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Original Article

Embryo splitting can increase the quantity but not the quality of blastocysts

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Abstract

Objective: In this study, we investigated the developmental potential of single blastomeres that were obtained from 4-cell mice embryos that were split during the blastocyst stage.
Materials and Methods: Imprinting Control Region (ICR) mice (age: 6–8 weeks), were superovulated and mated with a single fertile male of the same strain. We obtained 2-cell embryos that were then cultured in 4 groups (×4) with Human tubal fluid (HTF) supplemented with 12% fetal bovine serum. When these embryos reached the 4-cell stage, their zonae pellucidae were removed and every single blastomere was isolated by repeated pipetting with Ca/Mg^{2+}-free medium. The isolated blastomeres (study group) and the intact embryos (control group) were then cultured to determine the blastocyst formation rate and quality.
Results: We collected a total of 936 embryos from 524 morphologically intact, top-grade embryos in the 4-cell stage from 80 stimulated mice. We used 356 of these embryos to isolate the blastomeres. The remaining 168 embryos were cultured as controls. A total of 1312 single blastomeres were obtained and cultured *in vitro*. Among these, 620 blastocysts were harvested from the original embryos compared with 136 blastocysts that were harvested from the control group. The overall blastocyst formation rate was 174.2% (620 blastocysts from 356 embryos) for the study group compared with 81.5% (136 blastocysts from 168 embryos) for the control group. The study group was 43.3% (268 of 620) top-grade blastocysts compared with 91% (152 of 168) of the control group. Taken together, the percentage of top-grade blastocysts obtained per original embryo in the split group was 75.4% (174.2% × 43.3%) compared with 74.2% (81.5% × 91%) for the control group.
Conclusions: Embryo splitting can increase the number of blastocysts. However, the percentage of available top-grade blastocysts is the same compared with nonsplit embryos. Embryo splitting may not be a cost-effective technique for the generation of high-quality mouse blastocysts.

Keywords: blastomeres; embryo biopsy; embryo splitting; in vitro fertilization

Introduction

Because early embryonic cells are totipotent [13,16], the possibility of splitting the blastomeres from cleavage-stage embryos in order to increase the number of embryos available for *in vitro* fertilization-embryo transfer (IVF-ET) treatment has recently been discussed [12,14]. Embryo splitting remains the fastest and most effective method for increasing the number of offspring obtained from an embryo transfer program. Because embryo splitting could lead to two or more embryos with the same genome, the term "cloning" has also been used to describe this practice [11,12]. Embryo splitting, however, does not involve any direct manipulation, transfer, or substitution of the entire genome as occurs during conventional cloning via nuclear substitution [9,10].

Splitting one embryo into two or more embryos could serve the needs of an infertile woman with a poor ovarian reserve

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during IVF treatment in several ways. Because these women usually have no emlbryos or a very limited number that can be transferred back into the uterus, embryo splitting could increase the number of embryos available for transfer. Because the IVF pregnancy rate increases with the number of embryos transferred, it is believed that embryo splitting, when only one or two embryos are produced, may result in a pregnancy that would not have occurred otherwise [6,15]. For couples who produce more than enough embryos for one transfer cycle, embryo splitting may provide a sufficient number of embryos for subsequent transfers without having to go through another retrieval cycle, thus lessening the physical burdens and costs of IVF treatment [8]. In addition, for women at an advanced maternal age or with hereditary family disorders, embryo splitting can increase the chance of precise preimplantation genetic diagnosis (PGD) [14].

The aim of our study was to test the clinical value of embryo splitting. Here, we compare the quality and quantity of blastocysts cultured *in vitro* from split blastomeres and nonsplit embryos.

