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Effects of oocyte age, cumulus cells and injection methods on *in vitro* development of intracytoplasmic sperm injection rabbit embryos

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Summary

This study assessed the effects of oocyte age, cumulus cells and injection methods on *in vitro* development of intracytoplasmic sperm injection (ICSI) rabbit embryos. Oocytes were recovered from female rabbits superovulated with PMSG and hCG, and epididymal sperm were collected from a fertile male rabbit. The oocyte was positioned with the first polar body at 12 o'clock position, and a microinjection needle containing a sperm was inserted into the oocyte at 3 o'clock. Oolemma breakage was achieved by aspirating ooplasm, and the aspirated ooplasm and sperm were re-injected into the oocyte. The injected oocytes were cultured in M199 medium containing 10% fetal calf serum at 38 °C with 5% CO_2 in air. The results showed that oocytes injected at 1 h post-collection produced a higher ($p < 0.05$) fertilization rate than those injected at 4 or 7 h post-collection. Blastocyst rate in the 1 h group was higher ($p < 0.05$) than in the 7 h group. Denuded oocytes (group A) and oocytes with cumulus cells (group B) were injected, respectively. Rates of fertilization and development of ICSI embryos were not significantly different ($p > 0.05$) between the two groups. Four ICSI methods were applied in this experiment. In methods 1 and 2, the needle tip was pushed across half the diameter of the oocyte, and oolemma breakage was achieved by either a single aspiration (method 1) or repeated aspiration and expulsion (method 2) of ooplasm. In methods 3 and 4, the needle tip was pushed to the oocyte periphery opposite the puncture site, and oolemma breakage was achieved by either a single aspiration (method 3) or repeated aspiration and expulsion (method 4) of ooplasm. Fertilization rate in method 2 was significantly higher ($p < 0.05$) than in methods 1 and 3. Blastocyst rates were not significantly different ($p > 0.05$) among methods 1, 3 and 4, but method 2 produced a higher ($p < 0.05$) blastocyst rate than method 3.

Keywords: Cumulus cells, Injection method, Intracytoplasmic sperm injection, Oocyte age, Rabbit oocyte

Introduction

Intracytoplasmic sperm injection (ICSI) has been widely used as a method to study oocyte fertilization and embryo development. The fertilization efficiency is related to the quality of spermatozoa (Nagy *et al*., 1998; Strassburger *et al*., 2000), activation of oocytes (Nakagawa *et al*., 2001; Ock *et al*., 2003) and injection techniques (Dozortsev *et al*., 1998; Hlinka *et al*., 1998; Van der Westerlaken *et al*., 1999). Rabbit oocytes can be easily collected, and are a useful model for studying ICSI. Precise timing of the introduction of sperm into the oocyte is under control during ICSI, and effects of oocyte age post-collection on fertilization and embryo development can be investigated.

Cumulus cells have a positive influence on fertilization. They can provide a capacitation-inducing mechanism and facilitate the interaction between capacitated spermatozoa and the zona pellucida surface. In addition, cumulus cells can induce the acrosome reaction (Mattioli *et al*., 1998), increase oocyte penetrability (Romar *et al*., 2003) and improve embryo development (Nandi *et al*., 1998). Denuded oocytes were used for ICSI in previous studies. In this experiment, oocytes with cumulus cells were injected, and the efficiency of fertilization and development was tested.

Oocytes fertilized after ICSI show an oscillatory Ca^{2+} response (Tesarik & Sousa, 1994; Sato *et al*., 1999). Sperm cytosolic factor(s) play the role of oscillator,

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modulating the properties of the oocyte's intracellular calcium stores, whereas the role of trigger, normally realized by sperm–oocyte interactions at the level of their respective cell surfaces, can be supplemented in the conditions of ICSI by an artificial calcium influx generated by the procedure itself (Tesarik, 1998). Vigorous aspiration of oocyte cytoplasm may facilitate fertilization after ICSI by increasing the oocyte Ca^{2+} load at the time of injection (Tesarik & Sousa, 1995). Effects of cytoplasm aspiration methods on fertilization and development of ICSI embryos were examined in this study.

