In vitro blastocyst development from serially split mouse embryos and future implications for human assisted reproductive technologies

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Objective: To assess the efficacy of serial splitting of mouse embryos with respect to blastocyst development.
Design: Prospective study.
Setting: Commercial research facility.
Animal(s): Commercially available mouse embryos from B6C3F-1 × B6D2F-1.
Intervention(s): One, two, and three blastomeres were biopsied from two-, four-, and six-cell embryos, respectively, and were inserted into empty zona pellucida recipients (first split). These embryos were cultured to reach their original cell number status and then were split again (second split). Once these embryos regained their original cell status, they were split yet again (third split).
Main Outcome Measure(s): Blastocyst development of embryos split serially at the two-, four-, and six-cell stages.
Result(s): The blastocyst development rate for two-, four-, and six-cell embryos subjected to a first split was 74.3%, 75.0%, and 66.6%, respectively, as compared with 71.8%, 62.6%, and 48.4% (second split) and 48.4%, 38.1%, and 10.6% (third split).
Conclusion(s): First and second splitting of cleavage-stage embryos has yielded high efficiency rates for blastocyst development when compared with the third splitting, which did not provide any beneficial advantage for further embryo splitting and multiplication. This is the first study reporting on three serial embryo splittings in a mammalian species. Embryo splitting may have significant impact and applications in human assisted reproductive technology. (Fertil Steril® 2006;86(Suppl 3):1112–20. ©2006 by American Society for Reproductive Medicine.)

Key Words: Blastomere biopsy, culture in vitro, mouse preimplantation, multiple embryo splitting

Mammalian embryo splitting first was achieved in the mouse system by investigating the developmental potential of single blastomeres from early preimplantation embryos (1, 2). In chimeric mouse experiments, single blastomeres from the four-cell stage were aggregated with genetically different carrier embryos (3). After transfer of these embryo aggregates into surrogate females, live-born mice could be obtained that, in a few instances, appeared to originate exclusively from the isolated four-cell blastomere. In recently published studies on the potential of four-cell–stage mouse blastomeres contributing to the development of live-born chimeric mice, it was found that although all four blastomeres can have full developmental capacity for adult development, their development of individual blastomeres may differ according to their origin and special arrangement within the donor four-cell embryo (4, 5). Earlier studies showed that 65% of mouse hemi-embryos (split and transferred to foster mothers at the two-cell stage) developed to term (6). Furthermore, transfer of half mouse embryos derived from two-cell embryo splitting gave rise to healthy offspring similar in size to control live-born mice (7). In our recently published comparative and systematic study on mouse embryo splitting via blastomere biopsy, we have demonstrated that splitting at the two- and four-cell stages yielded superior success rates compared with the six- and eight-cell stages, as far as twin blastocyst development in vitro is concerned (8).

In farm animals, embryo splitting has been established successfully for several livestock species. In sheep, 36% of embryos split as two- and four-cell embryos via blastomere biopsy developed to term after transfer to recipients (9). In cattle, embryos split into separate blastomeres at the four-cell stage could develop to term, giving rise to multiple healthy calves (10). Bisected or biopsied early bovine embryos gave conception and pregnancy rates (50%–60%) similar to those obtained by using intact control embryos (55%–61%) and were, therefore, proposed for suitable application under field conditions (11). Cryopreservation of split bovine embryos allows live-born monozygotic calves of different ages to be produced (12). Monozygotic healthy twin kids have been produced from bisected early goat embryos (13). Also in goats, split early embryos, when transferred to genetically identical females, could develop to term in allogeneic pregnancies, being genetically identical twins to these foster females (14). In the pig, split embryos were capable of full-term development (twin piglets) (15, 16). In the horse, from split embryos created via blastomere

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biopsy at the two- and eight-cell stage and transferred in utero, healthy monozygotic foals were delivered (17).

Concerning embryo splitting in nonhuman primates, when rhesus monkey embryos were split at the two- and four-cell stages (18) and at the eight-cell stage (19) and were transferred in utero, only one female monkey was born originating from a quarter embryo biopsied at the eight-cell stage (19). Genetically identical rhesus monkeys would be very useful as models for the study of human-related twinning and tissue transplantation (20).

