Subzonal insemination with primary spermatocytes in mouse

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It is well known that oocytes, in general condition, can be penetrated by sperm rather than round spermatids. In 1993, Ogura[1] injected round spermatid nuclei into hamster oocytes and demonstrated that the spermatid nuclei could transform into pronuclei with DNA synthesis and that their chromosomes mingled with oocyte chromosomes immediately. In order to avoid the mechanical damage to the oocytes, an alternative method, cell-to-cell fusion, was used. Approximately 20%—40% and 30% of round spermatids could be fused with oocytes by electric stimulation in hamster and mouse, respectively. Some of the fused mouse oocytes could develop normally to term[2,3]. Secondary spermatocyte is 2 N haploid. After injection of a single secondary spermatocyte nucleus into an oocyte, both the oocyte and spermatocyte chromosomes completed their meiotic divisions, resulting in the formation of a haploid female and male pronuclei, which supported normal embryonic development. 24% of the embryos could develop into normal offspring after transfer[4]. Primary spermatocyte is 4 N diploid. Can it participate in syngamy? In 1995, Kimura[4] reported that when the oocyte into which a primary spermatocyte was injected was left unactivated for 2 h, the spermatocyte chromosomes condensed prematurely. These chromosomes as well as metaphase-Ⅱ oocyte chromosomes resumed meiosis upon electroactivation, culminating in the formation of two pronuclei, one of which was paternal origin and was diploid or polyploid. On the basis of our previous success in the microfertilization with round spermatids, the behavior and developmental potential of mouse primary spermatocyte nuclei fused into the oocytes are reported in this note.

1 Materials and methods

Kunming white mice were used as donor and recipient females and vas oligational males were purchased from the Experimental Animal Center, Institute of Genetics, Chinese Academy of Sciences. Donor male mice were C57BL/6J, black coats, purchased from the Experimental Animal Center, Beijing Medical University.

Collagenase-Ⅰ, Trypsin-Ⅲ, Hyaluronidase, Hepes, BSA were purchased from Sigma and BDH Chemical Co.

Oocytes were collected from oviducts of PMSG (10 IU)-primed superovulated females 14—16 h after injection of hCG (10 IU). The oocytes were freed of cumulus cells by a 2-min treatment with 0.1% bovine testicular hyaluronidase in 10 mmol/L Hepes buffered T6 medium (HT6).

Testes of a mature male C57BL/6J mouse were excided one at a time. After removal of the tunica albuginea with fine forceps, seminiferous tubules were allowed to spread with 1 mg/mL collagenase in HT6. The dispersed seminiferous tubules were then exposed to

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2 mg/mL trypsin in HT6 and the spermatogenic cells were dispersed into the medium by gently aspirating in and out of the pipette. The cell suspension was washed three times with HT6. Most of the round nucleate cells thus isolated were identified as round spermatids and primary spermatocytes by interference contrast microscopy. Primary spermatocyte was distinguished from spermatid by its bigger size (16 μm) and denser nucleus (figure 1).

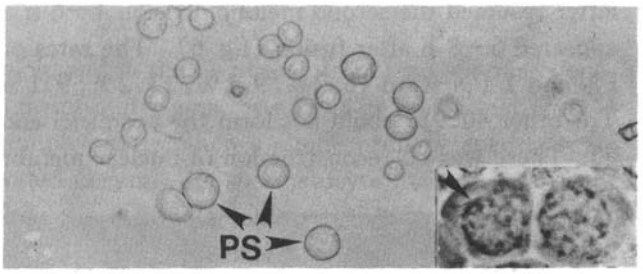

Fig. 1. Spermatogenic cells released from seminiferous tubles, the arrow indicates a primary spermatocyte (PS). Inset shows a PS dense nucleus under higher magnification (×1 300).

Mouse oocytes, free from cumulus cells, were transferred to HT6 in the manipulation glass dish. A primary spermatocyte was injected into the perivitelline space (fig. 2) using an Eppendoff 5124 micromanipulator. The internal diameter of the injection pipette was about 16 μm. The tip of the pipette had been beveled at a 35°—40° angle using a rotary grinding wheel (Narishige, PW-6, Japan). The primary spermatocyte-oocyte pairs (PS-O) were placed in HT6 for 10 min, then transferred to fusion medium (300 mmol/L mannitol, 0.05 mmol/L $CaCl_2$, 0.1 mmol/L $MgSO_4$) and exposed to a fusion DC pulse (3 700—3 800 v/cm, 25μs) preceded and followed by application of AC pulse (1MHz, 50 v/cm, 20—30 s) each. Electrofused oocytes were incubated in HT6 under 5% CO_2 in air for up to 30 h.

