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Effects of oocyte collection time and injection position on pronucleus formation and blastocyst development in round spermatid injection in mouse

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Summary

The injection of spermatozoa into mouse, human and rabbit oocytes at specific times and positions can result in different rates of viable embryo development. However, it is not clear how the timing and position of round spermatid injection (ROSI) affect pronucleus (PN) formation and blastocyst development of mice. First, we determined the changes in relative position of the first polar body and the spindle, carried out ROSI from 11.5 to 13 h post-hCG administration, then activated by Sr^{2+}, and finally compared the development of ROSI zygotes, including the formation of pronuclei and development of blastocyst. Between 11.5 and 13 h post-hCG administration, the rate of 2PN formation by ROSI at 3 o'clock was the highest among all treated oocytes. Moreover, the blastocyst rate of zygotes with two pronuclei (2PN) was up to 27.41%. These results suggest that the time and position of ROSI can significantly influence the formation of 2PN, that the rates of 2PN formation are closely correlated with blastocyst formation and that the formation of 2PN is necessary for later embryo development.

Keywords: Blastocyst, Mouse, Pronucleus, Round spermatid injection

Introduction

Viable offspring have been obtained after round spermatid injection (ROSI) in mouse, rat, rabbit, human and hamster ((Ogura *et al*., 1994; Hirabayashi *et al*., 2002; Sofikitis *et al*., 1994; Tesarik *et al*., 1995; Haigo *et al*., 2004). Recently, the technique has also been demonstrated to be a powerful tool for examining the developmental and genetic potential of spermatids (Meng *et al*., 2002; Kanatsu-Shinohara *et al*., 2003).

Round spermatids are immature haploid cells that have a decondensed nucleus; they transform slowly into elongated cells, and finally become spermatozoa. Moreover, similar to events following intracytoplasmic sperm injection (ICSI), the nuclei of round spermatids reportedly have an ability to transform into male pronuclei in ROSI oocytes.

It is additionally reported that in human and rabbit ICSI (Wang *et al*., 2001; Zheng *et al*., 2004), different injection positions and depths can significantly affect the early development of embryos following ROSI. However, it remains unclear whether it is necessary to inject round spermatids at a specific position, and whether the injection position influences the formation of two pronuclei (2PN) and the later development of embryos. The present study was carried out to determine the effects of oocyte age post-hCG administration on position of the first polar body, how the position of ROSI affects the production of pronuclei, and to test whether male pronuclei affect the rate of blastocyst development.

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Materials and methods

Animals

C57BL/6 mice were used to prepare spermatogenic cells and Kunming white mice were used as oocyte donors. All animals were maintained in accordance with the Animal Experiment Standard of State Key Laboratory of Reproductive Biology, Institute of Zoology, Chinese Academy of Sciences.

Collection of oocytes

Mature oocytes were collected from the oviducts of 8- to12-week-old Kunming white female mice that had been induced to superovulate with 7.5 IU pregnant mare serum gonadotropin (PMSG; Tianjin Animal Hormone Factory), followed by 7.5 IU human chorionic gonadotropin (hCG; Ningbo Animal Hormone Factory) 48 h later. Oocytes were collected from oviducts at 10, 11, 12, 13, 14 and 16 h after hCG injection respectively, placed in CZB medium (Chatot *et al*., 1989), and treated with 0.1% hyaluronidase in order to disperse cumulus cells. The oocytes were then placed in CZB medium with 5 mg/ml bovine serum albumin (BSA) and were used to examine the changes in the first polar body (Pb1) and for ROSI within 15 min.

Prearation of round spermatids

To collect spermatogenic cells, the seminiferous tubules of a C57BL/6 male mouse were minced as described previously (Kimura & Yanagimachi, 1995), and the cells suspended in 0.9% NaCl medium. One aliquot of spermatogenic cell suspension was mixed with approximately 10 aliquots of Hepes-CZB medium containing 12% (w/v) polyvinylpyrrolidone (PVP, Mr 360 kDa; Sigma) in a micromanipulation chamber.

Pretreatment and determination of the relative position of Pb1 and the spindle of mouse oocytes at different ages post-hCG

In order to examine the changes in Pb1 in mouse oocytes, the oocytes were stained with 2 mg/ml Hoechst 33342 for 10 min, and the position of Pb1 and the spindle observed under an inverted microscope (Nikon Eclipse TE300, Nikon, Japan).

