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Developmental capacity and implantation potential of the embryos with multinucleated blastomeres

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Abstract. The presence of multinucleated blastomeres (MNBs) in embryos is associated with poor developmental competence in assisted reproductive technologies. This phenomenon is observed not only in humans but also in other animal species. The purpose of the present study was to investigate the characteristics of embryos with MNBs (MNB embryos) that could be utilized in embryo transfer. The developmental rate of MNB embryos to the blastocyst stage (50.8%) was significantly lower than that of normal embryos (73.3%) (P < 0.05). The clinical pregnancy rates of fresh embryo transfer (ET) using day 2 or day 3 embryos were significantly lower in MNB embryos (5.1%) compared with normal embryos (24.0%) (P < 0.05). In the case of frozen-thawed ET using a single vitrified/warmed blastocyst, however, the clinical pregnancy rate of MNB embryos was close to that of normal embryos (59.1% *vs*. 52.8%). Thus, the findings of the present study suggest that the frozen-thawed ET of MNB embryos might improve the potential for implantation followed by successful pregnancy.
Key words: Blastocyst transfer, Human embryos, Implantation, Multinucleated blastomeres

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The presence of multinucleated blastomeres (MNBs) in embryos is associated with poor embryo development and IVF outcome in human assisted reproductive technologies (ART). Several researchers have already developed evidence-based scoring systems showing blastomere multinucleation as one of the most important parameters contributing to selection and successful transplantation of embryos [1–3]. A retrospective analysis of cleavage-stage embryos showed that multinucleated blastomeres were associated with impaired cleavage, increased fragmentation and low implantation rates [4, 5]. Furthermore, the rate of development up to the blastocyst stage declined in embryos with MNBs on day 2 and/or day 3 compared with embryos without MNBs [6]. Like embryo development, the rate of pregnancy after embryo transfer was inferior in the case of embryos with MNBs when compared with mononucleated embryos [7, 8].

Although most MNB embryos are reported in human ART, MNBs have also been reported in the embryos of other animal species, including mouse, rat and porcine embryos. Heindryckx *et al.* [9] reported that arrested 2- and 3-cell nuclear transfer embryos showed a higher rate of MNBs. In the case of *in vitro* produced porcine embryos, it was reported that 50–70% of 2- to 4-cell stage embryos

possessed morphological abnormalities, including MNBs (two nuclei in one or more than one blastomere) [10]. Grenier *et al.* [11] reported the paternal effects on formation of MNBs in rat embryos using cyclophosphamide, an anticancer alkylating agent. They also revealed that fertilization with cyclophosphamide-exposed sperm led to a dramatic elevation in MNBs in cleavage stage embryos. These reports suggested that the formation of embryos with MNBs (MNB embryos) is observed in many mammalian species universally and is not a phenomenon specific to human ART.

It is evident that the MNB embryos have inferior developmental ability and fertility. The development of oocyte donation systems tended to discourage the utilization of MNB embryos in ART, although oocyte donation systems do not seem to be as feasible as expected. An important point that should be noted is that exclusion of MNB embryos not only limits the number of available embryos but also suggests the possibility of a decreased number of successful pregnancies. Furthermore, in some countries where oocyte donation is substantially restricted, such as in East Asia, including Japan, use of MNB embryos is a real possibility. Thus, it is still important to understand the character of MNB embryos in detail so that they can be utilized for embryo transfer (ET) which can lead to improvement of the rate of successful embryo transfer. An alternative approach to utilization of MNB embryos deserves special attention so that the possibility of utilizing them in not only ART systems but also for efficient animal production using IVF, microinjection and nuclear transfer can be justified.

In the present study, we analyzed the clinical data for human embryos manipulated with ART, which reportedly contained a

Table 1. Descriptive data in patients which contain MNB embryos in a cycle

Parameter	Patient groups		
	Normal embryos	MNB embryos	Total
Cycles	8006	1245	9251
Patients	4304	1047	5351
Mean maternal age	36.9 ± 4.7	36.0 ± 4.1	36.8 ± 4.4
Mean no. of MII	5.3 ± 4.2	7.1 ± 4.8	5.7 ± 4.4
Normal fertilized embryos	23263	7600	30863
Multinucleated embryos	0 (0.0%)	1780 (23.4%)	1780 (5.8%)

relatively large number of MNB embryos. From the standpoint of exploring the possibility of utilizing MNB embryos, we investigated the developmental competence and implantation potential after transfer of embryos that showed multinucleation at 2 days after fertilization. We believe that the results will highlight the possibility of utilizing MNB embryos not only for human ART but also for the improved animal production in the future.