Materials and methods

Collection of 2-cell mouse embryos

ICR mice were cared for and used according to the Guide for the Care and Use of Laboratory Animals of the Chi-Mei Medical Center. They were housed under a 12-hour light/dark schedule. A total of 80 adult female mice (age: 6–8 weeks) were superovulated using a 10 IU injection of pregnant mare serum gonadotropin (PMSG; Intervet, Boxmeer, Holland), followed by another injection of 10 IU human

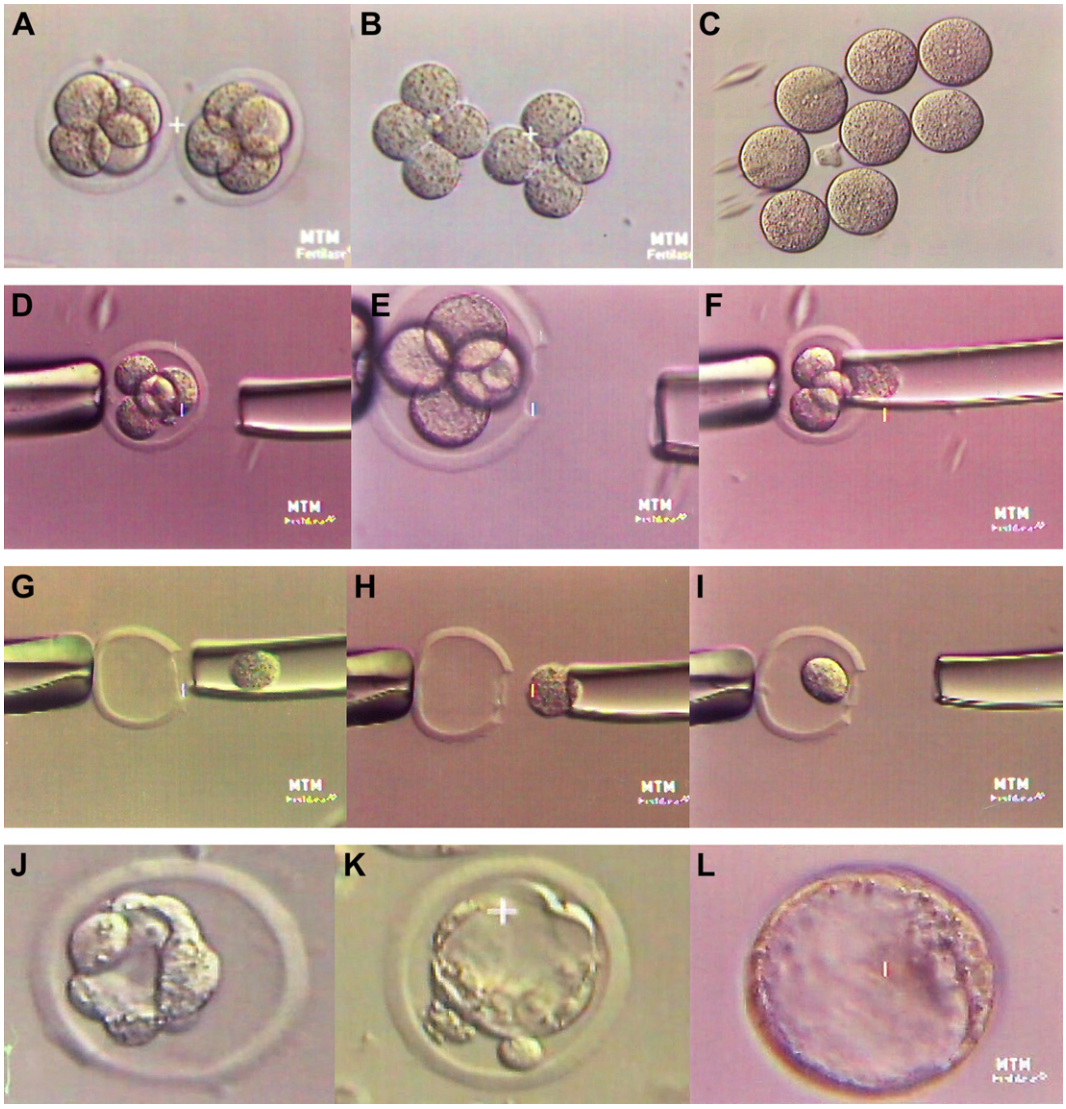

Fig. 1. Embryo splitting and *in vitro* culturing. **(A,B,C)** Blastomere separation. The embryo is split into individual cells. **(D,E,F)** Creation of an empty zona pellucida. The empty mouse zonae pellucidae were created by removing and discarding the blastomeres from the mouse embryos. **(G,H,I)** Microinjection of the blastomere. A single blastomere is shown in the transfer pipette, positioned for placement into a surrogate zona pellucida, and immobilized using a holding pipette. **(J,K,L)** *In vitro* culturing of a split embryo to a blastocyst.