With regard to human embryo splitting, the Ethics Committee of the American Society for Reproductive Medicine considered favorably any research on embryo splitting and stated in its report that "since embryo splitting has the potential to improve the efficacy of IVF treatments for infertility, research to investigate the technique is ethically acceptable" and further proposed that "splitting one embryo into two or more embryos could serve the needs of infertile couples in several ways" (21).

So far, to our best knowledge, consecutive and serial splitting and its consequences on further development has not been reported for any mammalian species. Therefore, our objectives for this study were to determine whether serial splitting of early mouse embryos is feasible and, if so, to assess the developmental potential of the multiple split embryos under in vitro culture conditions.

MATERIALS AND METHODS

Because no human material was used in this study, no institutional review board approval was required.

Mouse Embryos

Commercially available mouse embryos from B6C3F-1 × B6D2F-1 breedings cryopreserved at the two-cell stage, commonly used for quality control testing in IVF centers, were obtained from the same batch of delivery (Embryotech Laboratories, Wilmington, MA). These frozen two-cell mouse embryos were thawed according to the manufacturer's guidelines, washed carefully, and cultured in IVC-One medium (In Vitro Care, San Diego, CA), supplemented with 10% synthetic serum substitute (Irvine Scientific, Santa Ana, CA) at 5% CO_2 and 37°C.

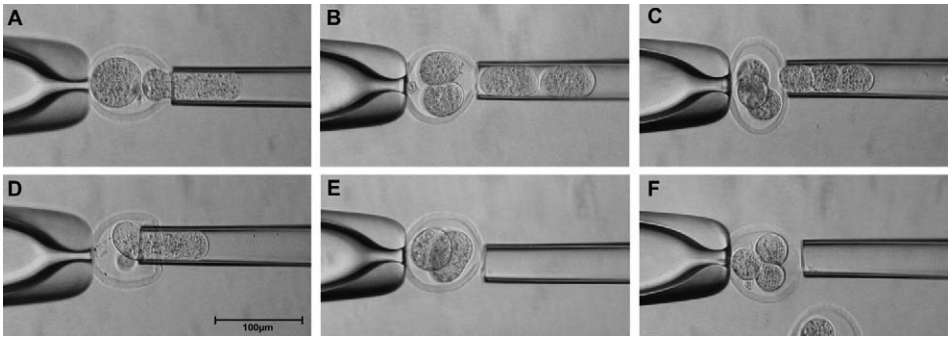
Microsurgical Biopsy

Biopsies of blastomeres from mouse embryos at the two-, four-, and six-cell stages were performed according to our published procedures (8). Before biopsy, mouse embryos from these three cleavage stages were preincubated in microdrops of embryo biopsy medium (Irvine Scientific) covered with equilibrated mineral oil (Sigma-Aldrich, St. Louis, MO) for 10 minutes at 5% CO_2 and 37°C to facilitate separation of blastomeres during biopsy. A modification from our previous protocol was not to add any cytochalasin B to the embryo biopsy medium.

The serial biopsies were performed as follows. In the first series, half the number of blastomeres from two-, four-, and six-cell donor embryos were removed by biopsy and inserted into empty zona pellucida (ZP) recipients. Both donor and recipient embryos were cultured in vitro up to the blastocyst stage (first split). In a second series, after one embryo-splitting procedure and subsequent cleavage, the embryos were split a second time once they again reached the two-, four-, or six-cell stage (second split) and were cultured in vitro. In a third series, after two embryo splitting procedures of two-, four-, and six-cell embryos and subsequent cleavage, these embryos were split yet again as described above (third split) and cultured in vitro (Fig. 1).

FIGURE 1

Microsurgical procedure of blastomere biopsy from early mouse embryos. One, two, and three blastomeres were biopsied from two-, four-, and six-cell donor embryos, respectively (**A, B, C**), and inserted into empty zona pellucida recipients (**D, E, F**).



Illmensee. Serial splitting of cleavage-stage mouse embryos. Fertil Steril 2006.

TABLE 1

Blastocyst development derived from first split of two-, four-, and six-cell mouse embryos.

