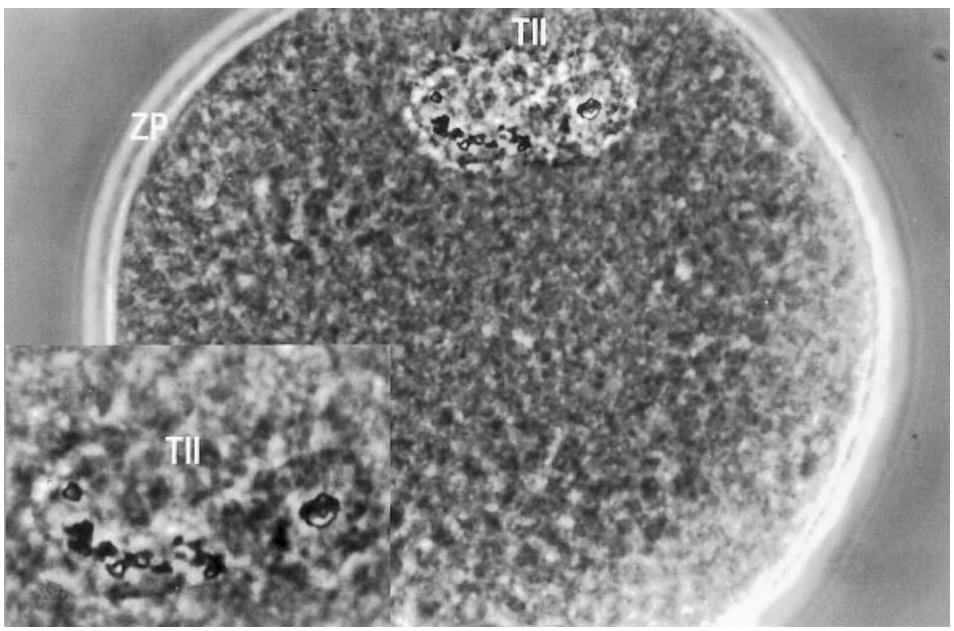

Figure 4 *In vitro* matured sheep oocyte fixed at 0.75 h after injection in late telophase II (TII). Homologous chromatids have separated and started decondensation to form a female pronucleus and second polar body. ZP, zona pellucida. × 400.

after IVF and swollen heads (decondensation; Fig 8) were first observed at 3 h after insemination and 2 h after anaphase II.

There was no correlation between the proportions of oocytes containing decondensed spermatozoa and of activated oocytes at anaphase II after ICSI and IVF (r^2 = 0.00). However, there was a linear correlation between the proportion of oocytes containing decondensed spermatozoa and the proportion of activated oocytes at telophase II after ICSI (r^2 = 82.2%) and IVF (r^2 = 30.4%).

Fertilisation and parthogenetic activation

The total oocyte activation after ICSI was significantly lower than after IVF ($p < 0.001$; Table 1). Of the total oocytes activated after ICSI (n = 225) and IVF (n = 413),

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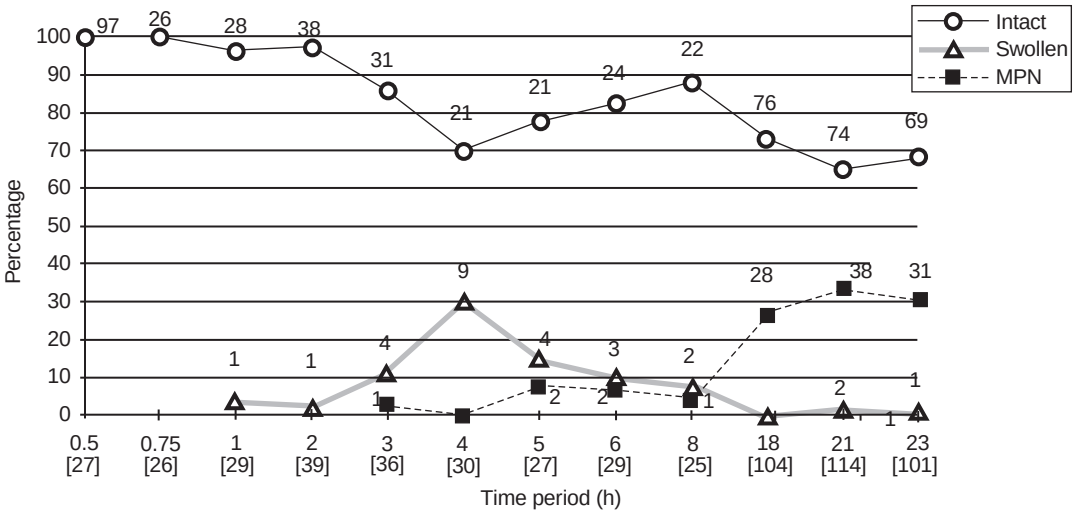