Descriptive data of patients are summarized in Table 1. Out of a total 9251 cycles, 1245 cycles had at least one embryo on day 2 in their cohort with multinucleation (13.5%). The rate of patients with MNB embryos in a cycle on day 2 and 3 after embryo transfer was 19.6%. The mean maternal ages were similar in groups of patients, that is, those with normal embryos (36.9 ± 4.7 years; normal group) and those with MNB embryos (36.0 ± 4.1 years; MNB group). Furthermore, there were no significant differences in mean number of retrieved MII oocytes per cycle between the normal and MNB groups. The rate of MNB embryos was 5.8% when considering the total number of normal fertilized embryos, and 23.4% of the embryos contained MNBs, which were retrieved and normally fertilized in the MNB group. Microscopic observation showed that normal embryos had only one nucleus in each blastomere (Fig. 1A). On the other hand, two categories of MNBs were observed, a binucleated type with two nuclei of equal sizes (Fig.1B) and a micronucleated type with more than two nuclei usually of different sizes (Fig. 1C).

A total of 30863 normal fertilized embryos were utilized in this study. From the total number of fertilized embryos, 5344 fresh embryos were transferred during 3506 cycles of ET on day 2 or day 3, 10763 embryos were used for culture to the blastocyst stage and the remaining embryos were frozen and stored for transfer in the future or rejected because they were found to be of poor grade in embryo evaluation. Out of the 10763 embryos used for the culture experiment, 2804 embryos were derived from MNB cycles; 611 of these embryos (21.8%) contained MNBs, and the other 2193 of these embryos, which did not contain MNBs, were considered sibling embryos. The remaining 7959 embryos were derived from normal cycles without MNBs and were used as a control. The rate of development to the blastocyst stage of the sibling embryos derived from MNB cycles (did not contain MNBs) was similar to that of the normal embryos, which were derived from normal cycles and did not contain MNBs (72.2% and 73.3%, respectively). On the other hand, the rate of development MNB embryos to the blastocyst stage (51.4%) was significantly lower than that of the normal and sibling embryos (P < 0.05), even when including the increased rate

of development at day 6 of culture in the case of the long culture period (Fig. 2). All of the evaluated blastocysts were frozen, and a total of 1831 embryos were used for frozen-thawed ET.

The results concerning clinical pregnancy for different systems of transfer are summarized in Fig. 3. The numbers of transfer cycles for fresh ET were 3085, 264 and 157, respectively, for normal, sibling and MNB embryos (Fig. 3A). The clinical pregnancy rate for normal embryos was 24.0% in fresh ET. The rate for sibling embryo transfer (25.8%) was not significantly different from that for normal embryo transfer. On the other hand, the pregnancy rates for MNB embryos (5.1%) in fresh ET was significantly lower than those of the other studied groups (P < 0.05). There were no significant differences in the abortion rates in the fresh ET groups, with the rates being 22.3%, 14.7% and 37.5%, respectively, for normal, sibling and MNB embryos. In frozen-thawed ET, the clinical pregnancy rates were higher than those for fresh ET in each category of embryos. The numbers of transfer cycles of frozen-thawed ET of normal, sibling and MNB embryos were 1363, 446 and 22, respectively (Fig. 3B). Since the developmental rate of MNB embryos to the blastocyst stage was significantly lower, as shown in Fig. 2, the number of MNB embryos that could be used for transfer to patients was very small. The pregnancy rates per embryo transfer were 52.8%, 52.5% and 59.1%, respectively, for normal, sibling and MNB embryos, and there were no statistical differences. The abortion rate for MNB embryos in frozen-thawed ET was 23.1%, and there were no significant differences compared with the other studied groups (25.7% in normal embryos and 24.8% in sibling embryos).