Microinjection of round spermatids (ROSI) into mouse oocytes

Microinjection was carried out, with some modifications, according to the methods (Zhou *et al*., 2002) for mouse ROSI. Round spermatids were easily recognized by their small size and a centrally located chromatin mass within the nucleus. In a series of experiments, single spermatids (about 10 μm in diameter) were sucked into an injection pipette (7–8 μm ID) and injected into oocytes at different injection positions including 0, 1.5, 3 and 6 o'clock (with the position of Pb1 as 0 o'clock) at 11.5–13 h post-hCG.

Activation and examination of pronucleus formation

ROSI oocytes were placed into Ca^{2+}-free CZB containing 5 mM $SrCl_2$ for 20 min (Bos-Mikich *et al*., 1995). After 6 h, the number of pronuclei in the injected oocytes was examined using an inverted microscope (Nikon Eclipse TE300, Nikon, Japan). A full-grown pronucleus close to the second polar body was assumed to be a female pronucleus. The injected oocytes were classified into two categories: oocytes with two pronuclei and one second polar body (2PN), and oocytes with one pronucleus and one second polar body (1PN). The rate of pronucleus formation (RPF) was calculated by dividing the number of 2PN oocytes and 1PN oocytes in the total number of injected oocytes; the resultant fraction was expressed as a percentage.

Embryo culture

Six hours after activation, 1PN- or 2PN-containing zygotes were separated into different drops and cultured in CZB medium for 24 or 96 h to examine their *in vitro* development.

Statistical analysis

Comparison of 2PN formation and embryo development rates following round spermatid injection at different time and positions was done using the chi-square test. A value of $p < 0.05$ was considered to be statistically significant.

Results

Changes in the relative positions of Pb1 and spindle in mouse oocytes at different ages post-hCG administration

As the time post-hCG injection increased, the angle between Pb1 and spindle of oocytes increased correspondingly, as shown in Fig. 1, Table 1 and Fig. 2, as did the number of oocytes with a large Pb1–spindle angle. These results suggest that the position and distance between Pb1 and the spindle of oocytes can gradually change with the time post-hCG injection; between 11.5 and 13 h post-hCG more than 74.58% (47.46% + 27.12%) have an angle of 0–45°.

Relationship between injection position and rate of pronucleus formation

Rates of pronucleus formation at different injection positions differed significantly. ROSI at 3 o'clock results

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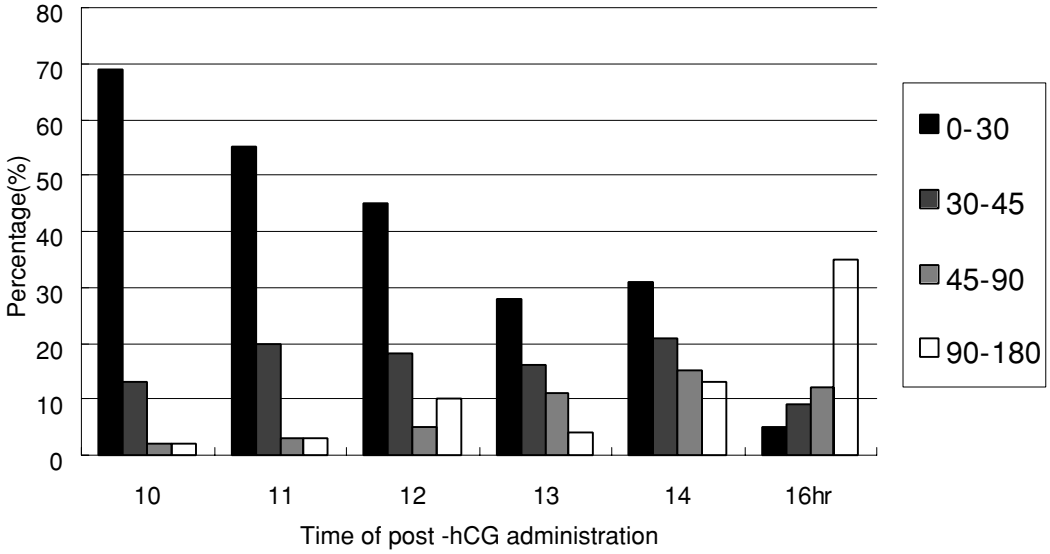

Figure 1 Effects of oocyte age post-hCG administration on PB1 position. *x*-axis, Time of oocyte post-hCG; *y*-axis, percentage of oocytes (number of oocytes with a certain Pb1 – spindle angle/total number of oocytes). Numbers in the right-hand key represent the angle between Pb1 and the spindle.

