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Microfilament disruption is required for enucleation and nuclear transfer in germinal vesicle but not metaphase II human oocytes

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Objective: To evaluate the usefulness of microfilament disruption before enucleation and nuclear transfer in human oocytes at different stages of maturation.

Design: Prospective experimental study.

Setting: Private clinics.

Patient(s): Infertile couples undergoing assisted reproduction attempts.

Intervention(s): Oocyte enucleation and nuclear transfer, activation of reconstructed oocytes.

Main Outcome Measure(s): Oocyte survival, nuclear transfer efficacy, activation outcomes.

Result(s): Survival rate and nuclear transfer efficacy of germinal vesicle oocytes exposed to the microfilament disrupting agent cytochalasin B before enucleation were 88% and 80%, respectively. These figures dropped, respectively, to 8% and 2% when cytochalasin treatment was omitted. By contrast, cytochalasin-treated and -untreated metaphase II oocytes showed similar survival rate (87% vs. 90%) and nuclear transfer efficacy (78% vs. 87%). This also applied to metaphase II oocytes matured in vitro from the germinal vesicle stage. Cytochalasin treatment did not affect activation rate of reconstructed oocytes, but it increased the occurrence of oocytes with multiple female pronuclei.

Conclusion(s): Microfilament disruption before enucleation is required for germinal vesicle oocytes but not for metaphase II oocytes. (Fertil Steril® 2003;79(Suppl 1):677–81. ©2003 by American Society for Reproductive Medicine.)

Key Words: Nuclear transfer, enucleation, microfilament disruption, cytochalasin B, human oocyte

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Over the past 5 years nuclear transfer techniques have been introduced to human reproductive medicine to help restore developmental potential of oocytes bearing cytoplasmic abnormalities compromising fertilization and early postfertilization development (1, 2). More recently, other nuclear transfer studies aimed at the development of techniques that would make it possible to replace the female (3, 4) and the male (5) gamete with an artificially haploidized somatic cell in the fertilization process (6). Methods used for nuclear transfer in human oocytes were derived from animal cloning experiments and involved previous mechanical enucleation of oocytes serving as nuclear donors and receptors, followed by the introduction of a foreign oocyte (1–3) or somatic cell (3, 4) nucleus into an enucleated oocyte (ooplast) by means of electrically (1, 3), chemically (2) or mechanically (2) driven fusion or by a direct nucleus injection into the ooplast (4). Similar to animal cloning, microfilament disrupting agents were used in these human studies in the enucleation phase of these procedures to facilitate the manipulation.

In the case of cloning, however, the main rationale for the use of microfilament or microtubule disrupting agents is the prevention of cytokinesis, which might lead to the extrusion of chromosomes to a pseudo-second polar body after the injection of a diploid nucleus to

a metaphase II ooplast. Yet, contrary to the case in conventional cloning, the extrusion of somatic cell chromosomes to a pseudo-second polar body is not a condition to be avoided in somatic cell haploidization but represents an important part of the mechanism by which a somatic cell nucleus may be remodeled to behave as a gamete nucleus in the fertilization process. That is why the term *reproductive semicloning* has been suggested to characterize the newly emerging techniques of assisted reproduction with haploidized somatic cells (6).

In addition to the possible interference with pseudo-second polar body extrusion, some authors have pointed out the cytotoxicity of microtubule and microfilament disrupting agents and their harmful effects on early embryo development (7–9). This study was undertaken to determine whether microfilament disruption with cytochalasin B, the microfilament disrupting agent most frequently used with human oocytes, is useful for enucleation and nuclear transfer at two different stages of human oocyte maturation. The impact of this treatment on subsequent activation of the reconstructed oocytes is also addressed.

MATERIALS AND METHODS

Source of Oocytes

Oocytes to be used in this study were supernumerary oocytes from assisted reproduction treatment attempts that were donated for research purposes by consenting patients. Institutional review board approval was obtained for this study. The oocytes allocated to this study were either immature oocytes, at the germinal vesicle stage, recovered from patients undergoing an assisted reproduction treatment involving intracytoplasmic sperm injection (ICSI), or mature oocytes, at metaphase of the second meiotic division (metaphase II), obtained from patients treated by ICSI with testicular sperm extraction in whom the number of spermatozoa that were available for ICSI was insufficient to perform ICSI in all metaphase II oocytes obtained. Some germinal vesicle oocytes were included immediately in this study, whereas other germinal vesicle oocytes were matured in vitro to metaphase II stage before inclusion.