In the studied center, MNB embryos are usually not used for transfer as an institutional rule unless no mononucleated embryos or not enough mononucleated embryos are available. However, 22 frozen-thawed blastocysts developed from embryos with at least one MNB were transferred to patients in the present study. Out of these 22 embryos, 13 embryos resulted in pregnancy, and 9 of those 13 embryos resulted in birth of healthy babies (two boys and seven girls).

The MNB embryos observed at day 2 of ET were mainly classified into two categories: a binucleated type with two nuclei of equal size and a micronucleated type with more than two nuclei of different size. These categories of MNB embryos with variable nucleation are consistent with previous observations. Since we had no records regarding classification of MNB embryos for any patients, we could not distinguish the types of MNB in each embryo. So, it was challenging to examine the relationship between the individual types of MNB embryos and developmental competence or pregnancy. However,
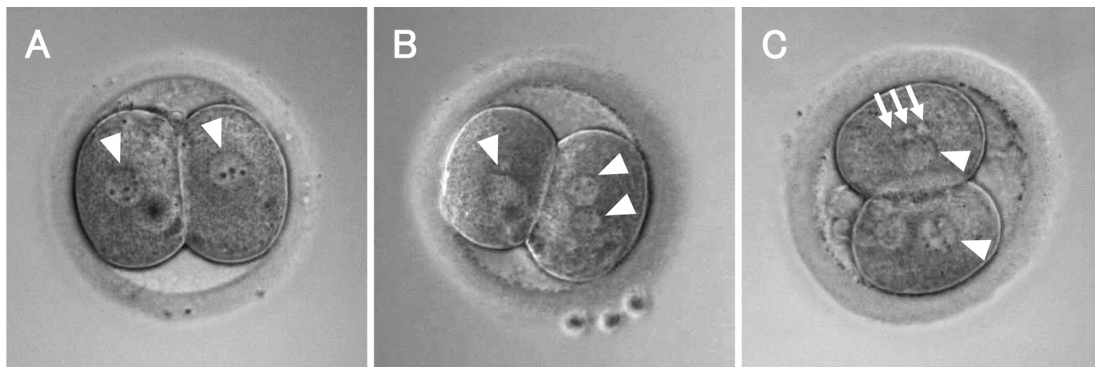
Fig. 1. Representative photographs from inverted microscopic examination (with Hoffman modulation) of human embryos that developed from normal zygotes. A: Normal 2-cell embryo with only one nucleus in each blastomere. B: 2-cell embryo with an MNB (binucleated type). C: 2-cell embryo with an MNB (micronucleated type). Arrowheads and arrows indicate the nucleus and micronucleus, respectively.
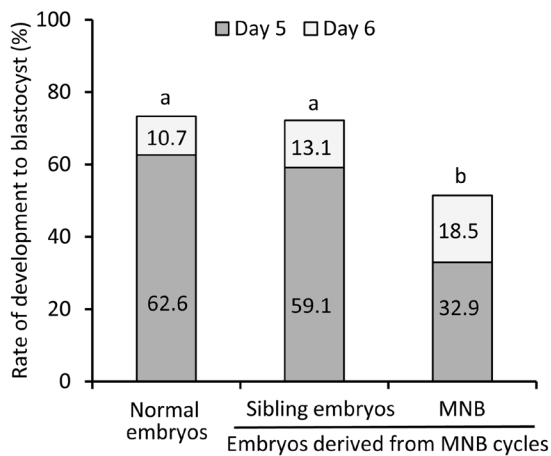

Fig. 2. Developmental rates of MNB embryos to the blastocyst stage. Embryos were obtained from a cycle containing MNB embryos and divided into those with or without MNB. The embryos derived from a cycle without MNBs were cultured as the control (normal embryos). The numbers in the bars in each column show the developmental rates (%). The total numbers of embryos cultured in each group were 7959 (normal), 2193 (sibling) and 611 (MNB), respectively. Different superscripts indicate significant differences between groups (P < 0.05).

