# Human embryo twinning with applications in reproductive medicine

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**Objective:** To assess the efficacy of human embryo twinning by blastomere biopsy at different early embryonic stages (splitting efficiency) and to determine the in vitro developmental capacity of twinned human embryos (developmental efficiency).
**Design:** Randomized comparative study.
**Setting:** Private IVF centers.
**Patient(s):** Couples undergoing IVF donating triploid embryos.
**Intervention(s):** Embryos at the 2- to 5- and 6- to 8-cell stage were split into twin embryos. Half the number of blastomeres from donor embryos were removed and inserted into recipient empty zonae pellucidae. After embryo splitting, donor and recipient embryos were cultured in vitro.
**Main Outcome Measure(s):** Development of twinned embryos to the blastocyst stage.
**Result(s):** The number of developing embryos obtained after splitting could be increased in comparison with the number of embryos available before splitting at the 6- to 8-cell stage but not at the 2- to 5-cell stage (splitting efficiency). Splitting of 6- to 8-cell embryos yielded superior rates of twin embryos developing to blastocysts (developmental efficiency). Twinning success was related to the superior morphological quality of embryos used for splitting.
**Conclusion(s):** This is the first report on twinned human embryos developing to blastocysts. This study exhibits the potential for novel applications in human assisted reproduction. (Fertil Steril® 2010;93:423–7. ©2010 by American Society for Reproductive Medicine.)

**Key Words:** Assisted reproductive technology (ART), female infertility, human embryology, blastomere biopsy, in vitro culture, embryo splitting, monozygotic twin embryos

Mammalian embryo splitting was first achieved in the mouse system by investigating the developmental potential of blastomeres isolated from early preimplantation embryos at the 4- and 8-cell stage (1). Further studies in the mouse showed that 65% of embryos split at the 2-cell stage and transferred to foster mothers developed to term (2). Moreover, transfer of embryos derived from 2-cell embryo splitting gave rise to healthy offspring very similar in size and morphology to control live-born mice originating from normal embryos. The investigators concluded that experimental embryo twinning did not interfere with normal adult development (3).

In farm animals, embryo splitting has successfully been established for several livestock species. In sheep, 36% of embryos split as 2- and 4- cell embryos developed to term after transfer to recipient females (4). In cattle, embryos split into individual blastomeres at the 4-cell stage could develop to term, giving rise to multiple monozygotic healthy calves (5). Bisected or biopsied early bovine embryos gave pregnancy rates similar to those obtained from intact control embryos. Thousands of twin calves resulting from embryo splitting have been born worldwide, and there have been no reports of abnormalities in the offspring due to the splitting procedure. Embryo twinning was therefore proposed for safe and efficient applications under commercial field conditions (6). Furthermore, cryopreserved split bovine embryos after their time-separated thawing and intrauterine transfer gave rise to healthy monozygotic calves of different ages (7). In the goat, monozygotic twin kids were produced from bisected early embryos (8). In addition, split embryos that were transferred to genetically identical females could develop to term in allogenic pregnancies, being genetically identical twins to these foster females (9). In the pig, split embryos were capable of full-term development giving rise to healthy twin piglets (10). In the horse, from split embryos created via blastomere biopsy at the 2- or 8-cell stage, healthy monozygotic foals were delivered at term pregnancy (11).

In nonhuman primates, however, embryo splitting has given only inferior results without leading to twin babies. The splitting of rhesus monkey embryos at the 8-cell stage resulted in one live-born monkey (12). Embryo twinning in the rhesus monkey has also been attempted by blastomere separation at the 2- and 4-cell stage and has led to two twin pregnancies but with no birth of monozygotic twins (13). Further investigations are needed to reveal the reasons for these limited results. Genetically identical rhesus monkeys would be very useful for the study of human-related twinning and tissue transplantation and may serve as a model system to

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investigate the epigenetic effects caused by the maternal environment during pregnancy (14).

Human embryo splitting carried out on genetically abnormal embryos has so far been presented only in a preliminary report (15). These embryos were obtained from IVF cycles and were donated for research. They were split at early cleavage stages, coated with artificial zona pellucida (ZP), and cultured in vitro. However, these split embryos were arrested in development after a few cell divisions at the most. In a commentary referring to these preliminary experiments and to embryo splitting in general, the merits of these attempts were acknowledged for future applications in reproductive medicine (16).