Ovarian Stimulation and Oocyte Recovery

All patients included in this study were stimulated with the use of recombinant FSH (Puregon; Organon, Oss, The Netherlands or Gonal F; Serono, Rome, Italy) after pituitary down-regulation with a gonadotropin-releasing hormone agonist (buserelin acetate, Suprefact; Hoechst, Marion Roussel, Milan, Italy; or triptorelin, Decapeptyl; Ipsen Pharma, Barcelona, Spain) administered in the luteal phase of the previous cycle. The development of ovarian follicles was monitored by vaginal ultrasound examination and by evaluating serum estradiol concentration. When at least three follicles had reached the diameter of 18 mm, ovulation was induced by 10,000 IU of human chorionic gonadotropin (Profasi; Serono). Follicle puncture and oocyte aspiration was performed by vaginal route under ultrasound guidance. Details of these procedures have been published elsewhere (10).

Oocyte Culture and In Vitro Maturation

Within 3 hours after recovery, oocytes were released from the cumulus oophorus and corona radiata by a brief incubation (20–30 seconds) at 37°C in Gamete 100 medium containing 20 IU/mL hyaluronidase (both purchased from Vitrolife, Goteborg, Sweden), followed by repeated aspiration into a finely drawn glass Pasteur pipette (SAGE Biopharma, Bedminster, NJ). The oocyte maturity status was determined at that time. Metaphase II oocytes that were allocated to this study were incubated in IVF-50 medium (Vitrolife) equilibrated with 5% CO_2 in air for 2–6 hours before manipulation. Germinal vesicle oocytes were incubated in the same medium for 24 hours, and those of them that reached metaphase II within this time period were included in this study.

Treatment With Cytochalasin B

Cytochalasin B was purchased from Sigma (St. Louis, MO) and dissolved in dimethyl sulfoxide (DMSO; cell culture grade, Sigma) to a concentration of 1 mg/mL. The drug was then serially diluted in IVF-50 medium (Vitrolife) previously equilibrated with 5% CO_2 in air to a final concentration of 5 μg/mL. Accordingly, the final solution to which oocytes were exposed also contained 0.5% (vol/vol) of DMSO. Oocytes were incubated with cytochalasin B at 37°C for 10 minutes immediately before enucleation. Control oocytes were incubated, under the same conditions, with 0.5% DMSO in IVF-50 medium alone. Immediately after enucleation, karyoplasts and ooplasts were washed in IVF-50 medium and kept at 37°C under 5% CO_2 in air for 10–30 minutes before further manipulation.

Micromanipulation Techniques

Oocyte enucleation, karyoplast transfer to the perivitelline space of ooplasts, and chemical fusion of the karyoplasts with the ooplasts with the use of polyethylene glycol (Sigma) as fusogenic agent were performed as described elsewhere (2). Each ooplast was fused with a karyoplast originating from another oocyte from the same group. Reconstructed oocytes were kept in culture in equilibrated IVF-50 medium at 37°C for 1–2 hours before an attempt at activation with calcium ionophore.

Activation of Reconstructed Oocytes With Calcium Ionophore

Reconstructed oocytes were treated with ionophore A23187 (Sigma, St. Louis, MO) as described elsewhere (11). The ionophore was dissolved in DMSO (cell culture tested, Sigma) at a concentration of 1 mg/mL. The final solution of 10 μM ionophore A23187 was prepared by diluting the stock solution of ionophore in IVF-50 medium equilibrated with 5% CO_2 in air. The final solution was used immediately

TABLE 1

Effect of microfilament disruption with cytochalasin B on survival and nuclear transfer (NT) efficacy of human oocytes at different stages of maturation.

Stage	Cytochalasin treatment	No. (%) of oocytes[a] Total	Surviving enucleation	With successful NT
GV	Yes	50 (100)	44 (88)	40 (80)
GV	No	50 (100)	4 (8)	1 (2)
MII	Yes	63 (100)	55 (87)	49 (78)
MII	No	62 (100)	56 (90)	54 (87)
GV→MII	Yes	46 (100)	40 (87)	32 (70)
GV→MII	No	46 (100)	39 (85)	31 (67)

Note: GV = oocytes recovered at germinal vesicle stage; MII = oocytes recovered at metaphase II stage; GV→MII = oocytes recovered at germinal vesicle stage and matured to metaphase II during 24 hours of in vitro incubation.