Figure 5 Sperm decondensation observed at different time intervals after ICSI. MPN, male pronucleus. Numbers are total oocytes by period of time.

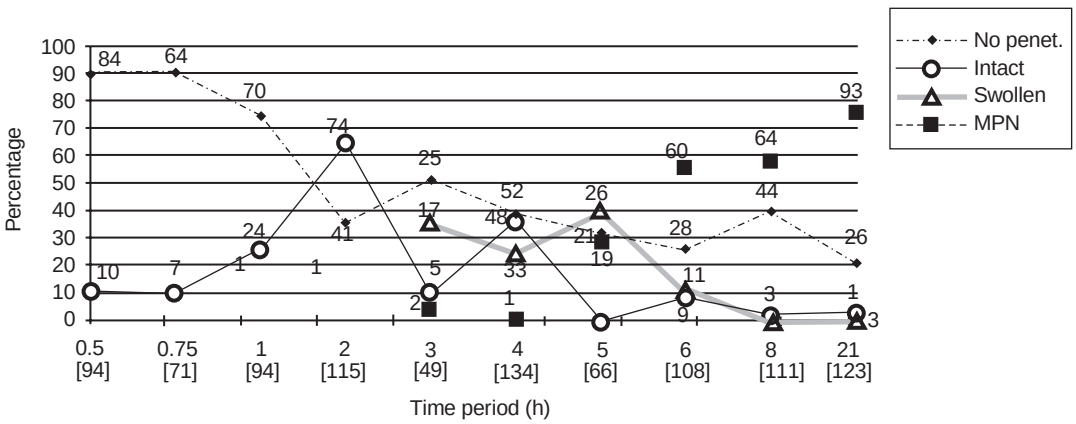

Figure 6 Sperm decondensation observation at different time intervals after IVF. Abbrevations as in Fig. 5.

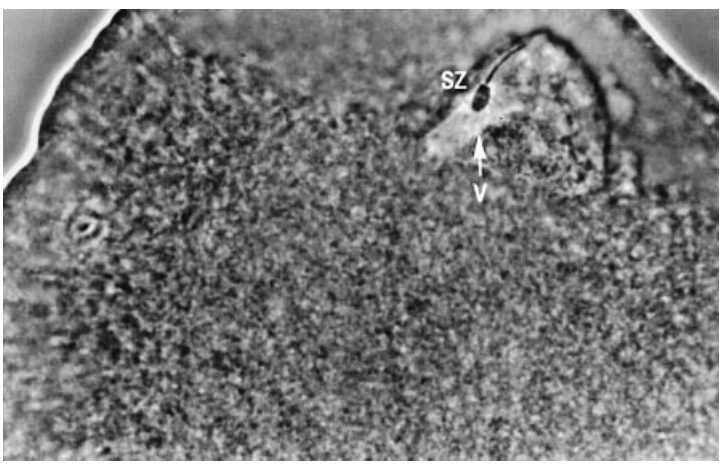

Figure 7 Spermatozoon (SZ) in a vacuole (V). The spermatozoon did not penetrate the oocyte because the plasmalemma was not broken during the injection procedure. × 400.

