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Sucrose Pretreatment for Enucleation: An Efficient and Non-Damage Method for Removing the Spindle of the Mouse MII Oocyte

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ABSTRACT Oocytes enucleated at metaphase II stage can support reprogramming of transferred nucleus and further developing to term. However, the first polar body in mice sometimes migrates away from the original place of expulsion, so the chromosomes of the oocyte will displace from the first polar body. Thus, it is not always possible to successfully enucleate according to the position of the first polar body. Here we use sucrose treatment to visualize metaphase spindle fibers and chromosomes with standard light microscopy. In the manipulation medium containing 3% sucrose, oocytes of poor quality become shrunken, deformed or fragmented, while oocytes of good quality in the same medium would show a swelling around the metaphase chromosomes and a transparent spindle area, shaped like "∞" and "0". So it is easy to remove the well-distinguished spindle and chromosomes in oocytes of good quality. Re-examined by Hoechst 33342 stain under the UV light, the enucleation rate was 100%. There was no significant difference in IVF and cleavage rates between the sucrose treatment and the control group. In conclusion, this study demonstrated that 3% sucrose pretreatment can give a method for evaluating embryo quality and more importantly, it can, under a common microscope, allow the visualization of the spindle and chromosomes in oocytes of good quality and hence efficiently improve enucleation rate without any harm. *Mol. Reprod. Dev. 58:432–436, 2001.* © 2001 Wiley-Liss, Inc.

Key Words: mouse oocytes; sucrose; enucleation; spindle; fertilization

INTRODUCTION

Mice are one of the most useful experimental animal models for developmental biology, particularly in nuclear transfer. Since the first report of successful nuclear transfer in mice (McGrath and Solter, 1983), normal pups have been obtained from 4 to 8-cell embryos (Cheong et al., 1993), morulae (Tsunoda and Kato, 1997), inner cell mass (Tsunoda and Kato, 1998), trophectoderm (Tsunoda and Kato, 1998), and somatic cells (Wakayama et al., 1998). In previous studies, enucleated zygotes (McGrath and Solter, 1983), enucleated 2-cell stage embryos (Tsunoda et al., 1987; Wang et al., 1997), and enucleated metaphase II (MII) oocytes (Kono et al., 1991) were used as recipients for nuclear transfer. Among them, enucleated oocytes were the most common recipients (Campbell et al., 1996). However, the nucleus in MII mouse oocytes cannot be clearly distinguished from the cytoplasm under a phase contrast or interference microscope (Tsunoda et al., 1988). Metaphase II oocytes are generally enucleated blindly by aspirating the first polar body (PBI) and the adjacent cytoplasm, presumably containing nuclear material (Mohamed Nour and Takahashi, 1999). The position of the PB1 in mouse oocytes randomly migrates with time after the injection of human chorionic gonadotropin (hCG). Only 10% of MII nuclei are beneath PB1 (Kono et al., 1991). Many attempts had been made for enucleation of MII oocyte in mice. Those attempts included anaphase enucleation (Kono et al., 1991), chemical enucleation (Fulka and Moor, 1993), Hoechst 33342 stain and then enucleation under ultraviolet (UV) light (Tsunoda et al., 1988), identification of spindle with differential interference microscope (Kono et al., 1993), Nomarski optics (Tsunoda and Kato, 1995), and so on. Recently, polarization microscope (polscope) was used to check the position of MII spindle in hamster oocytes (Silva et al., 1999). But these methods do have some limitations. For example, Hoechst stain and UV light were harmful to oocytes, and polscope is not so popular in common labs.

Here we report an efficient and non-damaging method for removing the spindle (chromosome) of MII mouse oocytes by treatment of 3% sucrose medium under common inverted microscope.

MATERIALS AND METHODS

Media

Five media were used as following: (1) $M2^-$ medium (Wang et al., 2000a), i.e., M2 medium (Hogan et al.,

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1986) excluded glucose and phosphate, was used for oocyte collection and washing; (2) M2 medium was only used in control group in Experiment 2; (3) The micromanipulation medium was $M2^-$ containing 3% (w/v) sucrose, 20% fetal bovine serum (FBS; Chuanye Co., Tianjin, China), and 7.5 μg/ml cytochalasin B (CB; Sigma, St. Louis, MO); (4) Control medium was same as (2), only sucrose was excluded; (5) M16 medium (Hogan et al., 1986) supplemented with 100 μM EDTA and 2.5 mM taurine (M16 + ET), was used in IVF and embryo culture (Wang et al., 2000b).