[a] Percentages are calculated for total oocyte numbers in each line.

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after preparation to avoid ionophore interaction with proteins contained in the medium. The incubation time was 10 minutes, and the temperature was 37°C.

Statistics

Percentages of reconstructed oocytes surviving enucleation, with successful nuclear transfer, and having undergone in vitro maturation and activation in different treatment groups were compared by χ^2 test.

RESULTS

The effect of oocyte exposure to the microfilament disrupting agent cytochalasin B on the efficacy of nuclear transfer was strongly conditioned by the stage of oocyte maturation at which nuclear transfer was performed (Table 1). The exposure to cytochalasin B was clearly beneficial when nuclear transfer was performed at the germinal vesicle stage, because an overwhelming majority of germinal vesicle oocytes undergoing enucleation in the absence of cytochalasin B did not survive the manipulation, and only 1 of 50 oocytes forming this group incorporated the foreign nucleus (Table 1).

In contrast, when oocytes were subjected to the same micromanipulation procedures at the metaphase II stage, no difference in the percentages of oocytes surviving enucleation and of those incorporating the foreign nucleus after nuclear transfer was observed between the cytochalasin-treated and -untreated group (Table 1), despite relatively low deformability of the oolemma observed during karyoplast preparation (Fig. 1). Similarly, when oocytes recovered at the germinal vesicle stage and matured in vitro to metaphase II were manipulated, the exposure to cytochalasin B did not produce any effect on oocyte survival and nuclear transfer efficacy (Table 1).

In vitro maturation after previous nuclear transfer at the germinal vesicle stage was successful in 73% of the cytochalasin-treated oocytes as well as in the only oocyte that survived enucleation and nuclear transfer without exposure to cytochalasin (Table 2). In both in vivo and in vitro matured metaphase II oocytes, the exposure to cytochalasin B during the enucleation procedure did not influence the proportion of oocytes that became activated (as assessed by the development of one or several pronuclei) in response to calcium ionophore, but it increased the proportion of activated oocytes with multiple pronuclei and decreased the proportion of oocytes showing a single female pronucleus (Table 2).

TABLE 2

Effect of microfilament disruption with cytochalasin B at the time of nuclear transfer (NT) on in vitro maturation (IVM) and activation of human oocytes after NT at different stages of maturation.

Stage	Cytochalasin treatment	Total NT oocytes	No. (%) of NT oocytes showing IVM/activation[a] IVM	No activation	1 PN	>1 PN
GV	Yes	40 (100)	29 (73)	6 (15)	19 (48)	4 (10)
GV	No	1 (100)	1 (100)	0 (0)	1 (100)	0 (0)
MII	Yes	49 (100)	—	5 (10)	23 (47)[b]	21 (43)[b]
MII	No	54 (100)	—	7 (13)	42 (78)[b]	5 (9)[b]
GV→MII	Yes	32 (100)	—	5 (16)	8 (25)[b]	19 (59)[b]
GV→MII	No	31 (100)	—	6 (19)	21 (68)[b]	4 (13)[b]

Note: GV = oocytes recovered at germinal vesicle stage; MII = oocytes recovered at metaphase II stage; GV→MII = oocytes recovered at germinal vesicle stage and matured to metaphase II during 24 hours of in vitro incubation; PN = pronuclei.

[a] Percentages are calculated for total NT oocyte numbers in each line.

[b] Values with the same superscript within a column are significantly different from each other ($P<.05$).