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Table 1 Oocyte activation after intracytoplasmic sperm injection (ICSI) and *in vitro* fertilisation (IVF)

Treatment	Total oocyte activation	Fertilisation		Parthenogenetic activation
		Normal	Abnormal	
ICSI	225 / 587 (38.3)[a]	108 / 123 (87.8)[a]	15 / 123 (12.2)[a]	49 / 172 (28.5)[a]
IVF	413 / 800 (51.6)[b]	323 / 382 (84.5)[a]	59 / 382 (15.6)[a]	5 / 387 (1.3)[b]

Values with different superscripts within columns differ significantly ($p < 0.05$).

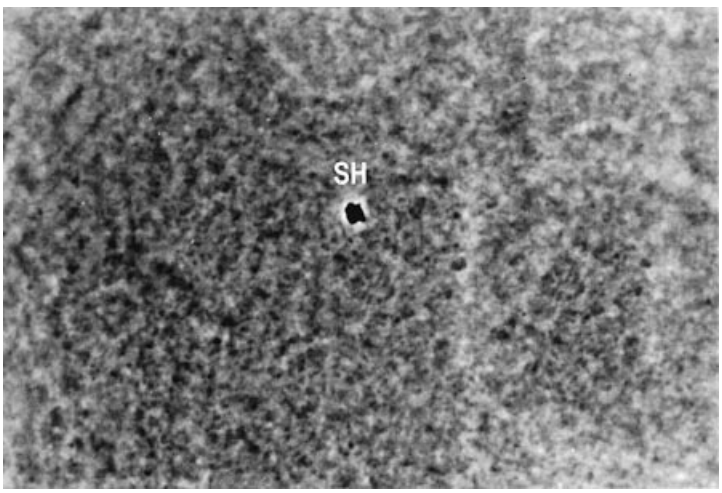

Figure 8 *In vitro* matured sheep oocyte fixed 1 h after ICSI. The swollen head (SH) of the spermatozoon is starting decondensation. × 400.

53 and 26 from the ICSI and IVF groups respectively were not included in the analysis (those oocytes fixed 0.5–3 h after ICSI and 1–2 h after IVF, because we considered that during this time oocytes that underwent early activation and contained undecondensed spermatozoa could not be classified yet as fertilised or parthogenetically activated). The rate of parthenogetic activation was higher after ICSI than IVF ($p < 0.001$), whereas the total activation rate was higher after IVF than ICSI ($p < 0.001$; Table 1). The normal and abnormal fertilisation rates were not significantly different between ICSI and IVF ($p > 0.05$). A normal fertilised oocyte is shown in Fig. 9.

The overall fertilisation rate after ICSI was 28.6% (123 / 430) between 4 and 23 h, which was significantly lower than the total fertilisation after IVF between 4 and 21 h (70.4%, 382 / 542; $p < 0.001$).

Eleven of 15 oocytes that were fertilised abnormally after ICSI contained three pronuclei, one polar body and a spermatozoon tail, and 4 oocytes contained one pronucleus, one polar body and a decondensing spermatozoon (delayed). The 59 oocytes that were abnormally fertilised after IVF contained three or more pronuclei, two polar bodies and more than two tails (polyspermy).

The distribution of the parthenogenetic oocytes after ICSI was as follows: 3 oocytes remained in anaphase II, 9 oocytes in telophase II, 12 oocytes contained one pronucleus and two polar bodies, 4 contained one pronucleus and one polar body, 2 contained two pronuclei and one polar body and 19 oocytes cleaved to 2–4 cells (Fig. 10). All the oocytes contained an undecondensed spermatozoon in the cytoplasm.

Discussion

This is the first study of the timing of nuclear progression in sheep oocytes after ICSI. The oocyte activation events were more advanced for ICSI than IVF, using injection and insemination times as reference points as has been reported after ICSI in human (Nagy *et al.*, 1994; Dorzortsev *et al.*, 1995). The fertilisation events such as sperm decondensation were more advanced for ICSI than IVF, but the time of male pronuclear formation did not differ, using early oocyte activation as a reference point. The delay in IVF oocytes in this study was between 1 and 2 h, which was different from that reported in the human 2–4 h; Nagy *et al.*, 1994). It is probable that the difference was caused by the