Table 1 Changes in PB1 position post-hCG administration

		No. (%) of oocytes with different PB1 positions[a]			
Oocyte age post-hCG (h)	No. of oocytes	0–30°	30–45°	45–90°	90–180°
10	86	69 (80.23)	13 (15.12)	2 (2.33)	2 (2.33)
11	81	55 (67.90)	20 (24.69)	3 (3.70)	3 (3.70)
12	78	45 (57.69)	18 (23.08)	5 (6.41)	10 (12.82)
13	59	28 (47.46)	16 (27.12)	11 (18.64)	4 (6.78)
14	80	31 (38.75)	21 (26.25)	15 (18.75)	13 (16.25)
16	61	5 (8.20)	9 (14.75)	12 (19.67)	35 (57.38)

[a] Angle between Pb1 and spindle.

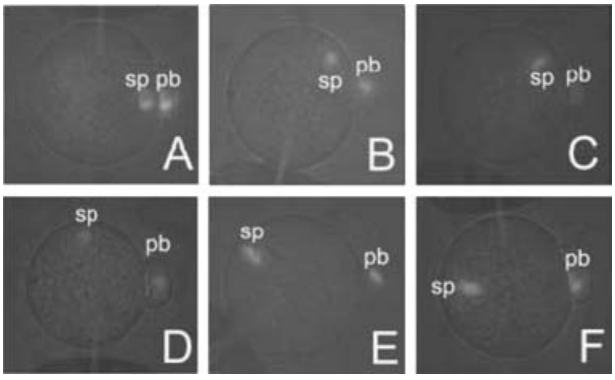

Figure 2 The changes in angle between Pb1 and the spindle at different ages post-hCG. (*A*), (*B*), (*C*), (*D*), (*E*) and (*F*) individually illustrate 0°, 30°, 45°, 90°, 135° and 180°. Sp, spindle; Pb, first polar body.

in optimal fertilization and number of 2PN zygotes, but the results using other positions are fairly poor ($p < 0.05$) as shown in Table 2. This suggests that the position

Table 2 Effects of different injection position on pronucleus formation in mouse zygotes

Injection position (Pb1 = 0 o'clock)	No. of oocytes	No. (%) of 1PN	No. (%) of 2PN
0 o'clock	186	41 (22.40)[a]	43 (32.12)[a]
1.5 o'clock	181	31 (17.13)[a]	32 (17.68)[a]
3 o'clock	188	28 (14.89)[a]	91 (48.40)[b]
6 o'clock	161	30 (18.63)[a]	32 (19.86)[a]

[a,b] Data within the same column with different superscripts are significantly different ($p < 0.01$).

of ROSI can significantly influence the formation of 2PN rather than 1PN in zygotes.

Relationship between rate of pronucleus formation and early-stage development

As shown in Table 3 and Fig. 3, the rate of 2PN formation for ROSI is closely related to the formation

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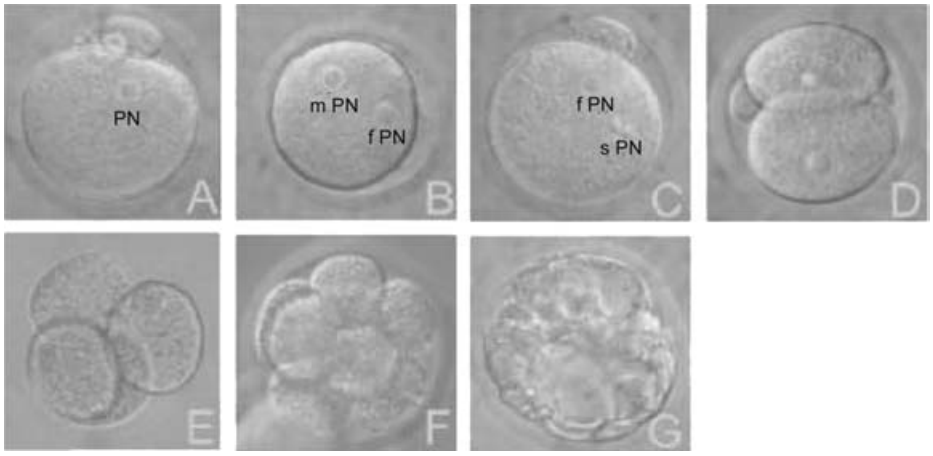

Figure 3 Pronuclei and development of ROSI mouse zygotes. Zygotes with a single pronucleus (1PN, *A*), 2PN with equal-sized male and female pronuclei (*B*), 2PN with a normal-sized female pronucleus and a small male pronucleus (*C*), and mouse embryos developed to the 2-cell (*D*), 4-cell (*E*), 8-cell (*F*) and blastocyst (*G*) stages. PN, pronucleus; fPN, female pronucleus; mPN, male pronucleus; sPN, small pronucleus.