Materials and methods

Sperm preparation

Epididymal sperm were collected from a male Japanese Big Eared White rabbit (6 months old) by aspiration. Briefly, sperm in the caudal epididymis were aspirated into a 1 ml syringe containing 0.1 ml of M2 medium by applying a negative pressure. The sperm concentration was adjusted to approximately $1–2 \times 10^6$/ml, and 10 μl of sperm suspension was diluted with 90 μl of 10% polyvinylpyrrolidone (PVP, Sigma).

Collection of mature rabbit oocytes

Female Japanese Big Eared White rabbits (4–6 months old) were superovulated with 80 IU pregnant mare-serum gonadotropin (PMSG, Tianjin Center of Experimental Animals, China) followed by 80 IU human chorionic gonadotropin (hCG, Tianjin Center of Experimental Animals, China) 72 h later. Oocytes were collected from Fallopian tubes at 15 h after hCG injection, and cumulus cells were removed by incubation for 30 s in M2 medium containing 0.1% hyaluronidase (Sigma). Denuded oocytes were rinsed three times and subjected to ICSI.

ICSI procedure

The holding pipette had an outer diameter of 150 μm and an inner diameter of 20 μm. The microinjection pipette was beveled to 30° with an inner diameter of 6–8 μm. The sperm was immobilized by touching the tail with the injection pipette, and was aspirated into the injection pipette. The oocyte was positioned with the first polar body at 12 o'clock. The insertion of the microinjection needle was performed with the sperm located as close as possible to the tip of the needle. Oolemma breakage was achieved by aspirating ooplasm into the microinjection needle, and the aspirated ooplasm and sperm were gently re-injected into the oocyte.

Embryo culture

The injected oocytes were washed three times, and then cultured in M199 medium containing 10% fetal calf serum (FCS) at 38 °C with 5% CO_2 in air. Fertilization was checked at 8–10 h after ICSI. Normally fertilized oocytes were defined as those with two pronuclei and a second polar body (Fig. 1A), and were selected for further culture in the same medium. They were monitored for *in vitro* development until the blastocyst stage (Fig. 1B).

Experimental design

Experiment 1
Mature oocytes were divided into three groups and injected at 1, 4 or 7 h post-collection. Oocyte fertilization and embryonic development were compared among these groups.

Experiment 2
Mature oocytes were divided into two groups after collection. Group A was treated with 0.1% hyaluronidase to remove cumulus cells completely. In group B, one or two levels of cumulus cells were retained on the oocytes, but the first polar body could be seen during ICSI. To observe pronuclei, cumulus cells in group B were removed by hyaluronidase at 8 h after injection.

Experiment 3
Four different injection methods were applied in this experiment:

Method 1: The injection needle was pushed across half of the diameter of the oocyte, and ooplasm was drawn into the pipette using a single aspiration until oolemma breakage. The needle tip was pushed to the oocyte periphery opposite to the puncture site, and the aspirated ooplasm and sperm were re-injected into the cytoplasm.
Method 2: The injection needle was pushed across half of the diameter of the oocyte, and oolemma breakage was achieved by repeated aspiration and expulsion of ooplasm (Fig. 1C). The needle tip was pushed to the oocyte periphery, and the aspirated ooplasm and sperm were re-injected into the cytoplasm (Fig. 1D).
Method 3: The injection needle was pushed to the oocyte periphery opposite the puncture site, and ooplasm was drawn into the pipette using a single aspiration until oolemma breakage. The aspirated ooplasm and sperm were re-injected into the cytoplasm.
Method 4: The injection needle was pushed to the oocyte periphery, and oolemma breakage was achieved by repeated aspiration and expulsion of ooplasm. The