Embryo stage	Embryos biopsied	Embryos, first split[b]	Blastocysts developed, % (n)		Blastocysts hatched, n (%)
			Of split	Of original[c]	
Two cell	42	78 (84)	74.3 (58/78)	138 (58/42)	70.5 (55/78)
Four cell	55	104 (110)	75.0 (78/104)	141 (78/55)	73.1 (76/104)
Six cell	56	102 (112)	66.6 (68/102)	121 (68/56)	61.8 (63/102)
Controls[a]	70		91.5 (64/70)		80.0 (56/70)

[a] Nonsplit two-cell embryos.
[b] Number in parentheses represents the theoretically expected number of embryos obtained after splitting. The difference between theoretically expected and practically obtained numbers of split embryos for clonal culture resulted from either lysis or apoptosis of some of the blastomeres during or after biopsy. (For biopsy procedure, see Figure 1).
[c] Multiplication rates for blastocysts derived from the original donor embryos used for splitting.

Illmensee. Serial splitting of cleavage-stage mouse embryos. Fertil Steril 2006.

Culture In Vitro

After first, second, and third embryo splitting, the resulting donor embryos (those from which blastomeres were biopsied and the remaining ones left in their original ZP) and recipient embryos (those with biopsied blastomeres that were inserted into empty ZP) were removed from the embryo biopsy medium, washed carefully, and cultured separately as two different groups in microdrops of IVC-One medium supplemented with 10% synthetic serum substitute, covered with equilibrated mineral oil at 5% CO_2 and 37°C. Development of split embryos was registered every 12 hours, up to the blastocyst stage, including hatching ability, and was photographically documented. Cell counts on developing split embryos were performed at their cleavage stages and for blastocysts by using Hoffman modulation phase contrast optics. By rotating the split embryos with a holding micropipette of 95-μm diameter (Conception Technologies, San Diego, CA), these conditions provided sufficient microscopic quantification for cell counts, and therefore, the use of fixed split embryo spreads for cell counts was not considered for this study.

Nonsplit two-cell mouse embryos were treated similarly to the biopsied embryos and cultured under the same in vitro conditions.

RESULTS

A total of 206 mouse embryos (59 at the 2-cell stage, 68 at the 4-cell stage, and 79 at the 6-cell stage) were used in this study for serial splitting purposes. Because donor embryos (those with blastomeres left in their original ZP) and recipient embryos (those with blastomeres biopsied and moved to new, empty ZP) both yielded very similar rates of blastocyst development and hatching for all three cleavage stages biopsied, the data obtained were pooled for both donor and recipient embryos.

Blastocyst formation by embryos split once, at the two-, four-, and six-cell stages, was 74.3%, 75.0%, and 66.6%, respectively. The blastocyst hatching rates were 70.5%, 73.1%, and 61.8% (Table 1). After this first split, the blastocyst siblings derived from their original donor embryos developed very comparably in rate and morphology, irrespective of whether blastomeres were left in their own ZP or moved to a new recipient or host ZP.

From the two-, four-, and six-cell stage embryos undergoing a second split, 71.8%, 62.6%, and 48.4% developed into blastocysts, respectively, of which 65.9%, 58.4%, and 41.4% hatched (Table 2). After the second split, the blastocyst siblings derived from their original donor embryos showed morphological differences in blastocyst formation but not in blastocyst formation success rate, irrespective of whether blastomeres were left in their original ZP or transferred into a new ZP.

After the third split of the two-, four-, and six-cell stage embryos, only 48.4%, 38.1%, and 10.6% of them reached the blastocyst stage, of which only 36.4%, 28.6%, and 0 hatched (Table 3). Moreover, the third split of six-cell stage embryos yielded fewer blastocysts than the original number of embryos used for splitting. After the third such consecutive split, the blastocyst siblings exhibited a very pronounced morphological and developmental heterogeneity, irrespective of the cleavage stage used for blastomere biopsy and of whether cultured with their own or new (host) ZP.