Oocyte Collection

Female Kunming Strain (white) mice (Institute of Genetics, Academia Sinica, Beijing, China), 8–12 weeks old, were superovulated with 10 IU of equine chorionic gonadotropin (eCG; s.c.; Tianjin Experimental Animal Center, Tianjin, China) followed by 10 IU hCG (i.p.; Institute of Zoology, Academia Sinica, Beijing, China) 48 hr later. Matured oocytes were collected from the ampullae of oviducts 14–17 hr after hCG injection and placed in 200 μl of $M2^-$ medium containing 300 IU/ml hyaluronidase (Sigma). After complete removal of cumulus cells from the oocytes, they were washed three times in $M2^-$ medium and then transferred for manipulation or cultured in 20 μl drops of M16 + ET, under mineral oil in a humidified atmosphere of 5% CO_2 and 95% air at 37°C until further use.

Enucleation of MII Spindle

In Experiment 1, the efficiency of showing and enucleating of spindle by 3% sucrose treatment was compared with that of control. Cumulus-free MII oocytes were randomly divided into two groups and put into two drops of 3% sucrose containing micromanipulation medium and control medium, respectively. We used the swelling and transparent area (Fig. 1a, c) (for details see Results) as the position for enucleation in the sucrose treatment (SUC) group, while PB1 as the position for enucleation in the control (CON) group. In SUC group, the zona pellucida was slit with a glass needle along 1/5–1/4 of its circumference (Tsunoda