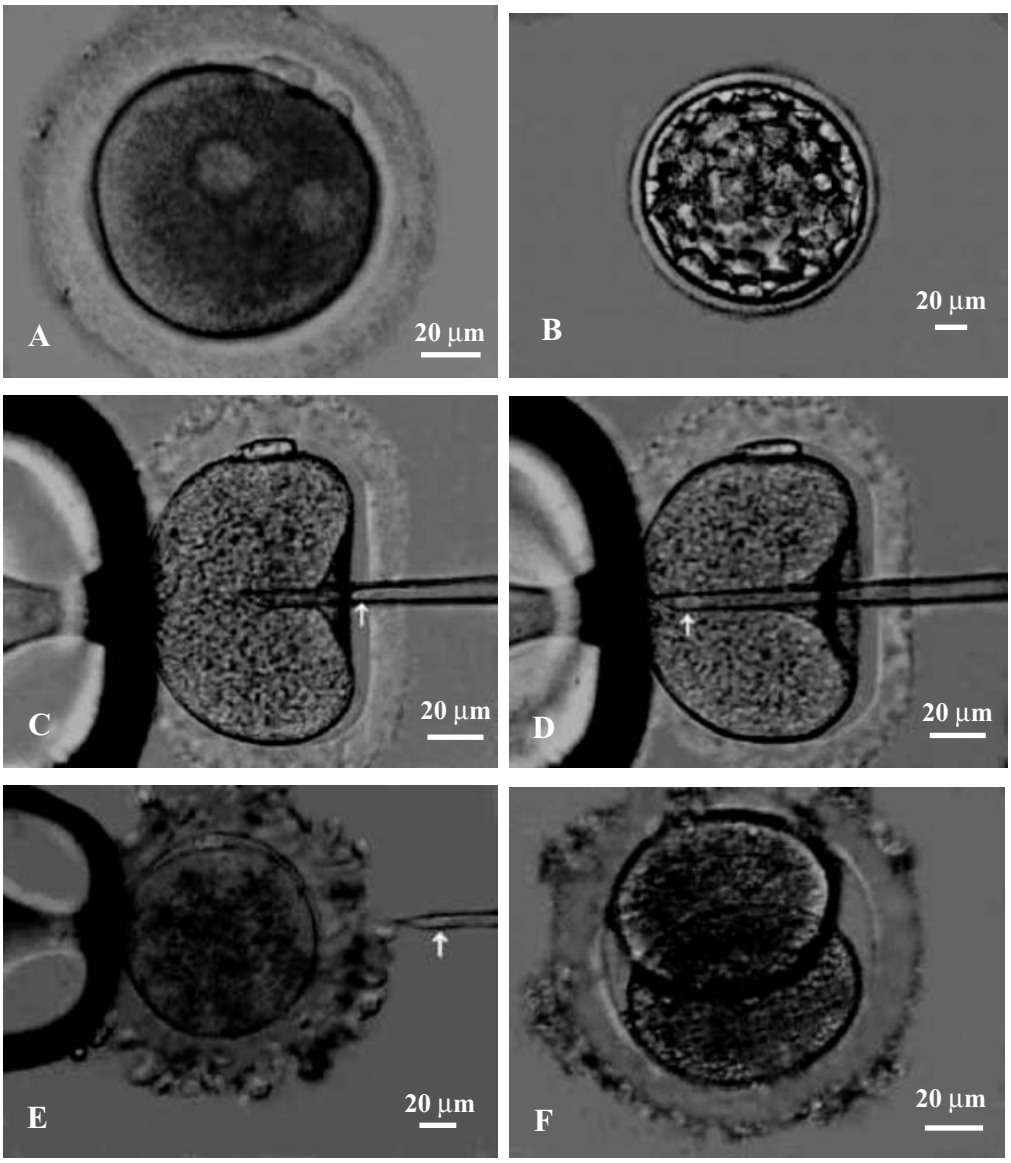

Figure 1 Rabbit intracytoplasmic sperm injection (ICSI). (A) A fertilized oocyte with two pronuclei and a second polar body after ICSI. (B) An ICSI blastocyst after 3 days of culture *in vitro*. (C) The injection needle containing a sperm (arrow) was pushed across half of the diameter of the oocyte, and oolemma breakage was achieved by repeated aspiration and expulsion of ooplasm. (D) The needle tip was pushed to the oocyte periphery, and the aspirated ooplasm and sperm (arrow) were re-injected into the cytoplasm. (E) An oocyte with cumulus cells was injected with a sperm (arrow). (F) Some cumulus cells were still attached to a 2-cell ICSI embryo.

aspirated ooplasm and sperm were re-injected into the cytoplasm.

Statistical analysis

Percentages were subjected to arcsine transformation before being analysed. Fertilization and development rates were analysed by *t*-test (experiment 2) and ANOVA followed by multiple comparisons (experiments 1 and 3). Differences were considered significant when $p < 0.05$.

Results

In experiment 1, oocytes were injected at 1, 4 or 7 h post-collection, respectively. Oocytes in the 1 h group had a higher ($p < 0.05$) fertilization rate than those in the 4 and 7 h groups. Cleavage rates were not significantly different ($p > 0.05$) among the three groups, but oocytes in the 1 h group had a higher ($p < 0.05$) blastocyst rate than those in the 7 h group (Table 1).