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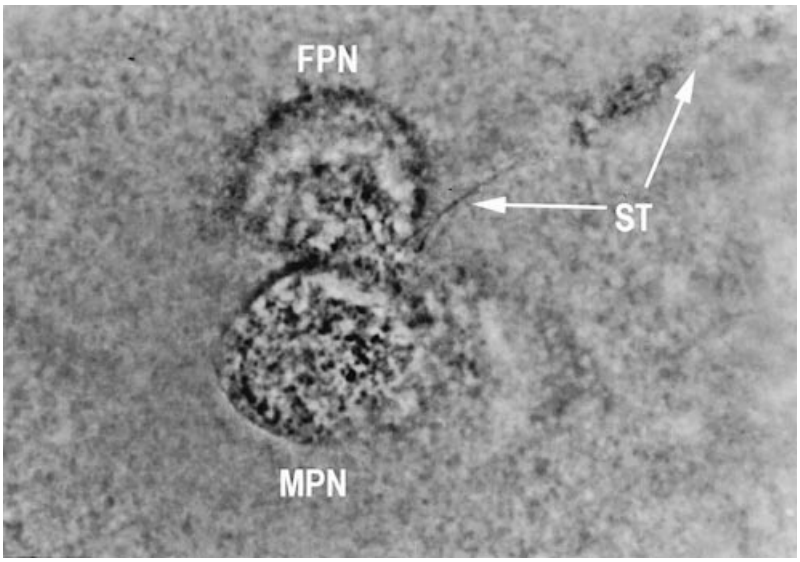

Figure 9 Normal fertilised activated oocyte fixed 18 h after ICSI. Male and female pronucleus (MPN, and FPN) sperm tail (ST) are visible in the oocyte cytoplasm. × 400.

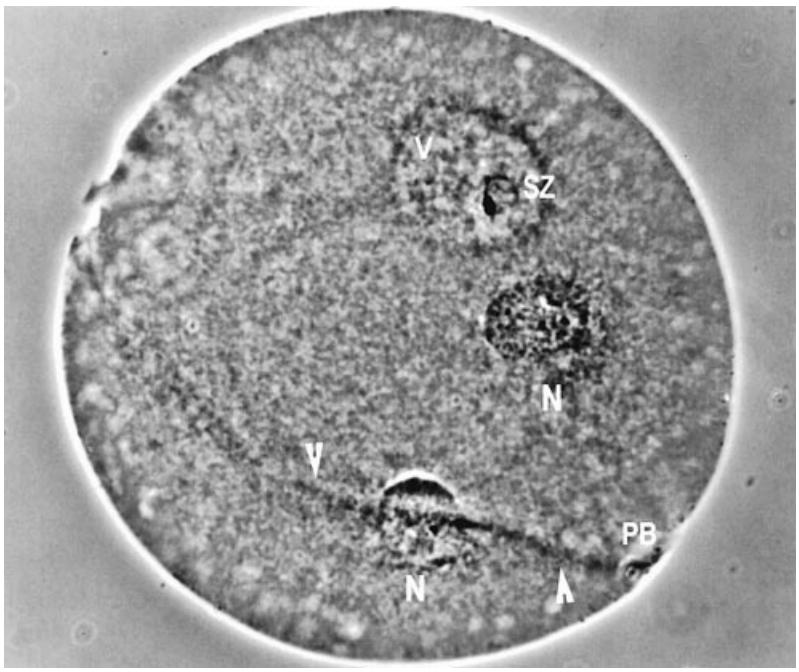

Figure 10 Parthenogenetic activated oocyte fixed 21 h after ICSI. Two nuclei (N) are separated by a membrane (arrows); spermatozoon (SZ) in a vacuole (V) and a polar body (PB) are visible in the cytoplasm. × 400.

stimulus that the oocyte received during the mechanical injection, which induced oocyte activation, than by the presence of the spermatozoon after injection (unpublished data). During ICSI, oocyte activation is induced by the injection pipette (Gómez *et al.*, 1997) and this was reflected in this study by quicker and earlier oocyte activation after ICSI than IVF.