Nonsplit two-cell stage embryos served as controls for the first, second, and third embryo split and developed into blastocysts at a rate of 91.5%, 90.0%, and 91.1%, with a hatching rate of 80.0%, 78.0%, and 77.7%, respectively (Tables 1–3).

To further investigate the hatching ability of blastocysts derived from the three serial splitting procedures, we com-

TABLE 2

Blastocyst development derived from second split of two-, four-, and six-cell mouse embryos.

Embryo stage	Embryos biopsied	Embryos split[b]		Blastocysts developed, % (n)		Blastocysts hatched, % (n)
		First split	Second split	Of split	Of original[c]	
Two cell	10	18 (20)	32 (36)	71.8 (23/32)	230 (23/10)	65.9 (21/32)
Four cell	8	14 (16)	24 (28)	62.6 (15/24)	188 (15/8)	58.4 (14/24)
Six cell	10	17 (20)	29 (34)	48.4 (14/29)	140 (14/10)	41.4 (12/29)
Controls[a]		50		90.0 (45/50)		78.0 (39/50)

[a] Nonsplit two-cell embryos.
[b] Number in parentheses represents the theoretically expected number of embryos obtained after splitting. The difference between theoretically expected and practically obtained numbers of split embryos for clonal culture resulted from either lysis or apoptosis of some of the blastomeres during or after biopsy. (For biopsy procedure, see Figure 1).
[c] Multiplication rates for blastocysts derived from the original donor embryos used for splitting.

Illmensee. Serial splitting of cleavage-stage mouse embryos. Fertil Steril 2006.

pared the ratio of blastocysts hatched versus blastocysts developed (Table 4). There was no statistical difference observed in the hatching ability of blastocysts obtained after the first and second embryo split. However, the blastocyst hatching ability was much lower after the third split and was statistically different to that obtained after the first split for the two- ($P=.06$), four- ($P=.05$), and six-cell stages ($P<.0001$). Moreover, the elevated hatching ability for blastocysts derived from the first and second embryo splittings, when compared with nonsplit control embryos, resulted from the assisted-hatching effect provided by the Tyrode-prepared opening of the ZP required for blastomere biopsy. However, for blastocysts derived from a third consecutive embryo splitting, hatching ability dropped below that of control blastocysts. This inability to hatch was most likely a result of the small size of these abnormally developed blastocysts, despite the presence of the Tyrode-prepared opening in the ZP.

The blastocysts derived from the first, second, or third split showed distinct morphological differences. Blastocysts derived from the first split, irrespective of the embryonic stage at splitting, exhibited a normal size, as judged by their diameter, and were composed of normal cell numbers, as quantified microscopically from cell counts. Furthermore, their morphology showed a pronounced cluster of inner cell mass (ICM) cells and a regularly developed trophoblast (Fig. 2), very comparable to nonsplit control blastocysts. From the second split, blastocysts derived from two- and four-cell stage embryos still showed an apparently normal morphology and size, whereas blastocysts derived from the split six-cell stage embryos were reduced in size and con-

TABLE 3

Blastocyst development derived from third split of two-, four-, and six-cell mouse embryos.

Embryo stage	Embryos biopsied	Embryos split[b]			Blastocysts developed, % (n)		Blastocysts hatched, % (n)
		First split	Second split	Third split	Of split	Of original[c]	
Two cell	7	12 (14)	20 (24)	33 (40)	48.4 (16/33)	228 (16/7)	36.4 (12/33)
Four cell	5	8 (10)	13 (16)	21 (26)	38.1 (8/21)	160 (8/5)	28.6 (6/21)
Six cell	13	22 (26)	36 (44)	47 (72)	10.6 (5/47)	38 (5/13)	0.0 (0/5)
Controls[a]		45			91.1 (41/45)		77.7 (35/45)

[a] Nonsplit two-cell embryos.
[b] Number in parentheses represents the theoretically expected number of embryos obtained after splitting. The difference between theoretically expected and practically obtained numbers of split embryos for clonal culture resulted from either lysis or apoptosis of some of the blastomeres during or after biopsy. (For biopsy procedure, see Figure 1).
[c] Multiplication rates for blastocysts derived from the original donor embryos used for splitting.