In experiment 2, denuded oocytes (group A) and oocytes with cumulus cells (group B) were injected, respectively, and fertilization and development of

Table 1 Effects of oocyte age post-collection on fertilization and development of ICSI rabbit embryos

Oocyte age (h)	No. of oocytes	No. (%) fertilized	No. (%) cleaved	No. (%) of blastocysts
1	102	73 (71.6)[a]	54 (74)	23 (31.5)[a]
4	90	51 (56.7)[b]	36 (70.6)	12 (23.5)[a,b]
7	65	28 (43.1)[c]	19 (67.9)	5 (17.9)[b]

[a,b,c] Data within the same column with different superscripts are significantly different ($p < 0.05$).

Table 2 Effects of cumulus cells on fertilization and development of ICSI rabbit embryos

Group*	No. of oocytes	No. (%) fertilized	No. (%) cleaved	No. (%) of blastocysts
A	105	78 (74.3)	60 (76.9)	26 (33.3)
B	83	64 (77.1)	52 (81.2)	23 (35.9)

* Groups A and B are referred to as denuded oocytes and oocytes with cumulus cells, respectively.
Data within the same columns are not significantly different ($p > 0.05$).

embryos in the two groups were compared. Percentages of fertilization, cleavage and blastocyst formation were not significantly different ($p > 0.05$) between the two groups (Table 2).

In experiment 3, effects of four ICSI methods on fertilization and development of embryos were compared. Method 2 resulted in a higher fertilization rate ($p > 0.05$) than methods 1 and 3. Blastocyst rates in methods 1, 3 and 4 were not significantly different, but method 2 had a significantly higher ($p < 0.05$) blastocyst rate than method 3 (Table 3).

Table 3 Effects of ICSI methods on fertilization and development of ICSI rabbit embryos

Injection methods*	No. of oocytes	No. (%) fertilized	No. (%) cleaved	No (%) of blastocysts
Method 1	77	50 (64.9)[a]	37 (74)	15 (30)[a,b]
Method 2	92	70 (76.1)[b]	55 (78.6)	25 (35.7)[a]
Method 3	69	43 (62.3)[a]	30 (69.8)	11 (25.6)[b]
Method 4	85	59 (69.4)[a,b]	43 (72.9)	17 (28.8)[a,b]

* In methods 1 and 2, the needle tip was pushed across half of the diameter of oocyte, and oolemma breakage was achieved by either a single aspiration (method 1) or repeated aspiration and expulsion (method 2) of ooplasm. In methods 3 and 4, the needle tip was pushed to the oocyte periphery opposite the puncture site, and oolemma breakage was achieved by either a single aspiration (method 3) or repeated aspiration and expulsion (method 4) of ooplasm.
[a,b] Data within the same column with different superscripts are significantly different ($p < 0.05$).

Discussion

Fertilization can be viewed as a phenomenon that remodels the markedly differing chromatin of the male and female gametes. After sperm penetration, the sperm undergoes chromatin decondensation (Ahmadi & Ng, 1997; Katayama *et al*., 2002), nuclear swelling (Dozortsev *et al*., 1998) and pronuclear formation (Ahmadi & Ng, 1997; Katayama *et al*., 2002). Decondensation of the sperm nucleus and formation of the pronucleus are essential for embryonic development. The failure of male pronucleus formation is related to low activity of glutathione in the oocyte (Yoshida *et al*., 1993), and this activity is acquired during maturation and declines with oocyte ageing. Insufficient uptake of glutathione during *in vitro* maturation (IVM) or its depletion during post-maturation aging may impair the development of the male pronucleus (Sutovsky & Schatten, 1997). In experiment 1, oocytes injected at 1 h post-collection produced a higher fertilization rate than those injected at 4 or 7 h post-collection. Delayed injection may lead to oocyte ageing and failure of male pronucleus formation, thus reducing the fertilization rate. Oocytes in the 1 h group had a higher blastocyst rate than those in the 7 h group, suggesting that development of ICSI embryos decreased with the increase of oocytes age post-collection. In addition, the meiotic spindle is directly adjacent to the first polar body in newly matured oocytes, and its position is changed during oocyte aging. A delayed injection may increase the chance of disrupting the spindle, which would reduce the developmental potential of ICSI embryos.