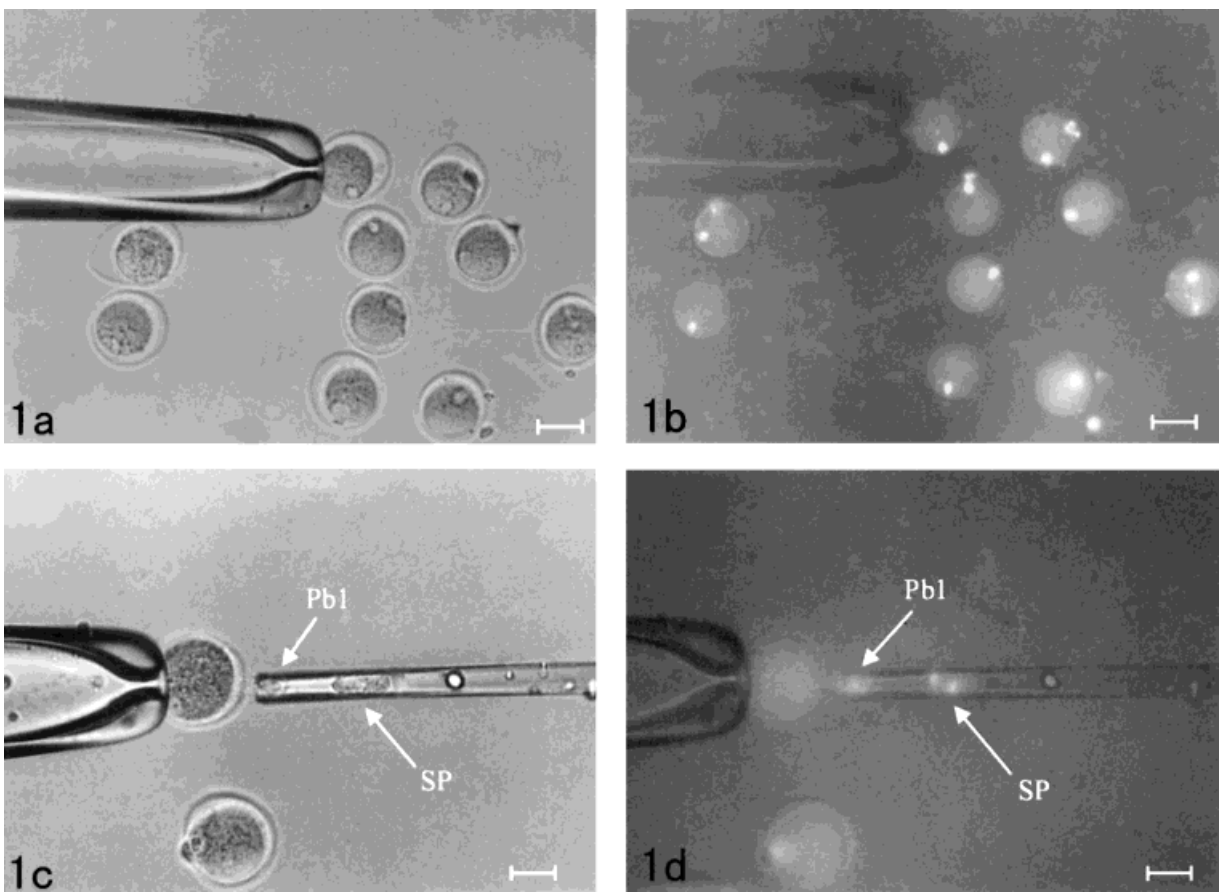

Fig. 1. Enucleating of MII spindle and chromosomes in mouse oocytes by sucrose treatment. **a**: The spindle (arrow) can be distinguished clearly from cytoplasm in manipulation medium containing 3% sucrose under common inverted microscope. **b**: Observation of the oocytes under UV light by staining in Hoechst 33342 with the same field as (a). **c**: Processing of enucleation in medium containing 3% sucrose and **d**: examined the removed PB1 and spindle in pipette under UV light by staining in Hoechst 33342. Pb1, first polar body; SP, spindle; Bar(s) in a–d, 50 μm.

et al., 1986; Wang et al., 1997) close to the position of the swelling or spindle. The MII chromosomes of all recipients were removed with an enucleation pipette with a non-bevel tip (Fig. 1c). In CON group, the zona pellucida of the oocyte was slit close to the position of presumable MII chromosomes (sometimes it showed a bit of difference from the other place under common inverted microscope) or PB1 if there was no indication from the oocyte. The enucleation rates in both groups were checked by Hoechst 33342 (5 μg/ml, Sigma) under UV light (Tsunoda et al., 1988).

In Vitro Fertilization of Oocytes After Zona Cutting in 3% Sucrose Medium

In Experiment 2, the influence of zona cutting in 3% sucrose containing medium on IVF was examined. The caudal epididymis of male Kunming mouse (12–15 weeks old) was cut and cleaned. Each caudal epididymis was cut into 4–6 pieces and transferred into 200 μl warmed M16 + ET covered by mineral oil. The spermatozoa were released and incubated at 37°C in a humidified atmosphere of 5% CO_2 in air for 1 hr for capacitation. Then about 10 μl capacitated spermatozoa were added into 50 μl M16 + ET medium drop that contained the oocytes in which zona pellucida was slit as in Experiment 1 within 1 hr. In CON group, the zona pellucida of the oocyte was slit in $M2^-$ medium with or without CB before being used for IVF. The concentration of spermatozoa for IVF was $0.5–10 \times 10^5$ /ml. The fertilized eggs were picked out 15 hr after IVF and washed three times in $M2^-$ medium before being transferred into another 20 μl fresh M16 + ET medium for culture. Cleavage of embryos was recorded at 24-hour intervals. Fifteen and 7 replicates were made in Experiment 1 and Experiment 2, respectively.

Statistical Analysis

The data were analyzed with Student's *t*-test.

RESULTS

After being treated with 3% sucrose, the cytoplasm surrounding the meiotic apparatus of MII oocyte swelled and could be well observed under common inverted microscope. Moreover, the meiotic apparatus (the spindle) became transparent and took a shape of "∞" or "0", well distinguished from the other part of cytoplasm (Fig. 1a). Whether or not the aspirated karyoplast contained MII spindle could also be identified by this method under common inverted microscope (Figs. 1c, 2a). Further detection by Hoechst 33342 under the UV light showed that the transparent area was indeed the meiotic apparatus and the enucleation rate by the sucrose method was 100% (Figs. 1b,d, 2b). In contrast, the rate of enucleation in CON group without sucrose treatment was only 28%, significantly lower than that in SUC group (Table 1).