Table 3 Effect of pronucleus number on development of ROSI mouse embryos

No. of pronuclei	No. of oocytes cultured	No. of 2-cell embryos (%)	No. of morulae (%)	No. of blastocysts (%)
1PN	130	124 (95.38)[a]	49 (37.69)[a]	6 (4.62)[a]
2PN	197	189 (95.94)[a]	108 (54.82)[a]	54 (27.41)[b]

[a,b]Data within the same column with different superscripts are significantly different ($p < 0.01$).

of a blastocyst. Although the rate of formation of 2-cell embryos and morulae is not markedly different between 1PN and 2PN zygotes, the rate of blastocyst formation from 2PN zygotes is significantly greater than that from 1PN zygotes (27.41% vs 4.62%; $p < 0.01$), suggesting that 2PN are necessary for the later development of mouse embryos.

Discussion

This study investigated how the relative position of Pb1 and the spindle changed following the elongation of oocytes post-hCG administration, whether the injection position could affect the rate of 2PN and 1PN zygotes, and how both 2PN and 1PN influence the *in vitro* development of mouse zygotes.

Our results indicate that the angle between Pb1 and the spindle is significantly affected by the time post-hCG, i.e. the position and distance between Pb1 and the spindle obviously change, and the optimal time for ROSI is from 11.5 to 13 h post-hCG while the angle between Pb1 and the spindle of most oocytes (67–85%; Table 1) is between 0° and 45°. As regards ROSI at different injection positions, injection at 3 o'clock results in an optimal rate of 2PN formation while the rate of 1PN formation is not affected by injection position; however, the rate of 2PN formation at 0, 1.5 and 6 o'clock is rather lower than that at 3 o'clock.

In general, during *in vivo* fertilization and/or assisted reproduction, most zygotes can form a male pronucleus and a female pronucleus, the size of the two pronuclei is not equal, and the morphology and pronucleus score shows (Kahraman *et al.*, 2002) correlations with implantation and development to the blastocyst stage. A normal pronucleus has a cytoplasmic halo, and presence/absence of a halo had a significant effect on the rate of development and the overall embryo morphology score. Therefore the determination of the pronucleus can help predict the developmental potential of embryos, aid in early selection and may indicate the health of the oocyte.

However, the present study shows that the rate of 2PN in ROSI zygotes is lower than that in normally fertilized oocytes. Many zygotes have a single pronucleus (1PN; Fig. 3A), and pronuclear morphology and the size of the two pronuclei in ROSI zygotes can differ: the size of male and female pronucleus may be almost equal (Fig. 3B), or the male pronucleus may be a great deal smaller than the female one (Fig. 3C), the formation of a small pronucleus perhaps resulting from insufficient decondensation of the round spermatid. Furthermore, importantly, although round spermatids have the ability to form a pronucleus in oocytes, and several kinds of animals (Ogura *et al.*, 1994; Hirabayashi *et al.*, 2002; Sofikitis *et al.*, 1994; Tesarik *et al.*, 1995; Haigo *et al.*, 2004) have been produced, mouse oocytes are easily activated and the relative position of Pb1 and the spindle can significantly change *in vitro*. Therefore, it

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would appear that the time restriction that applies to ROSI limits the ability of haploid male cells to become incorporated into a zygote, or (and) the ROSI procedure may also damage the spindle due to changes in the spindle, which can decrease the rate of 2PN formation and increase oocyte death. The results of our study reveal that a higher rate of male pronucleus formation is maintained if injections are carried out at 3 o'clock at 11.5–13 h post-hCG injection.

In addition, the results of the present study indicate that male pronuclei could support *in vitro* development of mouse zygotes. ROSI zygotes with 2PN can develop to the blastocyst stage more successfully than those with 1PN, which indicates that the function of a male pronucleus is necessary for the later development of mouse zygotes.