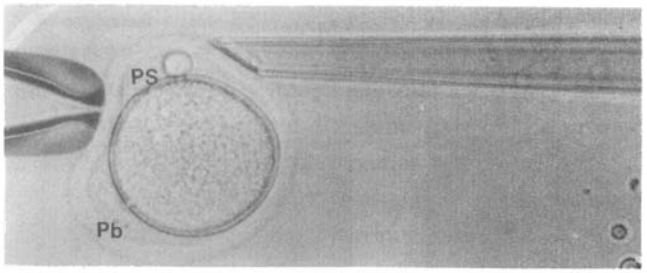

Fig. 2. Subzonal injection of primary spermatocyte (×620). Pb: First polar body.

Recipient female mice were mated with vasectomized males (2:1) at the time of hCG injection. A total of 10—20 embryos at pronucleus stage or 2—4 cell stage were transferred surgically into one side of the oviducts of pseudopregnant mice with vaginal plug.

At various times (1, 2, 3, 4, 6, 8 and 10 h) after application of the fusion pulse, oocytes were removed from the culture dish, mounted and compressed between a slide and a coverslip, then fixed with 10% formalin, stained with 1% acetic carmine, and finally decolorized before examination with a phase-contrast microscope. The decolorant was glycerol: acetic acid: water (1:1:3).

2 Results

A total of 500 oocytes were injected, of which 421 (84.2%) recovered their normal morphology. 2 h after fusion, 346 of the 421 PS-O (82.2%) were survival. 223 (64.5%) of survival PS-O pairs were fused. 16.1% of the fused eggs cleaved (figure 10).

Oocytes were examined at 1—10 h after application of fusion pulse. The primary spermatocytes could be fused at 1 h and their nuclear density was decreased (fig. 3). 2 h latter, they completed the first meiotic division and formed two secondary spermatocytes (fig. 4), then the

latter resumed the second meiotic division 4—6 h after fusion (fig. 5). 4 male pronuclei (PN) appeared 6—8 h after fusion (fig. 6). The rates of eggs developed to 5 PN, 4 PN, 3 PN, 2 PN and 1 PN were 18.2%, 9.1%, 13.2%, 9.1% and 9.1% (figs. 7 and 8) respectively. The other 40.9% could not form the pronuclei and always extruded several polar bodies (fig. 9). There was no reconstruction of nuclear membrane during meiotic division.

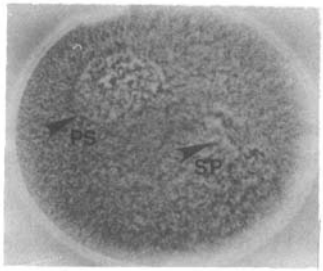

Fig. 3. PS was fused into oocyte 1 h after fusion pulse (× 680). SP, Spindle.

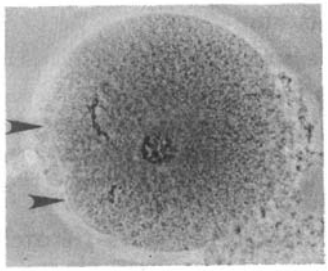

Fig. 4. PS was in telophase of the first meiotic division 2 h after fusion pulse (×680).

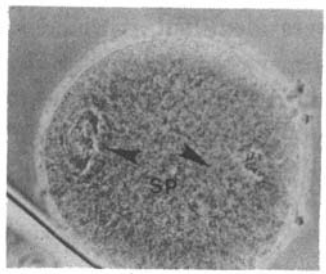

Fig. 5. PS was in metaphase of the second meiotic division 6 h after fusion pulse (×680).

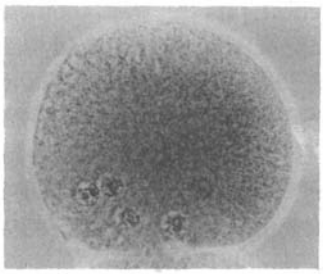

Fig. 6. One female and 4 male pronuclei appeared 8 h after fusion pulse (×680).

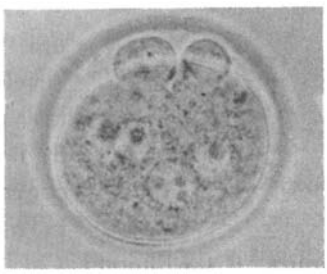

Fig. 7. Four pronuclei and 2 Pb appeared 8 h after fusion pulse (× 620).

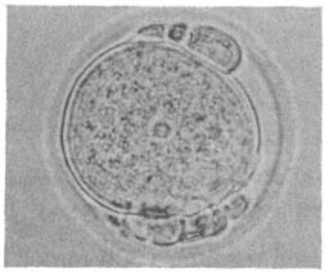

Fig. 8. One pronucleus and poly-polar bodies 8 h after fusion pulse (×620).

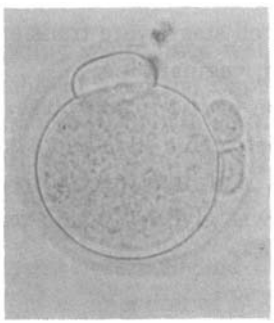

Fig. 9. No pronucleus and poly-polar bodies appeared 8 h after fusion pulse (×620).