With regard to human embryo splitting, the Ethics Committee of the American Society for Reproductive Medicine (ASRM) considered favorably research on embryo splitting and stated in its report "since embryo splitting has the potential to improve the efficacy of IVF treatments for infertility, research to investigate the technique is ethically acceptable" (17). According to these recommendations, we have first established efficient blastomere biopsy for embryo splitting in the mouse as an experimental model system (18, 19).

Our objectives for this current study were to apply the newly developed technology to human embryo splitting to evaluate its efficacy at different early embryonic stages (splitting efficiency) and to determine the best success rates of twin embryo development to the blastocyst stage under in vitro culture conditions (developmental efficiency).

## MATERIALS AND METHODS

### Institutional Review Board Approval

This study was approved by an independent Institutional Review Board (IRB-ID 5.18.08–1/Part I).

### Patients

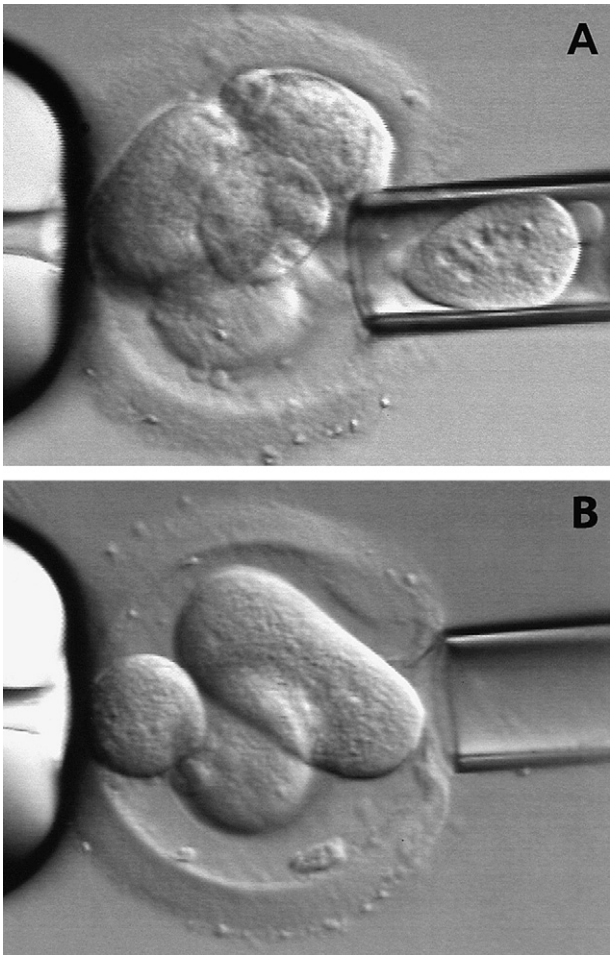
Couples enrolled in IVF programs gave written consent for using genetically abnormal oocytes and embryos in our study.

### Oocytes and Embryos

From IVF and intracytoplasmic sperm injection (ICSI) cycles, oocytes with three pronuclei and triploid embryos at early cleavage stages were donated for this study. These genetically abnormal oocytes and embryos are discarded during assisted reproductive technology (ART) programs. Triploid oocytes were cultured under standard IVF conditions to the 2- to 5- and 6- to 8-cell stage. These embryos as well as triploid embryos that already developed to the 2- to 5- and 6- to 8-cell stage were used for embryo twinning. Before splitting, embryo morphology was evaluated according to standard IVF criteria (grades 1 and 2 versus grades 3 and 4). A number of triploid embryos were randomly assigned as nonsplit controls but did not originate from embryo siblings used for splitting.

**FIGURE 1**

Microsurgical splitting of triploid human embryo at the 6-cell stage to create twin embryos. (**A**) Biopsy pipette is inserted through an artificial opening in the ZP and carefully positioned for blastomere removal. (**B**) Three biopsied blastomeres are transferred to a previously emptied ZP. Transferred blastomeres are gently pushed together to facilitate cellular contact. Twin embryos are cultured during preimplantation development to the blastocyst stage.