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TABLE 4

Hatching ability of mouse blastocysts derived from consecutive, serial embryo splitting at various cleavage stages.

Embryo stage	Blastocysts hatched from blastocysts developed, % (n)		
	First split	Second split	Third split
Two cell	94.8 (55/58)	91.3 (21/23)	75.0 (12/16)[b]
Four cell	97.4 (76/78)	93.3 (14/15)	75.0 (6/8)[c]
Six cell	92.6 (63/68)	85.7 (12/14)	0.0 (0/5)[d]
Controls[a]	87.5 (56/64)	86.6 (39/45)	85.4 (35/41)

[a] Nonsplit two-cell embryos.
[b] $P=.06$ when compared with the first split.
[c] $P=.05$ when compared with the first split.
[d] $P<.0001$ when compared with the first split.

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tained a smaller ICM (Fig. 3). From the third split, blastocysts derived from the two- and four-cell stage embryos consistently were smaller in size when compared with control blastocysts. The ICM was distinguishable only as a small cluster of cells, as determined microscopically. The trophoblasts showed morphological abnormalities with respect to shape and number of cells as evaluated from cell counts (Fig. 4). After the third split of six-cell stage embryos, the few blastocysts that were formed were very small in size and composed of approximately 20 cells only, as determined from microscopic cell counts (Fig. 4). After the third split at the six-cell stage, these embryos underwent premature compaction once they reached the six-cell stage again approximately 16 hours after third split and began with abnormal cavitation approximately 24 hours thereafter (Fig. 5). A less pronounced aberrant morphogenetic development at the morula and blastocyst stage was observed for embryos split at the two- and four-cell stage after three consecutive splittings. With regard to timing of cell divisions, a delay of approximately 4–6 hours was noted for all three cleavage-stage embryos after the second and third split. These delays could be a result of a possible recovery period required for the biopsied blastomeres after splitting.

Taking into consideration these delays and the time schedules required between the splitting procedure, the total time taken to reach the blastocyst stage for the first, second, and third splits was approximately 82 hours, 98 hours, and 114 hours, respectively, as compared with the nonsplit controls that showed blastocyst formation at approximately 72 hours, with time = 0 being at first biopsy. There were no notable differences observed in the time delay, with regard to blastocyst formation, for the three cleavage stages analyzed.

To evaluate the efficacy of the first, second, and third embryo splitting with regard to blastocyst formation, we calculated the embryo formation or multiplication rate which

FIGURE 2

Blastocyst development obtained from first mouse embryo split. These blastocysts showed normal morphological features and regular hatching ability and were derived from two-, four-, and six-cell embryo splitting, respectively (**A, B, C**). *Arrows* point to the site of blastocyst hatching.