Fig. 3. Rate of clinical pregnancy per embryo transfer after fresh ET using day 2 or day 3 embryos (A) and frozen-thawed ET using a single vitrified/warmed blastocyst (B). Normal embryos, sibling embryos and MNB embryos were transferred to investigate the rate of pregnancy per ET (%). The numbers of transfer cycles of the fresh ET were 3085 (normal), 264 (sibling) and 157 (MNB), and the numbers of frozen-thawed ET were 1363 (normal), 446 (sibling) and 22 (MNB), respectively. Different superscripts in the same panel indicate significant differences between groups (P < 0.05).

Meriano *et al*. [7] reported that the blastocyst development rates of micronucleated embryos were low but that the developmental rates of binucleated embryos were similar to those of mononucleated embryos. Since the multinuclear properties and developmental competence of each type of MNB embryo were different, multiple factors might exist and trigger the unusual events of MNB formation. However, the triggering factors and the mechanisms underlying the formation of MNB embryos are still not clear. Recent studies suggested that several factors related to chromosome movement are indispensable for normal nucleus formation. Ohsugi *et al*. [12] reported that loss of chromokinesin Kid/kinesin-10-mediated anaphase chromosome
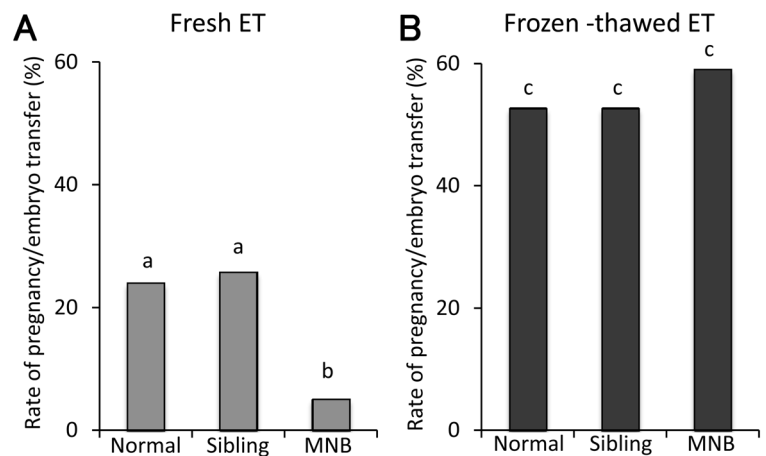
compaction often results in the formation of multinucleated cells. In a study using NTH 3T3 cells, Yasuda *et al*. [13] reported that chromosome misalignment and generation of multinucleated cells were induced by expression of a dominant-active mutant of Cdc42, a small GTPase of the Rho family. These results suggested the possibility of these factors participating in the mechanism of MNB formation. Thus, in order to regulate the incidence of MNB embryos, it is necessary to define the factors and uncover the mechanism of MNB formation.

The observed frequency of multinucleation of embryos in this study was almost the same as the frequencies observed by Van Royen *et al*. [4] and Mariano *et al*. [7], who reported incidences of multinucleation of 27.4% and 23.8%, respectively. However, in

our study, only 19.6% (1047/5351) of patients had multinucleated embryos, which is much lower than the rate reported in the earlier study (44% patients had MNB embryos) of Balakier and Cadesky [14]. In another study, Van Royen *et al.* [4] reported 87.0% patients had multinucleated embryos, which was remarkably higher than the frequency in the current study. Thus it is evident that there are remarkable disparities in the frequency or incidence of MNB embryos and that they might be due to the differences in the scheme of embryo assessment. In our study, embryos were observed at day 2 of ET, which resulted in a lower frequency of MNB embryos, but a higher frequency was noticed when embryos were observed and categorized at days 2 and 3 [4]. Additionally, the incidences of MNB embryos tend to be higher in patients from which a higher number of oocytes can be retrieved which is potentially influenced by FSH stimulation [4, 15]. It is evident that the FSH stimulation scheme differs from clinic to clinic, and consequently disparities occur in terms of MNB embryo frequency.