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### Microsurgical Technology

From human triploid embryos at the 2- to 5-cell and 6- to 8-cell stage, blastomeres were biopsied following our previously published procedures (18, 19). However, additional adaptations and technical refinements had first to be established for further improving blastomere biopsies on human embryos. Acidified Tyrode's solution (SAGE, Trumbull, CT) was locally applied to the ZP of embryos assigned for splitting to prepare an opening for pipette penetration necessary for blastomere removal. In addition, arrested embryos were evacuated with the same technique to obtain empty ZPs to

be used as blastomere recipients. To facilitate the microsurgical biopsy of blastomeres, embryos were briefly preincubated in microdrops of $Ca^{2+}$, $Mg^{2+}$ free HTF-HEPES medium (SAGE), supplemented with 5% Plasmanate (TALECRIS, Biotherapeutics, NC) and covered with equilibrated mineral oil (SAGE) at 6% $CO_2$ and 37°C. Blastomere removal was carried out with blunt 40-$\mu$m pipettes (Conception Technologies, San Diego). Half the number of blastomeres from donor embryos were removed and inserted into empty recipient ZPs (Fig. 1). The openings of the ZPs had to be 40–50 $\mu$m in diameter to avoid protrusion of transferred blastomeres, artificial twinning, or premature embryo hatching.

### Culture In Vitro

After embryo splitting, the resulting donor and recipient embryos (those from which blastomeres were removed and those with blastomeres inserted, respectively) were washed and incubated for 30 minutes in microdrops of Quinn's Advantage Medium (SAGE), supplemented with 30% Plasmanate and covered with equilibrated mineral oil at 6% $CO_2$ and 37°C. Such preincubation in high protein concentrations supported cellular recovery of blastomeres from biopsy stress. Subsequently, twin embryos were transferred into microdrops of Quinn's Advantage Medium with 15% Plasmanate under mineral oil and cultured in vitro. Development of split embryos was recorded every 24 hours up to the blastocyst stage, including hatching ability. Molecular and chromosomal analysis of split embryos was not considered essential or appropriate for the outcome of this current study. Nonsplit embryos (controls) were cultured and observed under the same conditions.

### Statistical Analysis

Statistical analysis was performed using the SPSS statistical software (SPSS, Chicago). Specifically, Pearson's $\chi^2$-test was used to compare the splitting and developmental efficiency of embryos split at the 2- to 5- and the 6- to 8-cell stage, the effect of embryo morphology (grade 1–2 versus grade 3–4) before splitting on the splitting and developmental efficiency, and the developmental capacity of split versus nonsplit embryos.

## RESULTS

A total of 70 triploid human embryos were used in this study. Twenty-five of those were randomly assigned as nonsplit embryos (controls). Of the remaining 45 embryos, 21 were split at the 2- to 5- cell stage and 24 were split at the 6- to 8-cell stage. First, the splitting efficiency of embryos at the 2- to 5-cell stage was compared with the splitting efficiency at the 6- to 8-cell stage. Second, the developmental efficiency was evaluated for embryos split at the 2- to 5-cell and 6- to 8-cell stage and compared with nonsplit embryos. Third, the twinning efficiency of grade 1 and 2 embryos was compared with that of grade 3 and 4 embryos.

From 13 embryos (grade 1–2) split at the 2- to 5-cell stage, five embryos, and from 12 embryos (grade 1–2) split at the 6- to 8-cell stage, 16 embryos, developed to morulae and blastocysts (Table 1). Embryos used for splitting at the 6- to 8-cell stage provided a much higher splitting efficiency than embryos split at the 2- to 5-cell stage (133.3% or 16 of 12 embryos split at the 6- to 8-cell stage versus 38.4% or five of 13 embryos split at the 2- to 5-cell stage ($P<.05$, Pearson's $\chi^2$-test). Grade 1–2 embryos split at the 6- to 8-cell stage produced more viable embryos (morulae and blastocysts) than were originally available before splitting (see Table 1).