The fertilization rate in zona cut oocytes was compared in all groups and there were no significant differences of IVF and cleavage in both groups (Table 2).

DISCUSSION

The results of the present study demonstrated that treatment of mouse MII oocytes with medium containing 3% sucrose was non-toxic, non-damaging, and highly efficient for the removal of MII meiosis apparatus or spindle. Sucrose has been widely used for a long time, not only in embryo cryopreservation, but also in nuclear transfer in cattle and its safety for further embryo development had been proven (Rall, 1987; Collas and Barnes, 1994; Saito et al., 1994). We have also observed about 11% of oocytes of poor quality, e.g., large zona perivitelline space or rough plasma membrane just after recovery from oviducts, or deformed, but no oocytes of good quality showed such changes and they always maintained a smooth membrane and no significant changes in perivitelline space before or after

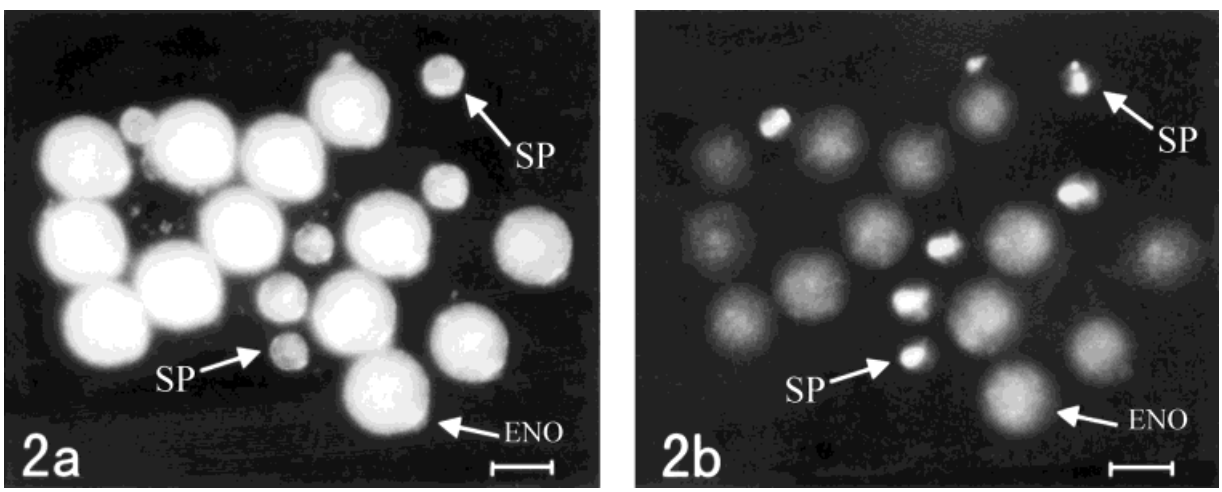

Fig. 2. Observation and examination of the enucleated oocytes in common inverted microscope and in UV light. **a**: Enucleated oocytes and removed spindle (arrow) under dark field microscope and **b**: UV light by staining in Hoechst 33342. SP, spindle; ENO, enucleated oocyte; Bar(s) in a–b, 50 μm.

TABLE 1. Efficiency of Enucleation MII Oocytes After Pretreatment by 3% Sucrose Containing Medium

Groups	Number of oocytes used for treatment	Number of oocytes abnormal (%)	Number of oocytes used for enucleation	Number of oocytes enucleated/ manipulated (%)
3% sucrose and CB contained $M2^-$ medium (SUC)	375	33(11)	342	317/317(100)[a]
CB contained $M2^-$ medium(CON)	163	16(10)	132	28/101(28)[b]

SUC, sucrose treatment; CON, control.
Superscripts (a,b) within the column differ significantly ($P < 0.01$).

TABLE 2. Influence of Pretreatment Oocytes in Sucrose Containing Medium on IVF

Groups	Number of oocytes used for treatment	Number of oocytes used for IVF	Number of oocytes fertilized (%)	Number of oocytes cleavage (%)
3% sucrose and CB contained $M2^-$ medium (SUC)	155	148	143(97)[a]	130(91)[a]
CB contained $M2^-$ medium (CON 1)	45	43	38(88)[a]	38(88)[a]
M2 medium(CON 2)	57	55	49(89)[a]	46(82)[a]

SUC, sucrose treatment; CON 1, control 1; CON 2, control 2.
No significant difference within columns.

treatment with 3% sucrose medium. Thus the use of 3% sucrose can provide a method for judging oocyte quality, so that only oocytes of good quality will be selected for further micromanipulation in a shorter time.