Table 1
Blastocyst formation rate of each group.

	Split group	Nonsplit group	*p*
Blastocyst formation rate	47.2 % (620 of 1312)	81.5% (136 of 168)	0.05
Overall blastocyst formation rate	174.2% (620 of 356)	81.5% (136 of 168)	0.01

Table 3
Embryonic characteristics of the split and control groups.

	Split group	Control group	*p*
Grade 1 blastocyst	43.3% (268 of 620)	91% (152 of 168)	0.04
Grade 2 blastocyst	36.7% (228 of 620)	9% (16 of 168)	0.06
Grade 3 blastocyst	20% (124 of 620)	0%	< 0.001

chorionic gonadotropin (hCG; Merck Sereno, Geneva, Switzerland) hormone 48 hours later to trigger ovulation. Following the hCG injection, one or two females were caged overnight with ICR male mice (8–10 weeks old). The following morning, mating was confirmed by the presence of the vaginal plug. To collect the 2-cell embryos, the pregnant females were sacrificed by cervical dislocation at 48-hours post-hCG injection. By flushing the uterine horn and fallopian tubes, the 2-cell embryos were obtained on the afternoon of Day 2 of gestation. After removing the fallopian tube, the embryos were flushed with human tubal fluid (HTF) medium that had been supplemented with 0.4% bovine serum albumin (BSA; Sigma, Deisenhofen, Germany). Then, the embryos were washed, divided into 4 groups (×4), and incubated in HTF culture medium supplemented with 12% BSA and mineral oil (Sigma, Deisenhofen, Germany) in a humidified atmosphere with 5% CO_2 at 37°C. The development of the cleavage stage in the 4-cell embryos was monitored 24 hours later. Morphologically intact embryos were used in all experiments.

Blastomere isolation

At this stage, the zonae pellucidae were removed using a 1–2 minute treatment of 1% pronase (Protease; Sigma, St Louis, MO, USA). Individual blastomeres were mechanically isolated by repeatedly pipetting (using a flame-polished micropipette with an inner diameter of 55–60 μm) the zona-free, 4-cell embryos with Ca/Mg^{2+}-free HTF medium. The empty zona pellucida was created by removing and discarding the blastomeres from the mouse embryos. Isolated single blastomeres were then individually inserted into empty zonae pellucidae (study group). The split embryos were then removed from the embryo biopsy medium, carefully washed, and placed in 20-μL droplets of culture medium under mineral oil.

In vitro culturing and evaluation of the development of the isolated blastomeres

After embryo splitting, the resulting split embryos were placed in HTF medium supplemented with 12% BSA, covered with equilibrated mineral oil, and incubated in an atmosphere with 5% CO_2 at 37°C. The development of the split embryos was assessed daily through the blastocyst stage, including hatching ability in order to determine the blastocyst formation rate. This process was photographically documented. Confirmation of blastocyst development from the split blastomere was determined by the formation of a blastocyst with a visible inner cell mass (ICM) and a distinct trophoblast (Fig. 1).

The nonsplit, 4-cell mouse embryos were treated in a similar manner as the split embryos and cultured under the same *in vitro* conditions. In this study, about 18% of the embryos were left intact to serve as controls.

Statistical analysis

Differences in developmental rates, blastocyst formation, and characteristic grading between both groups were analyzed using the χ^2 test. Significance was defined as $p < 0.05$.