Although during IVF spermatozoa have to undergo capacitation and the acrosome reaction before penetration of the oocytes, the spermatozoa were found as early as 0.5 h after insemination in the oocyte cytoplasm. Oocyte activation only started (anaphase II) 1 h after insemination, or 0.5 h after penetration of the spermatozoon. This suggests that oocyte activation after ICSI is induced by mechanical injection and after IVF by penetration of the spermatozoon. In spite of this difference in the time of oocyte activation in the two treatments, sheep oocytes seem to take the same time

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(about 30 min) after ICSI and IVF to reach anaphase II after the initial trigger.

It is difficult to explain the differences in the duration of anaphase II and telophase II stages of activation after ICSI and IVF. It seem probable that after ICSI the mechanical injection induced a pattern of oocyte activation that alters the cell cycle in comparison with IVF. In the human, Edwards (1995) reported that cell cycle disorders may occur after ICSI since the technique involves the fusion of a sperm nucleus in G_2 phase with an oocyte in M phase. This resulted in either prematurely condensed chromosomes (PCC) in spermatozoa or the formation of a single male pronucleus and metaphase chromosomes within the egg. Although abnormal oocyte activation and fertilisation were observed in the present study, no PCC or male pronuclei and metaphase chromosomes were observed after ICSI, suggesting that cell cycle disorders are present after ICSI of sheep oocytes but that the cause is related to improper oocyte activation.

Swollen heads of spermatozoa have been found in equal proportions in non-activated and activated human oocytes after ICSI (Dozortsev *et al.*, 1994; Flaherty *et al.*, 1995), suggesting that initial swelling is independent of oocyte activation. Swollen heads are rarely found in unactivated oocytes after IVF (Edirisinghe *et al.*, 1992), suggesting that the presence of swollen heads after ICSI is an abnormal condition. In the present study, swollen spermatozoa were found after ICSI and IVF only in activated oocytes, and a strong relationship was found between oocyte activation (telophase II) and sperm decondensation, suggesting that decondensation of the spermatozoon is dependent on oocyte activation in the sheep.

The earliest sperm deconstruction after ICSI was found 1 h after the injection, which is earlier than after IVF. Although sperm decondensation was earlier after ICSI, male pronuclear formation was present at the same time (3 h) after either ICSI or IVF, suggesting that formation of the male pronucleus is controlled by some factor in the oocyte that is time dependent (Nagy *et al.*, 1994). Furthermore, pronuclear formation after ICSI was asynchronous, whereas after IVF it was synchronous. The reason for this asynchrony after ICSI is not clear. One explanation might be the differences in oocyte activation between ICSI and IVF. Mechanical injection of sheep oocytes induces the release of cortical granules and female pronuclear formation earlier than male pronuclear formation (Gómez *et al.*, 1996), whereas after IVF the events occur synchronously after penetration of the spermatozoon.

Abnormal fertilisation has been found after ICSI in the human (Cohen *et al.*, 1995; Staessen & Van Steriteghem, 1997). In the present study, abnormal fertilisation was mainly in the form of tripronucleate eggs, possibly due to inhibition of the extrusion of the second polar body resulting in the formation of two female pronuclei and a single sperm pronucleus. This is a different mechanism from that of IVF, where tripronuclear formation is due to multiple sperm penetration. Abnormal fertilisation after ICSI in sheep oocytes, combined with inadequate oocyte activation, are responsible for the low rate of blastocyst development, which found in a high proportion of embryos arrested on day 4 after cleavage (Gómez *et al.*, 1998).

Cohen *et al.* (1995) described three patterns of parthenogenetic activation that occur in human oocytes after ICSI and IVF, and similar patterns were observed in sheep oocytes after ICSI in the present study. The first and the most common pattern was extrusion of the second polar body and the formation of a single female pronucleus (24.5% of the total parthogenetic activation). The second, seen in a lower proportion oocytes (8.2%), was failure of extrusion of the second polar body and formation of a single diploid pronucleus. The third, and least common, form of parthenogenesis (4.0%) was the completion of telophase II without extrusion of the second polar body, resulting in the formation of two female pronuclei. The rate of parthenogenetic activation was lower after IFV compared with ICSI. IVF parthenogenetic oocytes showed only one pattern of oocyte activation: extrusion of a second polar body and the formation of one pronucleus. These differences in oocyte activation suggest that the abnormalities of fertilisation and activation observed after ICSI are due to the injection process.