The use of sucrose can increase the concentration of cytoplasmic protein, which induced a slight permeation to the cell, and shrinkage of the cell (Saito et al., 1994). The components in the spindle are different from the components in the cytoplasm (Longo, 1997). This might be the reason that sucrose treatment on oocyte can induce, or enhance, the spindle to undergo different changes from other components in the cytoplasm, which then enable the spindle easily found under common inverted microscope. The higher survival rate in sucrose treatment group than that of control was, maybe mostly, due to the little increased perivitelline space, thus avoiding the trauma to the oocyte membrane during the zona cutting operation.

Treatment of oocytes in micromanipulation medium containing 3% sucrose was simple and convenient because no more micromanipulation procedure was needed. Previous reports suggested that the metaphase meiosis II nucleus or spindle could be shown under differential interference microscope as a translucent region (Kono et al., 1993) or a small swelling around the chromosomes (Tsunoda and Kato, 1995). We found less than 30% of oocytes could be distinguished and enucleated under common inverted microscope. In contrast, 100% of oocytes could be distinguished by 3% sucrose treatment. The oocytes treated by 3% sucrose had high efficiency and there was no damage to IVF and further cleavage of the embryo. In another experiment, combined with the method of sucrose-pretreated enucleation, two offspring were obtained by IVF after transfer spindle from C57BL/6 mouse (black) into enucleated oocytes of Kunming mouse and fused by electrofusion (Wang et al., 2000c).

The anaphase enucleation is time-consuming because the oocytes were collected from the ovarian follicles and needed to be matured in vitro (Kono et al., 1991). In Hoechst UV stain enucleation, longer than 15-second exposure in UV light is harmful to the development of treated embryos (Tsunoda et al., 1988). It certified that the exposure to UV rays was damaging to chromosomes (Ebert et al., 1985); ultraviolet light, including low energy UVA (320–400 nm) radiation, as well as high intensity near-infrared (NIR) laser radiation may induce cell damage (Konig et al., 1996). It is unclear whether treatment by different chemicals is harmful to further development of the embryo (Fulka and Moor, 1993). Recently, it was reported that the polarized microscope could be used to noninvasively evaluate the spindle architecture (Silva et al., 1999; Liu et al., 2000). But there are a few limitations of using a polarized microscope, such as the addition of Pol-Optics, smaller operation space, and high cost. We also demonstrated that pretreating rat oocytes with 3% sucrose-containing medium was also helpful for the further enucleation (Wang et al., unpublished results). With the advantage of simplicity and non-damage to ooctyes and embryos,

the sucrose pretreatment for the enucleation method may thus be used in microoperation in other animals and even in humans.

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REFERENCES

Campbell KHS, Loi P, Otaegui P, Wilmut I. 1996. Cell cycle coordination embryo cloning by nuclear transfer. Rev Reprod 1: 40–46.

Cheong HT, Takahashi Y, Kanagawa H. 1993. Birth of mice after transplantation of early cell-cycle-stage embryonic nuclei into enucleated oocytes. Biol Reprod 48:958–963.

Collas P, Barnes FL. 1994. Nuclear transplantation by microinjection of inner cell mass and granulosa cell nuclei. Mol Reprod Dev 38:264–267.

Ebert KM, Hammer RE, Papaioannou VE. 1985. A simple method for counting nuclei in the preimplantation mouse embryo. Experientia 41:1207–1209.

Fulka J Jr, Moor RM. 1993. Noninvasive chemical enucleation of mouse oocytes. Mol Reprod Dev 34:427–430.

Hogan B, Costatini F, Lacy E. 1986. In vitro culture of eggs, embryos and teratocarcinoma cells. In: Hogan B, Costatini F, Lacy E, editors, Manipulating the mouse embryo: a laboratory manual. New York: Cold Spring Harbor Laboratory Press. p 246–267.