Results

A total of 936 embryos were collected from 80 stimulated mice, and 524 of these were considered morphologically intact, top-grade embryos at the 4-cell stage. Among these, 356 embryos were used for splitting and 168 embryos in the same stage were used as controls. A total of 1312 isolated blastomeres from the split embryos were obtained and cultured *in vitro*. Six hundred and twenty blastocysts were harvested from the original embryos in the split group compared with 136 blastocysts that were harvested from the control group. The overall blastocyst formation rate was 174.2% (620 blastocysts from 356 embryos) per embryo in the study group compared with 81.5% (136 of 168) per embryo in the control group (Table 1).

In order to compare the quality of each blastocyst, the blastocysts were classified into three groups according to their ICM, trophectoderm, and blastocele, as shown in Table 2. In the spit group, 43.3% (268 of 620) were classified as grade 1 blastocysts, 36.7% (228 of 620) as grade 2 blastocysts, and 20% (124 of 620) as grade 3 blastocysts. In the control group, 91% (152 of 168) were classified as grade 1 blastocysts and 9% (16of 168) as grade 2 blastocysts. There were no grade 3 blastocysts in the control group (Table 3). Taken together, the

Table 2
Blastocyst classification.

Blastocyst	Grade 1	Grade 2	Grade 3
Inner cell mass	Numerous and tightly packed	Several and loosely packed	Few cells
Trophectoderm	Many cells organized in the epithelium	Several cells organized in loose epithelium	Few cells
Blastocele	Blastocele fills the blastocyst	Blastocele fills more than half of the blastocyst	Blastocele fills less than half of the blastocyst

Table 4
Final embryonic characteristics of the split and control groups.

	Split group	Control group	p
Overall blastocyst formation rate	174.2%	81.5%	0.01
Good blastocyst formation rate	75.4% (174.2% × 43.3%)	74.2% (81.5% × 91%)	0.04

percentage of top-grade blastocysts per original embryo in the split group is 75.4% (174.2% × 43.3%) compared with 74.2% (81.5% × 91%) for the control group (Table 4).

Discussion

Based on the final data of this study, it appears that embryo splitting could increase the number of blastocysts (174.2% in the split group vs. 81.5% in the control group; $p = 0.01$). However, when the quality of the blastocysts was compared between the two groups, we found the percentage of top-grade blastocysts was significantly lower in the split group (43.3% in the split group vs. 91% in the control group; $p = 0.04$). In addition, 20% of the total blastocysts in the split group were classified as grade 3 blastocysts compared with 0% in the control group ($p < 0.001$). Taking everything into account, we found that although embryo splitting increases the number of blastocysts, it does not increase the number of clinically useful blastocysts (75.4% in split group vs. 74.2% in control group; $p = 0.04$).

Therefore, although we presume that the individual blastomeres of a single embryo are capable of independent growth [1–3], their developmental potentials are very different [4,5]. According to published reports [6,7], the implantation rate of blastocysts is about 50%, and almost all result from top-quality (grade 1) blastocysts. In other words, while we were able to increase the number of blastocysts through embryo splitting, we were unable to improve the final outcome regarding the total number of implanted embryos.

Several possibilities can be proposed to explain this phenomenon: 1) embryo splitting may induce traumatic changes in the embryo; 2) the condition and composition of the culture medium may be different between an intact embryo and a separated blastomere; and, 3) there is a preexisting difference in the developmental potential between the blastomeres of a single embryo. After several months of practice, we believe we have minimized the degree of trauma that occurs when we split an embryo. The fact that 40% of the embryos in the split group were still top quality makes the possibility of suboptimal culturing unlikely. Therefore, it seems that the most reasonable explanation for the results of this study is that there is a significant difference in the differentiating abilities of individual blastomeres [4,5].

According to our preliminary results, we believe that embryo splitting is not a cost-effective technique for the formation of high-quality mouse blastocysts. Although it can increase the quantity of blastocysts, the quality was suboptimal when compared with nonsplit embryos. Whether embryo splitting is clinically feasible, or if the expected benefits to infertile couples outweigh its risks, is still unknown (8) and cannot be determined without further research.

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