The developmental efficiency of grade 1–2 embryos split at the 6- to 8-cell stage was increased in comparison with embryos split at the 2- to 5-cell stage (66.6% or 16 of 24 embryos after splitting at the 6- to 8-cell stage versus 19.2% or five of

**TABLE 1**

**Microsurgical embryo twinning according to cell stage and embryo grading.**

| Embryo cell stage and grade | Embryos available for twinning | Embryos created by twinning | Embryos developed | | Splitting efficiency % (n/N) | Developmental efficiency % (n/N) |
|---|---|---|---|---|---|---|
| | | | Morula | Blastocyst | | |
| 2–5, Grade 1–2 | 13 | 26 | 2 | 3 | 38.4 (5/13)[a] | 19.2 (5/26)[b] |
| 2–5, Grade 3–4 | 8 | 14 | 0 | 0 | 0 (0/8) | 0 (0/14) |
| 6–8, Grade 1–2 | 12 | 24 | 4 | 12 | 133.3 (16/12)[a] | 66.6 (16/24)[b] |
| 6–8, Grade 3–4 | 12 | 21 | 2 | 0 | 16.7 (2/12) | 9.5 (2/21) |
| Controls | 25 | | 2 | 5 | | 28.0 (7/25) |

*Note:* Splitting efficiency is defined as the ratio of morulae and blastocysts to embryos available for twinning. Developmental efficiency is defined as the ratio of morulae and blastocysts to embryos created by twinning. Nonsplit embryos were randomly assigned as controls.

[a] $P<.05$, Pearson's $\chi^2$-test.

[b] $P<.001$, Pearson's $\chi^2$-test.

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26 embryos after splitting at the 2- to 5-cell stage; $P<.001$, Pearson's $\chi^2$-test).

Successful embryo twinning was also related to superior morphological quality of embryos used for splitting, irrespective of embryo cell stage before splitting (42.0% or 21 of 50 embryos for grade 1–2 versus 5.7% or two of 35 embryos for grade 3–4; $P<.0005$, Pearson's $\chi^2$-test).

Twin blastocysts of good morphological quality developed and hatched in culture after 3–4 days postsplitting (Fig. 2). A total of 15 blastocysts developed and hatched, from split grade 1–2 embryos (three from embryos split at the 2- to 5-cell stage and 12 from embryos split at the 6- to 8-cell stage; see Table 1).

The microsurgical technique for embryo twinning did not affect the developmental efficiency of split embryos since their survival rate was not significantly different from that of nonsplit controls (27.0% or 23 of 85 split embryos versus 28.0% or seven of 25 nonsplit embryos; $P$ = nonsignificant, Pearson's $\chi^2$-test). In this study, genetically abnormal (triploid) embryos were used for twinning. These embryos are clearly inferior to normal diploid embryos regarding their developmental capacity.

## DISCUSSION

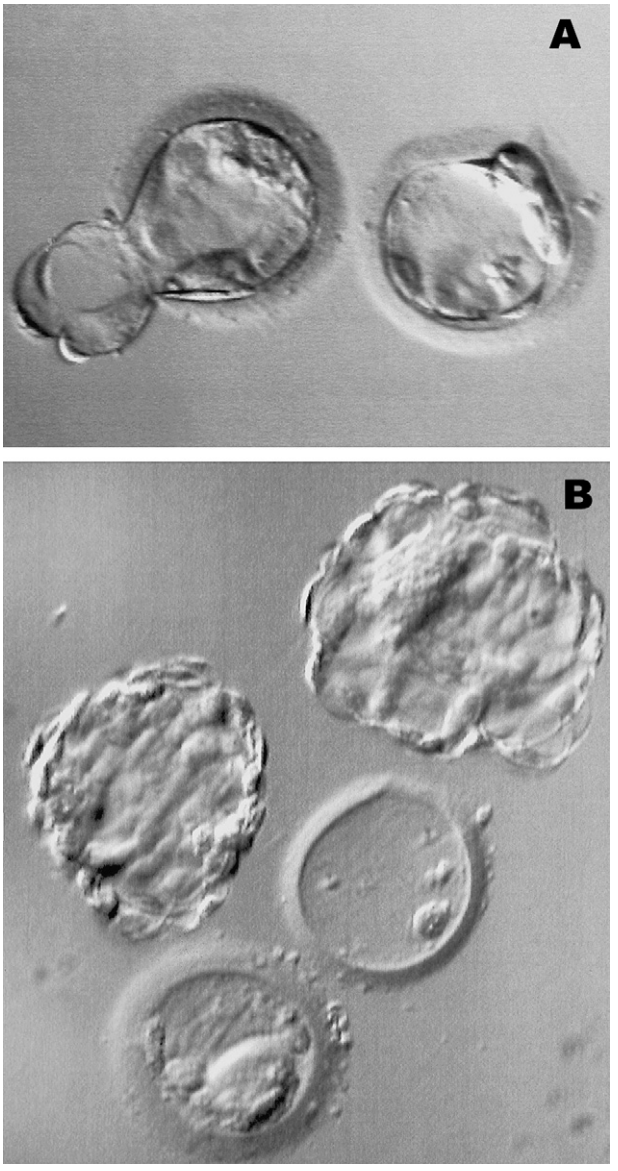
This study documented that it is possible to effectively split human embryos by blastomere biopsy. A significantly increased rate for embryo twinning was observed for the more advanced embryos split at the 6- to 8-cell stage than for those split at the 2- to 5-cell stage. When embryos were split at the 2- to 5-cell stage, it was not apparent which of those would have the potential to progress to the 6- to 8-cell stage or beyond. Of course, a similar argument could also be considered for the 6- to 8-cell embryos concerning their prospective developmental capacities. Further investigation is necessary to explain why embryo splitting at the 6- to 8-cell stage gave superior twinning results.