In the present study, the rate of development to the blastocyst stage was significantly lower for MNB embryos than for normal and sibling embryos without MNBs, which is in agreement with findings of earlier studies. Balakier and Cadesky [14] reported that 57% of MNB embryos cultured for 96–120 h arrested at the 2-cell to 15-cell stage. In another study, Jackson *et al.* [15] reported that MNB embryos showed a significant reduction in average cell number and significant increase in fragmentation. Further, the rate of blastocyst formation decreased in embryos with MNBs on day 2 and/or day 3 compared with embryos without MNBs [6, 16]. As one of the possible mechanisms of multinucleation, it has been suggested that MNBs can arise from karyokinesis in the absence of cytokinesis and associated with abnormal spindle formation or chromosome segregation can involve defects of microtubule function [17–19]. Although spindle formation and chromosome segregation were not examined in the present study, it was anticipated that these factors might decrease the developmental competence of MNB embryos.

Because of their lower developmental competence and implantation rate, there are no reported available concerning ET of blastocyst stage embryos developed from MNB embryos. To the best of our knowledge, this is the first study to analyze pregnancy and abortion in MNB embryos when transferring a single vitrified/warmed blastocyst per transfer cycle. Surprisingly, the pregnancy rate and abortion rate did not differ from those of normal and sibling embryos. Thus, it was suggested that good quality blastocysts have similar potential in terms of implantation/pregnancy regardless of the incidence of MNBs during culture. Recent studies suggested that the implantation rate of normal embryos without MNBs transferred at the blastocyst stage significantly higher than that of those transferred at the cleavage stage on day 2 and 3 [20–22]. Due to the existing differences of fresh and frozen-thawed embryos, it was not rational to compare directly each pregnancy rate after ET in the present study. However, frozen blastocysts are usually used in human ART, and the difference between the transfer systems showed the effectiveness of blastocyst transfer for MNB embryos. Conversely, the present study may indicate that culture of MNB embryos to the blastocyst stage resulted in spontaneous selection of blastocysts that had a pregnancy rate similar to that of normal embryos. Thus, further studies are necessary to develop a suitable culture system to optimize the developmental rate of MNB embryos.

In conclusion, a number of MNB embryos retained full developmental capacity and resulted in birth of healthy babies, and therefore, frozen-thawed ET of blastocysts may be a worthy option for human ART future. Thus, the findings of the present study suggest that MNB embryos might have the potential for development and implantation followed by successful pregnancy and thus might also be usable in animal production. Therefore, we believe that the results of the present study will not only contribute to the advancement of ART in humans but also might contribute to improvement of production using feasible assisted reproductive techniques.

Methods

Analysis was based on a total of 9251 cycles (IVF = 3492 cycles; ICSI = 5759 cycles) of 5351 patients and was carried out for a period of 12 years (between January 2000 and December 2012). Informed consent was obtained from all couples before the study was conducted, and the institutional Review Board of the Kuramoto Women's Clinic approved the experimental protocol.

IVF/ICSI protocols

The ovarian stimulation, oocyte collection, IVF, ICSI and embryo transfer protocols adopted in this study were in accordance with those reported earlier by Kuramoto *et al.* [22]. Briefly, all patients were administered to a GnRH agonist or GnRH antagonist treatment for controlled ovarian hyperstimulation. Oocytes were retrieved under transvaginal ultrasound guidance at 34 to 35 h after hCG injection. The choice between conventional IVF and ICSI as the fertilization approach depended on semen sample characteristics and the histories of the couples involved. Zygotes were cultured in 20 µl droplets of BlastAssist System medium (Origio, Jyllinge, Denmark) or G-Series™ medium (Vitrolife, Göteborg, Sweden) at 37 C in a humidified atmosphere of 6% CO_2, 5% O_2 and 89% N_2. In order to obtain blastocysts, cleavage stage embryos were subsequently placed in 20 µl droplets of BlastAssist System medium or G-Series™ medium and cultured for additional 72 h.