The results of the present study suggest that the events of oocyte activation and sperm decondensation take place earlier (1–2 h) after ICSI than after IVF. It can be concluded that the main reason for this difference is inadequate or abnormal oocyte activation. The pattern of oocyte activation after ICSI and IVF is also different. The main cause of this difference is the injection process, which induces abnormal fertilisation and oocyte activation. Further investigation is necessary to define the nature of abnormalities of activation and fertilisation after ICSI and to stringently assess the effects of the injection on such abnormalities. This might be achieved by detailed chromosome analysis of embryos produced by ICSI, using control IVF-produced embryos subjected to the same culture conditions as for ICSI but omitting the injection procedure.

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References

Cattl S.L., Catt, J.W., Gómez, M.C., Maxwell, W.M.C. & Evans, G. (1996). The birth of a male lamb derived from an *in vitro* matured oocyte fertilized by intra-cytoplasmic injection of a single presumptive 'male' sperm. *Vet. Rec.* **139**, 494–5.

Cohen, J., Levron, J., Palermo, G., Munné, S., Adler, A., Alikani, M., Schattman, G., Sultan, K. & Willadsen, S.M. (1995). Atypical activation and fertilization patterns in humans. *Theriogenology* **43**, 129–40.

Dozortsev, D., De Sutter, P. & Dhont, M. (1994). Behaviour of spermatozoa in human oocytes displaying no or one pronucleus after intracytoplasmic sperm injection. *Hum. Reprod.* **9**, 2139–44.

Dozortsev, D., De Sutter, P., Rybouchkin, A. & Dhont, M. (1995). Timing of sperm and oocyte nuclear progression after intracytoplasmic sperm injection. *Hum. Reprod.* **10**, 3012–17.

Edirisinghe, W.R., Murch, A.R. & Yovich, J.L. (1992). Cytogenetic analysis of human oocytes and embryos in an *in-vitro* fertilization programme. *Hum. Reprod.* **7**, 230–6.

Edwards, R.G. (1995). Cell cycle factors in the human oocyte and the intracytoplasmic injection of spermatozoa. *Reprod. Fertil. Dev.* **7**, 143–53.

Flaherty, S., Payne, D., Swann, N.J. & Matthews, C.D. (1995). Assessment of fertilisation failure and abnormal fertilisation after intracytoplasmic sperm injection (ICSI). *Reprod. Fertil. Dev.* **7**, 197–210.

Gómez, M.C., Catt, S. L., Gillan, L., Catt, J.W., Maxwell, W.M.C. & Evans, G. (1996). Time course of pronuclear formation after insemination *in vitro* and intracytoplasmic sperm injection of *in vitro* matured sheep oocytes. Proc 13th ICAR, Australia, **2**, 10–19 [Abstract].

Gómez, M.C., Catt, J.W., Gillan, L., Evans, G. & Maxwell, W.M.C. (1997). Effect of culture, incubation and acrosome reaction of fresh and frozen / thawed ram spermatozoa for *in vitro* fertilisation and intracytoplasmic sperm injection. *Reprod. Fertil. Dev.* **9**, 665–73.

Gómez, M.C., Catt, J.W., Evans, G. & Maxwell, W.M.C. (1998). Cleavage, development and competence of sheep embryos fertilized by intracytoplasmic sperm injection (ICSI) and *in vitro* fertilisation (IVF). *Theriogenology* **49**, 1143–54.

Nagy, Z., Liu, J., Horis, H., Devroey, P. & Van Steirteghem, A.C. (1994). Time-course of oocyte activation, pronucleus formation and cleavage in human oocytes fertilized by intracytoplasmic sperm injection. *Hum. Reprod.* **9**, 1743–8.

Staessen, C. & Van Steirteghem, A.C. (1997). The chromosomal constitution of embryos developing from abnormally fertilized oocytes after intracytoplasmic sperm injection and conventional *in vitro* fertilization. *Hum. Reprod.* **12**, 321–7.