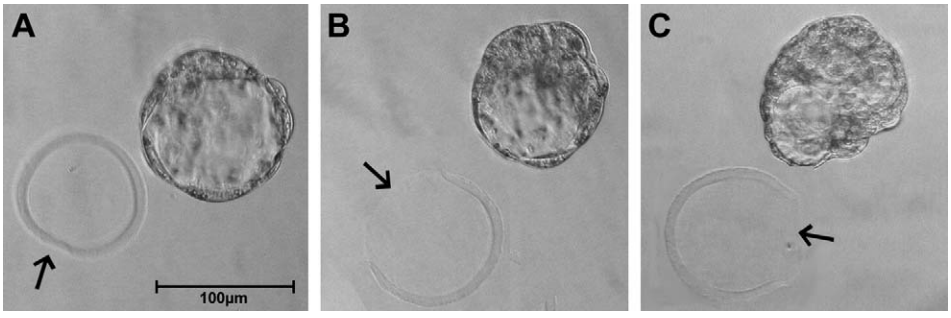

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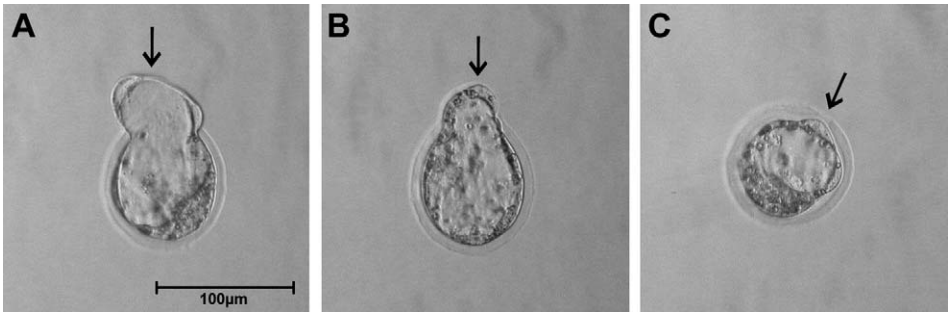
FIGURE 3

Blastocyst development obtained from second mouse embryo split. Blastocysts that were derived from two- and four-cell embryo splitting (**A, B**) showed an apparently normal morphology and size with regular hatching ability, whereas blastocysts originating from six-cell embryo splitting (**C**) exhibited a reduced size and hatching ability. *Arrows* point to the site of blastocyst hatching.



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is defined by the number of blastocysts derived from the original number of embryos split expressed in percentage form. The multiplication rate for two-, four-, and six-cell–stage embryo splitting was 138%, 141%, and 121% after the first split; 230%, 188%, and 140% after the second split; and 228%, 160%; and only 38% after the third split, respectively.

The nonsplit control embryos showed a consistent blastocyst developmental rate of 91.5%, 90.0%, and 91.1% for the first, second, and third split, respectively, and served as a baseline for comparing the efficiency of these three serial splitting procedures with regard to blastocyst multiplication.

DISCUSSION

Embryo splitting to create monozygotic twins or multiples has been reported successful for a variety of mammalian species (11, 22, 23). In various studies on farm animals, embryo splitting via blastomere biopsy from two- and four-cell–stage embryos most efficiently resulted in live-born twins (9, 10, 17). Blastomeres from these early embryonic stages are totipotent and can give rise to healthy and normal offspring.

However, when blastomeres from eight-cell embryos were used in embryo splitting, the success rates for live-born twins or multiples were significantly reduced in cattle (24). Such regression in developmental potential has been reported on mouse embryos derived from eight-cell blastomere splitting (6). Frequently, blastocysts originating from eight-cell split embryos were smaller in size and were composed of fewer cells forming ICM and trophoblast. When isolated blastomeres from eight-cell mouse embryos were investigated for their developmen-

FIGURE 4

Blastocyst development obtained from third mouse embryo split. Blastocysts derived from two- and four-cell embryos splitting (**A, B**) were smaller in size with abnormal morphology and were rarely able to hatch. *Arrow* points to the site of blastocyst hatching. Blastocysts derived from splitting of six-cell embryos (**C**) were unable to hatch because of their very small size.