Konig K, Tadir Y, Patrizio P, Berns MW, Tromberg BJ. 1996. Effects of ultraviolet exposure and near infrared laser tweezers on human spermatozoa. Hum Reprod 11:2162–2164.

Kono T, Kwon OY, Nakahara T. 1991. Development of enucleated mouse oocytes reconstituted with embryonic nuclei. J Reprod Fertil 93:165–172.

Kono T, Sotomaru Y, Sato Y, Nakahara T. 1993. Development of androgenetic mouse embryos produced by in vitro fertilization of enucleated oocytes. Mol Reprod Dev 34:43–46.

Liu L, Odlenbourg R, Trimarchi JR, Keefe DL. 2000. A reliable, noninvasive technique for spindle imaging and enucleation of mammalian oocytes. Nat Biotech 18:223–225.

Longo JF. 1997. Gamete fusion and sperm incorporation. In: Longo JF, editor. Fertilization. 2nd ed. London: Chapman & Hall. p 49–65.

McGrath J, Solter D. 1983. Nuclear transplantation in the mouse embryo by microsurgery and cell fusion. Science 220:1300–1302.

Mohamed Nour MS, Takahashi Y. 1999. Prepartion of young preactivated oocytes with high enucleation efficiency for bovine nuclear transfer. Theriogenology 51:661–666.

Rall WF. 1987. Factors affecting the suvival of mouse embryos cryopreserved by vitrification. Cryobiology 24:387–402.

Saito N, Imai K, Tomizawa M. 1994. Effect of sugars-addition on the survival of vitrified bovine blastocysts produced in vitro. Theriogenology 41:1053–1060.

Silva CP, Kommineni K, Oldenbourg R, Keefe DL. 1999. The first polar body does not predict accurately the location of the metaphase II meiotic spindle in mammalian oocytes. Fertil Steril 71:719–721.

Tsunoda Y, Kato Y. 1995. Development of enucleated mouse oocytes receiving PDGF or FGF treated fetal male germ cells after activation with electrical stimulation. J Reprod Dev 41:71–75.

Tsunoda Y, Kato Y. 1997. Full-term development after transfer of nuclei from 4-cell and compacted morula stage embryos to enucleated oocytes in the mouse. J Exp Zool 278:250–254.

Tsunoda Y, Kato Y. 1998. Not only inner cell mass cell nuclei but also trophectoderm nuclei of mouse blastocysts have a developmental totipotency. J Reprod Fertil 113:181–184.

Tsunoda Y, Yasui T, Nakamura K Uchida T, Sugie T. 1986. Effect of cutting the zona pellucida on the pronuclear transplantation in the mouse. J Exp Zool 240:119–125.

Tsunoda Y, Yasui T, Shioda Y, Nakamura K, Uchida T, Sugie T. 1987. Full-term development of mouse blastomere nuclei transplanted into enucleated two-cell embryos. J Exp Zool 242:147–151.

Tsunoda Y, Shioda Y, Onodera M, Nakamura K, Uchida T. 1988. Differential sensitivity of mouse pronuclei and zygote cytoplasm to Hoechst staining and ultraviolet irradiation. J Repod Fertil 82:173–178.

Wakayama T, Perry ACF, Zuccotti M, Johnson, Yanagimachi R. 1998. Full-term development of mice from enucleated oocytes injected with cumulus cell nuclei. Nature 394:369–374.

Wang MK, Kato Y, Tsunoda Y. 1997. Effects of several factors on the monozygotic twin production in the mouse. J Reprod Dev 43:91–95.

Wang MK, Zhang T, Wang XY, Lian L, Li JS, Chen DY. 2000a. Several modified culture media can support the embryos overcoming the 2-cell block and developing to blastocyst stage in Kunming mouse. Acta Zool Sin 46:81–87.

Wang MK, Liu JL, Zhang T, Li GP, Lian L, Jiang YP, Chen DY. 2000b. M16 added with taurine and EDTA can support the IVF and development to blastocyst of Kunming strain mouse. Hereditas (Beijing) 22:301–302.

Wang MK, Chen DY, Liu JL, Li GP, Sun QY. 2000c. In vitro fertilization of mouse oocytes reconstructed by transfer of metaphase 2 chromosomes results in live births. Zygote 8 (in press).