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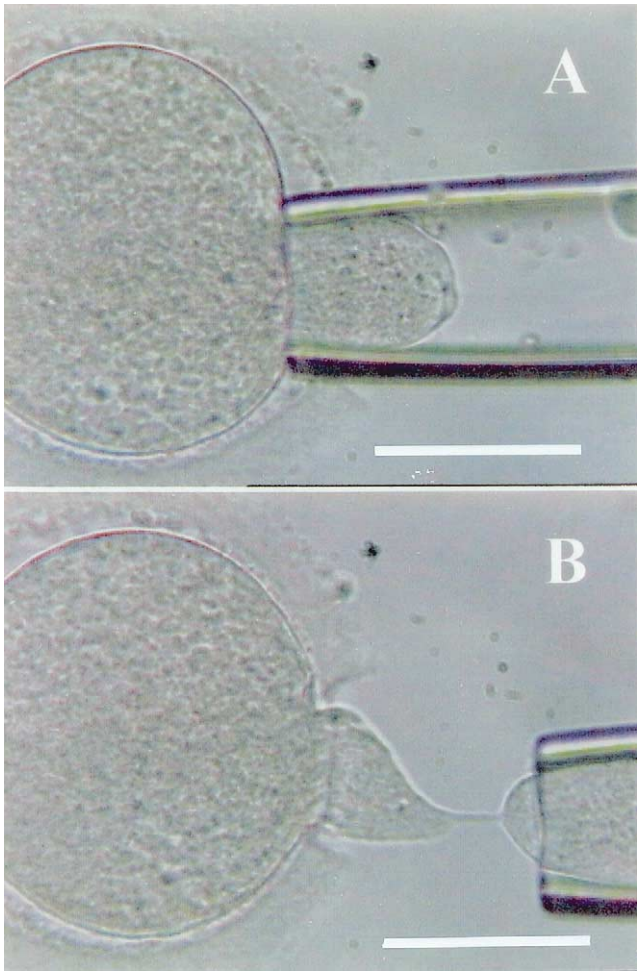
FIGURE 1

Sequential images taken during enucleation of an in vivo matured metaphase II oocyte. (**A**) Initial phase of karyoplast formation. Part of peripheral cytoplasm adjacent to polar body (previously removed) and containing oocyte chromosomes (not seen) enters the enucleation needle, which has been introduced through a laser-drilled hole in the zona pellucida. Bar = 50 μm. (**B**) Karyoplast in the enucleation needle just before definitive mechanical separation from the rest of the oocyte. Note relatively low degree of oolemma deformation in this final phase of enucleation. Bar = 50 μm.



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DISCUSSION

The results of this study justify the recourse to microfilament disruption when enucleation and nuclear transfer are performed at the germinal vesicle stage. Cytochalasins B and D and latrunculin are most commonly used to this end in animal nuclear transfer experiments (9), and cytochalasin B has been used in all previously published studies on nuclear transfer in human oocytes (1–4). On the other hand, this is the first study showing that the inclusion of microfilament disruption does not bring any advantage, in terms of oocyte survival and foreign nucleus incorporation, when enucleation and nuclear transfer are performed with human oocytes at the metaphase II stage.

It is not clear why germinal vesicle oocytes are more sensitive to manipulation procedures than metaphase II oocytes. However, this difference is likely to be related to maturation-dependent changes in the distribution of microfilaments in different oocyte regions because it disappears when oocytes are treated with a microfilament disrupting agent before the manipulation. Spatial rearrangements of microfilaments have been observed during oocyte maturation in several mammalian species (14, 15), including the human (16). These reports show that microfilaments are accumulated in a relatively large cortical and subcortical region of germinal vesicle oocytes, but they become increasingly associated with chromosomes during subsequent stages of oocyte maturation (14–16). Accordingly, differences in microfilament density and organization in the cortical and perinuclear area that are exposed to direct mechanical stress during karyoplast preparation may explain the difference in the requirement for microfilament disruption to facilitate this manipulation in germinal vesicle and metaphase II oocytes.

Interestingly, metaphase II oocytes that were exposed to cytochalasin B in the enucleation phase showed a higher frequency of the occurrence of multiple pronuclei after subsequent activation with calcium ionophore as compared with the case of oocytes enucleated without cytochalasin B, although the overall oocyte activation rate was not different in the cytochalasin-treated and -untreated group. The formation of surplus female pronuclei may result from the failure of some chromosomes to be extruded to the second polar body at anaphase II, which may be a consequence of a persisting impairment of actin microfilament function after cytochalasin treatment. Interestingly, cytochalasin B has been reported to be detrimental to embryonic development in cloning of mice (9) but to be beneficial to embryonic development in cloning of rabbits (12) and sheep (13). Interspecies differences may be at the origin of these conflicting observations.

As to human metaphase II oocytes, cytochalasin B concentration used in the present study was equal to that used in a previous study dealing with nuclear transfer from somatic cells (4) and lower than those used in studies on nuclear transfer from other metaphase II oocytes (1, 2). Further study is needed to determine whether the efficacy of these techniques can be improved by omitting oocyte exposure to microfilament disrupting agents at the enucleation step.

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