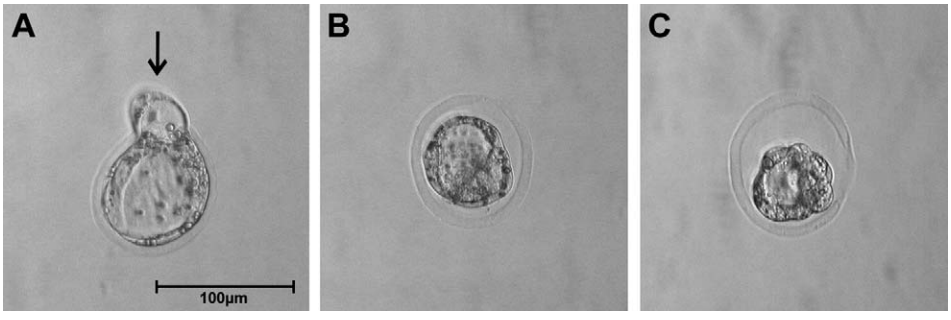

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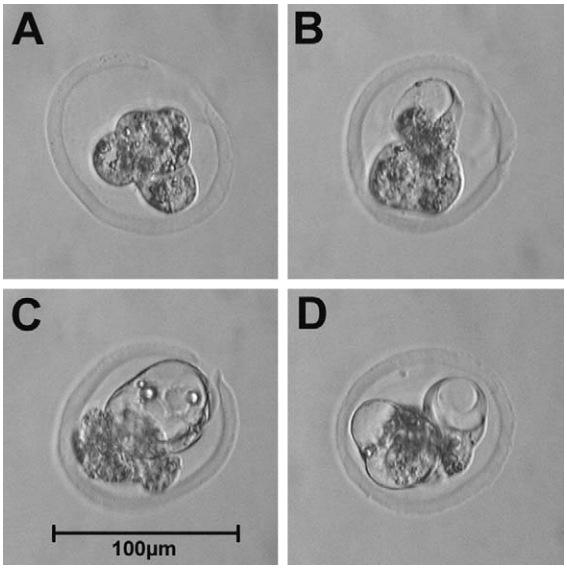
FIGURE 5

Abnormal preimplantation development after third split of six-cell mouse embryos. These embryos showed premature compaction (**A, B**) and aberrant cavitation (**C, D**) when progressing from the morula to blastocyst stage. These embryos consistently were very small, with reduced number of cells.



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tal potential in vivo, Rossant (25) discovered that preimplantation embryos originating from these isolated blastomeres after their transfer into surrogate females only occasionally implanted into the uterus and developed into postimplantation embryos with highly abnormal morphology. That investigator concluded from these results that abnormal blastocysts with reduced cell numbers formed from these isolated eight-cell–stage blastomeres were responsible for such abnormal and arrested embryogenesis in utero.

A similar phenomenon has been observed in our most recent studies on the in vitro development potential of single blastomeres derived from various stages of mouse preimplantation embryos (26). Individual blastomeres biopsied from two- and four-cell–stage embryos could develop to apparently normal blastocysts, whereas individual blastomeres biopsied from six- and eight-cell–stage embryos could only progress abnormally to blastocysts with aberrant morphology and structure.

To our knowledge, consecutive and serial embryo splitting has not previously been reported in any mammalian species. Our objectives in this study were therefore to assess in a systematic and comparative effort, the developmental consequences of the first, second, and third embryo splitting on subsequent development up to the blastocyst stage.

First, serial splitting of two-, four-, and six-cell mouse embryos was feasible. The techniques for blastomere biopsy and transfer into empty ZP recipients, as described in this study, permitted successful blastocyst development and subsequent blastocyst hatching and only a few embryos showed either lysis or apoptosis of some of the blastomeres during or after the biopsy procedure. Both the donor embryos (those with blastomeres left in their original ZP) and the recipient embryos (those with blastomeres biopsied and moved to new, empty ZP) yielded very similar rates of blastocyst development and hatching for all three cleavage stages biopsied. Therefore, the data obtained for both donor and recipient embryos were pooled in this study.

Second, there was a linear decline in blastocyst formation when applying consecutive splitting for the three embryonic stages investigated. Furthermore, a first and second embryo splitting at the two- and four-cell embryos resulted in superior success rates of blastocyst development when compared with those obtained from six-cell embryo splitting.