Cumulus cells play an important role in fertilization of oocytes. They can promote the acrosome reaction (Mattioli *et al*., 1998) and increase oocyte penetrability (Romar *et al*., 2003). But these processes are bypassed in ICSI, because the sperm is directly injected into the oocyte. In experiment 2, no significant effects of cumulus cells on fertilization and development of ICSI embryos were observed. In normal fertilization, cumulus cells are disengaged from oocytes after sperm penetration. The timing of the gap junction closure between cumulus cells and the oocyte may be a crucial temporal event in oocyte and embryo development (Host *et al*., 2002). In experiment 2, oocytes with one or two levels of cumulus cells were injected (Fig. 1E), and the cumulus cells showed fragmentation and disengagement from oocytes after ICSI. Broken cumulus cells could still be seen attached to the zona pellucida of some 2-cell embryos (Fig. 1F), but the embryos eventually lost the cumulus cells at the 4-cell stage.

Oocytes destroyed or not activated would undergo failure of fertilization and cleavage. It was reported that repeated movements of the microinjection needle in the oocyte during ICSI could produce a considerable influx of calcium ions from the culture medium into the oocyte, and mechanically disrupt the endoplasmic reticulum in the oocyte, which would contribute to the release of calcium stored in this organelle (Tesarik *et al*., 2002). However, an excessive influx of polyvinylpyrrolidone (PVP) or medium during ICSI may cause oocyte degeneration (Schwartz *et al*., 1996). The method of repeated movements of injection needle was not adopted in this experiment. In experiment 3, method 2 resulted in a significantly higher fertilization rate than methods 1 and 3. Repeated aspiration and expulsion of ooplasm may disrupt the endoplasmic reticulum in the oocyte, and the calcium released from this organelle may improve fertilization efficiency.

Injury to the meiotic spindle during ICSI may result in oocyte degeneration (Schwartz *et al*., 1996). Cytoplasm aspiration performed with the needle tip located in the central area of the oocyte can reduce the risk of disturbing the area occupied by oocyte chromosomes (Tesarik *et al*., 2002). In this experiment, blastocyst rates were not significantly different among methods 1, 3 and 4, but method 3 resulted in a significantly lower blastocyst rate than method 2. Aspiration of ooplasm in the oocyte periphery may increase the risk of disrupting the meiotic spindle and chromosomes, thus impairing the development of ICSI embryos. To achieve oolemma breakage, more ooplasm was aspirated into the pipette in the single aspiration method than in the repeated aspiration method, therefore a single aspiration of ooplasm may increase the chance of disrupting the spindle compared with repeated aspiration of ooplasm. Repeated aspiration and expulsion of ooplasm in the central area of the oocyte may be a more suitable method of breaking the oolemma during ICSI.

In conclusion, sperm injection at 1 h after oocyte collection can improve the efficiency of fertilization and blastocyst development of ICSI rabbit embryos. Cumulus cells on oocytes have no significant effects on fertilization and embryonic development after ICSI. The method of repeated aspiration and expulsion of ooplasm in the oocyte centre can enhance blastocyst development of ICSI embryos more than the method of a single aspiration of cytoplasm in the oocyte periphery.

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References

Ahmadi, A. & Ng, S.C. (1997). Sperm head decondensation, pronuclear formation, cleavage and embryonic development following intracytoplasmic injection of mitochondria-damaged sperm in mammals. *Zygote* **5**, 247–53.

Dozortsev, D., Wakaiama, T., Ermilov, A. & Yanagimachi, R. (1998). Intracytoplasmic sperm injection in the rat. *Zygote* **6**, 143–7.

Hlinka, D., Herman, M., Zesela, J., Hredzak, R., Horvath, S. & Pacin, J. (1998). A modified method of intracytoplasmic sperm injection without the use of polyvinylpyrrolidone. *Hum. Reprod*. **13**, 1922–7.

Host, E., Gabrielsen, A., Lindenberg, S. & Smidt-Jensen, S.A. (2002). Apoptosis in human cumulus cells in relation to zona pellucida thickness variation, maturation stage, and cleavage of the corresponding oocyte after intracytoplasmic sperm injection. *Fertil. Steril*. **77**, 511–15.