Therefore, we conclude that a specific time and injection position are required for injected round spermatids to become male pronuclei that are capable of supporting *in vitro* development; this may indicate male pronuclei need to be critically reprogrammed. Furthermore, during the development of embryos the formation of 2PN (a male pronucleus and a female one) is required, rather than 1PN, which suggests to some extent the male pronucleus is absolutely necessary for blastocyst formation and later production of offspring.

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References

Bos-Mikich, A., Swann, K. & Whittingham, D.G. (1995). Calcium oscillations and protein synthesis inhibition synergistically activate mouse oocytes. *Mol. Reprod. Dev.* **41**, 84–90.

Chatot, C.L., Ziomek, C.A., Bavister, B.D., Lewis, J.L. & Torres, I. (1989). An improved culture medium supports development of random-bred 1-cell mouse embryos *in vitro*. *J. Reprod. Fertil.* **86**, 679–88.

Haigo, H., Yamauchi, Y., Yazama, F., Yanagimachi, R. & Horiuchi, T. (2004). Full-term development of hamster embryos produced by injection of round spermatids into oocytes. *Biol. Reprod.* **71**, 194–8.

Hirabayashi, M., Kato, M., Aoto, T., Ueda, M. & Hochi, S. (2002). Rescue of infertile transgenic rat lines by intracytoplasmic injection of cryopreserved round spermatids. *Mol. Reprod. Dev.* **62**, 295–9.

Kahraman, S., Kumtepe, Y., Sertyel, S., Donmez, E., Benkhalifa, M., Findikli, N. & Vanderzwalmen, P. (2002). Pronuclear morphology scoring and chromosomal status of embryos in severe male infertility. *Hum. Reprod.* **17**, 3193–200.

Kanatsu-Shinohara, M., Ogonuki, N., Inoue, K., Ogura, A., Toyokuni, S., Honjo, T. & Shinohara, T. (2003). Allogeneic offspring produced by male germ line stem cell transplantation into infertile mouse testis. *Biol. Reprod.* **68**, 167–73.

Kanatsu-Shinohara, M., Ogura, A., Ikegawa, M., Inoue, K., Ogonuki, N., Tashiro, K., Toyokuni, S., Honjo, T. & Shinohara, T. (2002). Adenovirus-mediated gene delivery and *in vitro* microinsemination produce offspring from infertile male mice. *Proc. Natl. Acad. Sci. USA* **99**, 1383–8.

Kimura, Y. & Yanagimachi, R. (1995). Mouse oocytes injected with testicular spermatozoa or round spermatids can develop into normal offspring. *Development* **121**, 2397–405.

Meng, X., Akutsu, H., Schoene, K., Reifsteck, C., Fox, E.P., Olson, S., Sariola, H., Yanagimachi, R. & Baetscher, M. (2002). Transgene insertion induced dominant male sterility and rescue of male fertility using round spermatid injection. *Biol. Reprod.* **66**, 726–34.

Ogura, A., Matsuda, J. & Yanagimachi, R. (1994). Birth of normal young after electrofusion of mouse oocytes with round spermatids. *Proc. Natl. Acad. Sci. USA* **91**, 7460–2.

Sofikitis, N.V., Miyagawa, I., Agapitos, E., Pasyianos, P., Toda, T., Hellstrom, W.J. & Kawamura, H. (1994). Reproductive capacity of the nucleus of the male gamete after completion of meiosis. *J. Assist. Reprod. Genet.* **11**, 335–41.

Tesarik, J., Mendoza, C. & Testart, J. (1995). Viable embryos from injection of round spermatids into oocytes. *N. Engl. J. Med.* **333**, 525.

Wang, W.H., Meng, L., Hackett, R.J., Odenbourg, R. & Keefe, D.L. (2001). The spindle observation and its relationship with fertilization after intracytoplasmic sperm injection in living human oocytes. *Fertil. Steril.* **75**, 348–53.

Zheng, Y.L., Jiang, M.X., Zhang, Y.L., Sun, Q.Y. & Chen, D.Y. (2004). Effects of oocyte age, cumulus cells and injection methods on *in vitro* development of intracytoplasmic sperm injection rabbit embryos. *Zygote* **12**, 75–80.

Zhou, Q., Boulanger, L. & Renard, J.P. (2000). A simplified method for the reconstruction of fully competent mouse zygotes from adult somatic donor nuclei. *Cloning* **21**, 35–44.