Embryo assessment

Fertilization was verified at 16–19 h after insemination or microinjection. Normal fertilization was confirmed by the presence of two distinct pronuclei. The morphology of cleavage embryos from normally fertilized oocytes was evaluated using an inverted microscope equipped with Hoffman Modulation Contrast optics (Nikon TE2000) at × 400 magnification. In the present analysis, all embryos were evaluated based on the number of blastomeres, fragmentation and symmetry on day 2 (41–44 h) and 3 (66–71 h) of embryo development. The evaluation of embryos was initiated according to the procedure described below. Embryos were graded as good quality when embryos showed 3 to 5 even cells with less than 20% fragmentation on day 2 at least 6 to 9 even cells on day 3 with less than 25% fragmentation. Fair embryo quality was defined as embryos possessing 3 to 5 even or uneven cells on day 2, 5 to 9 even or uneven cells on day 3 with more than 10% but less than 25% fragmentation more than 10 cells with less than 25% fragmentation at the time of observation.

Embryos with two evenly sized nuclei (binucleated type), as well as embryos with more than two nuclei of uneven size (micronucleated type), were categorized as MNB embryos. Mononucleated embryos were defined as embryos having only blastomeres with no nuclear structures or only one nuclear structure. The number of MNB embryos in each cycle was recorded, and the embryos were then cultured in individual drops to observe and record their development.

Blastocysts were classified into four categories on days 5 and 6 according to blastocyst size, morphology of the inner cell mass (ICM), morphology of the trophectoderm (TE) and degree of fragmentation. Furthermore, blastocysts with a Gardner's score ≥ 2, excluding those with a grade of CC for the inner cell mass and trophectoderm, were selected on days 5 and 6 for cryopreservation.

Embryo transfer (ET)

ET was performed with two different timings, and then the rate of clinical pregnancy for each timing of ET (pregnancy rate per embryo transfer) was evaluated. Fresh ET was performed on 5344 embryos from 3506 transfer cycles at day 2 or 3. In frozen-thawed ET, in which vitrified/warmed blastocysts were transferred, single embryo transfer was performed in 1831 transfer cycles. The analysis population consisted of patients who failed to conceive after fresh ET and had a surplus of vitrified blastocyst, as well as patients who underwent elective cryopreservation of all embryos and received ET with vitrified blastocysts.

Blastocysts with a Gardner's score ≥ 2, excluding those with a grade of CC for the inner cell mass and trophectoderm on days 5 and 6, were frozen with a vitrification method. The cryopreservation and thawing procedure was adapted from a protocol described previously [23, 24]. Briefly, the base solution for both vitrification and thawing was Dulbecco's phosphate-buffer saline (DPBS; Irvine Scientific, Santa Ana, CA, USA), supplemented with 50 mg/ml HSA (Buminate 25%; Baxter, Tokyo, Japan) or 2.5 mg/ml rHA (G-MM; Vitrolife). Blastocysts were equilibrated in 10% ethylene glycol (EG) at 37 C for 12 to 15 min. After equilibration, the blastocysts were placed in the solution of 15% EG, 15% dimethyl sulfoxide (DMSO) and 0.5 mol/l sucrose for 1 min. During this exposure, the blastocysts were placed on a Cryotop carrier strip (Kitazato, Fuji, Japan), and the Cryotop was quickly plunged into LN_2. For warming, the cryopreserved Cryotop was quickly placed in 0.5 mol/l sucrose. After 1 min, the blastocysts were washed at 37 C following stepwise dilution: 2.5 min in 0.25 mol/l sucrose, 5 to 10 min in 0.1 mol/l sucrose and 5 to 10 min in the base solution without supplementation. Warmed blastocysts were cultured for at least 3 hours and assessed for survival based on the re-expansion of the blastocoel. Froze-thawed ET of survived single blastocyst was performed with hormone replacement cycles, and mechanically assisted hatching was carried out before ET.

After ET, micronized vaginal progesterone (400 mg/day) was used for luteal support. Clinical pregnancy was defined as a positive urine pregnancy test and the presence of a gestation sac.

Statistical analysis

Results were analyzed based on the statistical relationship between the origin of embryo development, clinical pregnancy rate and abortion rate. These relationships were evaluated by χ^2 tests and Fisher's exact test. Differences in patient age and number of MII oocytes were analyzed by Student's t-test. Differences were considered significant at the level of $P < 0.05$. Statistical analysis was performed using IBM SPSS Advanced Statistics 20 (IBM Corp., Armonk, NY, USA).

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