In general, split embryos hatched in advance of nonsplit embryos, suggesting an "assisted hatching effect." This was facilitated by the ZP opening that was required for blastomere biopsy. For ART, it has been proposed that assisted embryo hatching may provide a beneficial effect for facilitating embryo implantation in patients with advanced age, with repeated implantation failures after several IVF or ICSI cycles, or with cryopreserved-thawed ET cycles (20, 21).

Splitting of 6- to 8-cell embryos resulted in more developing embryos in comparison with the number of embryos available before splitting (Table 1). The numerical increase of embryos obtained after splitting would have obvious clinical advantages for patients enrolled in IVF programs (22). Embryo splitting in ART may be applicable and considered for those patients termed as "low responders" with only a few oocytes retrieved after controlled ovarian stimulation. However, embryo splitting should only be considered if the embryos are of high quality and reach the 6- to 8-cell stage after IVF, as our study indicates. In this case, embryo splitting may increase the likelihood for obtaining a pregnancy since more embryos could be made available for intrauterine transfer. Embryo splitting would not be of practical benefit for patients with poor-quality embryos that do not develop to the 6- to 8-cell stage by day 3 postfertilization. Relevant to this crucial transition during early embryonic development, it was documented at the molecular level that maternally derived mRNA stored during oogenesis is used for protein

**FIGURE 2**

Development of twin blastocysts derived from 6-cell embryo splitting with good morphological quality during in vitro culture. (**A**) Both blastocysts show first signs of hatching 3 days after splitting. (**B**) Twin blastocysts derived from another embryo splitting are hatched entirely from their ZP.

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synthesis from fertilization to the initial blastomere divisions. Thereafter, a new wave of embryonic mRNA is synthesized and is responsible for continuation of embryogenesis (23, 24). From these data on gene-expression profiling of early embryos it seems reasonable to consider the 6-cell stage as most optimal for embryo twinning.

For couples with few embryos of good quality available during one IVF cycle, embryo splitting may yield additional embryos to be cryopreserved for subsequent transfer, potentially increasing the likelihood of a pregnancy and even providing time-separated twins. The Ethics Committee of the ASRM has stated in its report that "splitting one embryo into two or more embryos could serve the needs of infertile couples in several ways. As long as a couple is fully informed of the risk of such an outcome, there would appear to be no major ethical objection" (17). It has previously been postulated that embryo splitting at the blastocyst stage may be of benefit for patients with only one or few normal embryos obtained by IVF techniques (25). Indeed, duplication of embryos by microsurgical splitting to improve the chance of pregnancy should not cause a medical or ethical objection since monozygotic twins can occasionally occur in natural conception as well as in IVF cycles (26). Although there is a concern that embryo splitting may result in unequal cell distribution to the twin embryos, such distribution does not seem to interfere with normal development. There is evidence for the unequal allocation of cells to the twin embryos, leading to some genetic and phenotypic differences among healthy monozygotic twins (27).

In our present study, we have established efficient techniques for human embryo splitting to create developing twin embryos, although they originated from genetically abnormal (triploid) embryos. We are currently conducting a randomized controlled prospective study under Institutional Review Board approval to further corroborate and extend these findings. In the future it should be anticipated that such novel twinning technology will find clinical use in reproductive medicine (28).

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