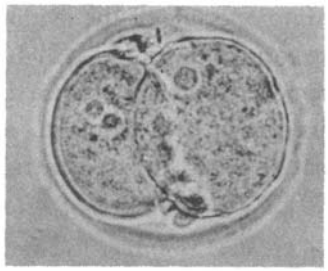

Fig. 10. 2-cell stage embryo appeared 28 h after fusion pulse (×620).

A total of 33 pronuclear eggs and 38 2-cell embryos were transferred surgically into 5 recipients. None of them was pregnant.

3 Discussion

The primary spermatocytes are 4 n diploid and in the G_2 phase. In normal spermatogenesis, a primary spermatocyte completes two meiotic divisions, resulting in the formation of 4 spermatozoa with a haploid chromosomal complement. When a single primary spermatocyte was injected into the ooplasm of an unactivated oocyte, the spermatocyte nucleus underwent premature chromosome condensation and then the meiotic spindle attached to chromosomes. When such oocytes were activated, both the oocyte and spermatocyte chromosomes completed meiotic division, resulting in the formation of two polar bodies; one was of maternal origin, the other paternal origin. Most of the zygotes were triploid or near triploid and could not develop normally[4]. In this experiment, the primary spermatocytes were electrofused with oocytes, and the electric pulses could also activate the oocytes at the same time, and made the oocyte's second meiotic division resume. The increase of the maturation promoting factor (MPF) activity could lead to the resumption of first meiotic division of primary spermatocyte. Although MPF activity decreased after oocyte completed the division[5], primary spermatocyte could continue the second meiotic division soon after the first. We can infer that the second meiotic division of primary spermatocyte may be regulated by itself.

During the meiotic division, part of the primary spermatocyte chromosomes could be extruded in the form of polar bodies which we called "male polar bodies (MPB)". Extruding of the MPBs at random during the first or/and second meiotic division of primary spermatocyte led to the formation of polypronuclear eggs. Fig. 11 illustrates the behavior of primary sperma-

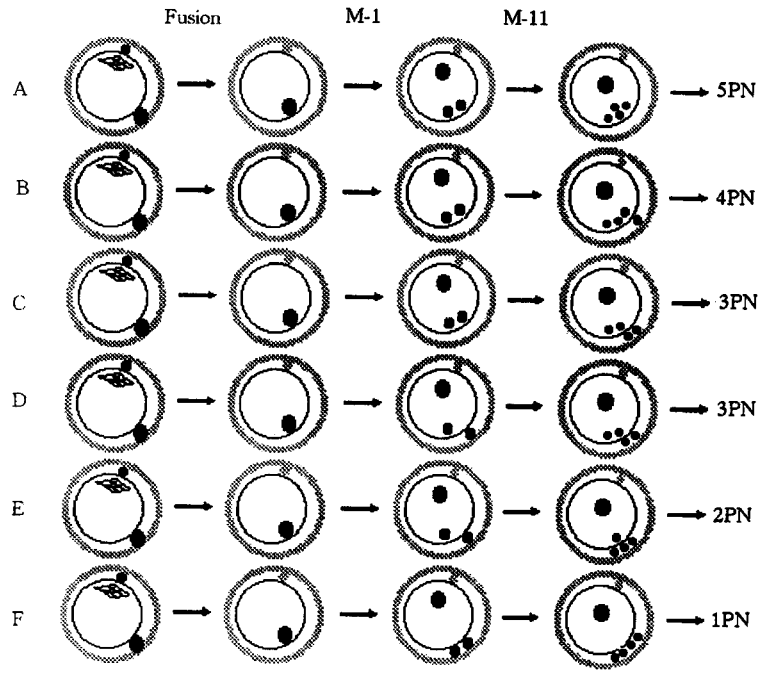

Fig. 11. Behavior of primary spermatocyte nuclei fused into oocytes. A, No MPB was extruded during the first or second division; B, one MPB was extruded during the second division; C, two MPBs were extruded during the second division; D, one MPB was extruded during the first division; E, one MPB was extruded during the first and second division respectively; F, whole spermatocyte nucleus was extruded.

tocyte nuclei fused into the oocytes. When MPBs failed to be extruded during the meiotic division, 5 pronuclei formed within one egg, one of which was of maternal origin, and the rest were of paternal origin (fig. 11A). When an MPB was extruded during both the first and the second meiotic division, an egg with a haploid male pronucleus formed, which could be expected to support normal embryonic development (fig. 11E). Some of oocytes could assemble the whole nuclei of primary spermatocytes and/or oocytes and extruded them as polar bodies. In this situation, eggs with one pronucleus (fig. 11F) or without pronucleus were obtained.

The results indicate that oocyte cell cycle may play an important role in primary spermatocyte meiotic division. We expect to obtain more normal two-pronuclei embryos (diploid) or offspring by regulating oocyte cell cycle. These results also provide a good idea for the treatment of male factors infertility and the production of transgene animals as well as the protection of wild animal's genetic resources.

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