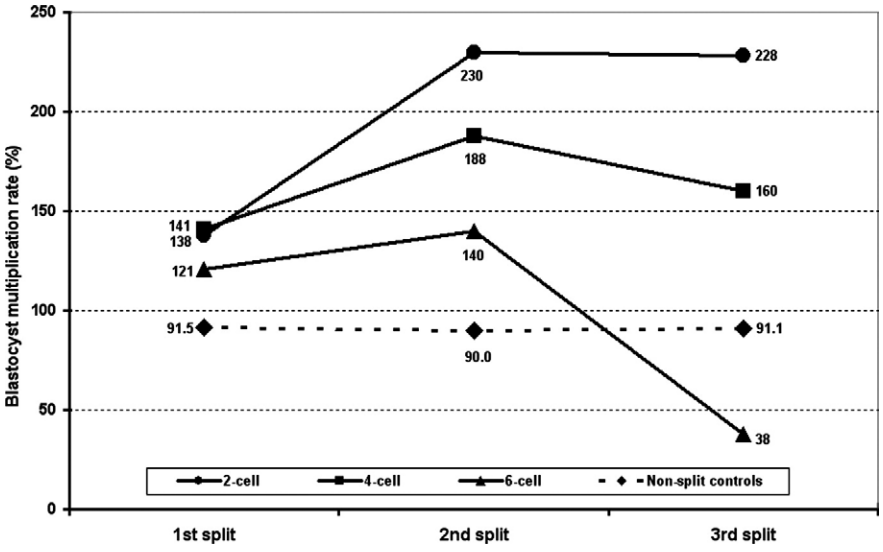
Third, after a third embryo splitting, the success rates for blastocyst formation dropped significantly, irrespective of the three cleavage stages analyzed. In particular, six-cell–stage embryos after a third split compacted prematurely and exhibited abnormal blastocyst formation with artificial cavitation. Similarly, but not as extensively pronounced, morphological aberrations were noted in blastocysts that were derived from a third split of two- and four-cell–stage embryos. We therefore assume that as a result of premature compaction, a reduced number of cell divisions leading to fewer cells was responsible for the small size of blastocysts accompanied with abnormal ICM and trophoblast formation. For the third embryo splitting, less time was available for cell divisions to occur before embryo compaction, and this would have an influence on the cytokinetic and morphogenetic program of those embryos. It appears that compaction occurred at a defined time during embryogenesis without accounting for the number of cells present, usually required for normal morula formation. This phenomenon has been discussed in the context of intrinsically programmed morphogenetic processes termed as *clock mechanism* and leading to embryonic compaction at the morula stage and cavitation at the blastocyst stage (27).

Furthermore, the small and abnormal blastocysts that did form were not able to hatch from their ZPs, even though Tyrode's solution had been used to prepare a hole in their ZP required for blastomere biopsy. In contrast, an assisted hatching effect was observed for normally sized blastocysts derived from first and second split, as also was reported elsewhere for twin blastocysts derived from split mouse embryos (8).

As a conclusion, we therefore assume that a third serial embryo split at the two-, four-, and six-cell stages did not provide any additional advantage for embryo multiplication and could create, instead, abnormal morphogenesis leading to defective blastocysts that would not result in normal

FIGURE 6

Graphic representation of multiplication rates for blastocysts developed from first, second, and third split of two-, four-, and six-cell mouse embryos.



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development in-utero. Furthermore, the extended time schedule noted for blastocyst formation after the third split (approximately 114 hours) does not make it a viable and efficient option for blastocyst multiplication.

Fourth, the blastocyst multiplication rate for the two-, four-, and six-cell–stage embryos was quite similar for the first split and increased after the second split to different degrees (Fig. 6). This increase in blastocyst multiplication was most pronounced for the two-cell–split embryos, less elevated for four-cell–split embryos, and least elevated for six-cell–split embryos. Furthermore, the third split did not show any further advantage over the second split in terms of blastocyst multiplication. In fact, the two-cell third split showed no improvement, whereas the four-cell third split showed a decline in efficiency. More significantly, the six-cell third split showed a drastic decline in multiplication rate, resulting in even fewer blastocysts than the original number of embryos used for this third split (Fig. 6).

Even though a meaningful statistical analysis could not be obtained because of the small sample size used in this study, the graphic representation of multiplication rates for blastocyst development showed a very definite biological trend. These data depict very clearly that the first and second splitting of two-, four-, and six-cell embryos increases the multiplication rates for successful blastocyst development and thereby increases the number of embryos available for potential transfer. However, an additional third split even created a negative and adverse effect on multiplying the number of blastocysts obtained. Blastocyst multiplication derived from two serial embryo splittings therefore may be considered for further investigations concerning the in vivo developmental potential of these blastocysts.

Early embryo splitting has been discussed in the context of future applications in human assisted reproductive technologies. About a decade ago, the merits of embryo splitting were acknowledged as a valuable future application in reproductive medicine (28). The Ethics Committee of the American Society for Reproductive Medicine clearly states in its report (21) that "for couples who can produce only one or two embryos, splitting embryos could increase the number of embryos available for transfer in a single IVF cycle." Such embryo-splitting technologies, as described in this study, may have significant impact and application in future use for human reproduction in the various assisted reproductive technology programs.

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