Katayama, M., Miyano, T., Miyake, M. & Kato, S. (2002). Progesterone treatment of boar spermatozoa improves male pronuclear formation after intracytoplasmic sperm injection into porcine oocytes. *Zygote* **10**, 95–104.

Mattioli, M., Lucidi, P. & Barboni, B. (1998). Expanded cumuli induce acrosome reaction in boar sperm. *Mol. Reprod. Dev*. **51**, 445–53.

Nagy, Z.P., Verheyen, G., Tournaye, H. & Van Steirteghem, A.C. (1998). Special applications of intracytoplasmic sperm injection: the influence of sperm count, motility, morphology, source and sperm antibody on the outcome of ICSI. *Hum. Reprod*. **13**, 143–54.

Nakagawa, K., Yamano, S., Moride, N., Yamashita, M., Yoshizawa, M. & Aono, T. (2001). Effect of activation with Ca ionophore A23187 and puromycin on the development of human oocytes that failed to fertilize after intracytoplasmic sperm injection. *Fertil. Steril*. **76**, 148–52.

Nandi, S., Chauhan, M.S. & Palta, P. (1998). Influence of cumulus cells and sperm concentration on cleavage rate and subsequent embryonic development of buffalo (*Bubalus bubalis*) oocytes matured and fertilized *in vitro*. *Theriogenology* **50**, 1251–62.

Ock, S.A., Bhak, J.S., Balasubramanian, S., Lee, H.J., Choe, S.Y. & Rho, G.J. (2003). Different activation treatments for successful development of bovine oocytes following intracytoplasmic sperm injection. *Zygote* **11**, 69–76.

Romar, R., Coy, P., Ruiz, S., Gadea, J. & Rath, D. (2003). Effects of oviductal and cumulus cells on *in vitro* fertilization and embryo development of porcine oocytes fertilized with epididymal spermatozoa. *Theriogenology* **59**, 975–86.

Sato, M.S., Yoshitomo, M., Mohri, T. & Miyazaki, S. (1999). Spatiotemporal analysis of $[Ca^{2+}]_i$ rises in mouse eggs after intracytoplasmic sperm injection (ICSI). *Cell Calcium* **26**, 49–58.

Schwartz, P., Magerkurth, C. & Michelmann, H.W. (1996). Scanning electron microscopy of the zona pellucida of

human oocytes during intracytoplasmic sperm injection (ICSI). *Hum. Reprod*. **11**, 2693–6.

Strassburger, D., Friedler, S., Raziel, A., Schachter, M., Kasterstein, E. & Ron-El, R. (2000). Very low sperm count affects the result of intracytoplasmic sperm injection. *J. Assist. Reprod. Genet*. **17**, 431–6.

Sutovsky, P. & Schatten, G. (1997). Depletion of glutathione during bovine oocyte maturation reversibly blocks the decondensation of the male pronucleus and pronuclear apposition during fertilization. *Biol. Reprod*. **56**, 1503–12.

Tesarik, J. (1998). Oocyte activation after intracytoplasmic injection of mature and immature sperm cells. *Hum. Reprod*. **13**, 117–27.

Tesarik, J. & Sousa, M. (1994). Comparison of Ca^{2+} responses in human oocytes fertilized by subzonal insemination and by intracytoplasmic sperm injection. *Fertil. Steril*. **62**, 1197–204.

Tesarik, J. & Sousa, M. (1995). Key elements of a highly efficient intracytoplasmic sperm injection technique: Ca^{2+} fluxes and oocyte cytoplasmic dislocation. *Fertil. Steril*. **64**, 770–6.

Tesarik, J., Rienzi, L., Ubaldi, F., Mendoza, C. & Greco, E. (2002). Use of a modified intracytoplasmic sperm injection technique to overcome sperm-borne and oocyte-borne oocyte activation failures. *Fertil. Steril*. **78**, 619–24.

Van der Westerlaken, L.A., Helmerhorst, F.M., Hermans, J. & Naaktgeboren, N. (1999). Intracytoplasmic sperm injection: position of the polar body affects pregnancy rate. *Hum. Reprod*. **14**, 2565–9.

Yoshida, M., Ishigaki, K., Nagai, T., Chikyu, M. & Pursel, V.G. (1993). Glutathione concentration during maturation and after fertilization in pig oocytes: relevance to the ability of the oocytes to form male pronucleus. *Biol. Reprod*. **49**, 89–94.