Artificial Twinning and Human Cloning

Josep Santaló
Universitat Autònoma de Barcelona

Abstract

Artificial twinning has been defined as a procedure to obtain two or more sibling embryos derived from a single one. It is based on the totipotency (full developmental capacity) of blastomeres (embryonic cells) in early stages of development before implantation. Although this is a method that, strictly speaking, could produce a limited number of clones from the same individual, cloning is not the ultimate aim of this procedure. It has been proposed as a method to multiply the number of embryos available to be transferred after an *in vitro* fertilization procedure, thus increasing the chances of having a pregnancy. However, this procedure implies some manipulations, such as reducing total cell number and removing the egg coat (zona pellucida), that can seriously compromise embryonic viability. According to that, it would be only advisable to use it in those couples where a very low number of eggs can be obtained and after a complete study of the benefits of the procedure.

Introduction

Definition

Artificial twinning is a technique that mimics the monozygotic twinning process that spontaneously takes places in humans. It is well known that in such twins, after fertilization takes place, each half of the embryo adopts an individual entity thus giving rise to two separate genetically identical individuals (clones).

Artificial twinning implies the isolation of individual blastomeres (embryonic cells) from a single human preembryo (between the 2- and 8-cell stage) to obtain a higher number of embryos to be transferred to the mother, therefore increasing the chances to obtain a pregnancy.

Cell totipotency

This technique is based on the capacity of a single blastomere to produce a whole organism (in this case a human being) before cells start differentiation, i.e. while they remain totipotent.

However, this capacity is limited in time since it only lasts until the embryonic genetic program starts to take the control of the development (between the 4 and 8-cell stage in humans; Braude et al., 1988). Before that, embryo development had been largely controlled by factors (mainly mRNAs and proteins) of maternal origin stocked during oocyte maturation. Shortly after this stage, a sort of differentiation takes place, and the cells reach a point of no return.

Techniques involved

The techniques involved in artificial twinning are so easy to perform that they are available to almost every center having a human assisted reproduction program. They imply dissolving out the zona pellucida (the external egg coat) by either an enzymatic treatment (for example pronase) or an acidic treatment (using acid Tyrode's solution at pH = 2.5). After removing the zona pellucida, blastomeres are isolated using a Ca^{2}/Mg^{2} free medium to artificially loosen the adhesive mechanisms that stick embryonic cells together, thus obtaining 1/2, 1/4 or even 1/8 embryos.

Is this procedure harmless enough to be safely applied to humans? Few data are available to answer this question, especially in humans, although some of them come from animal models.

For instance, it has been clearly demonstrated, both in animal models and in Preimplantation Genetic Diagnosis (PGD) programs, that culture in Ca^{2}/Mg^{2} free medium does not seriously affect embryo viability (Veiga et al., 1994; Santaló et al., 1996).

Considering the procedure as a whole, only data from animals are available, although some indirect results from the human species can also be considered. In most reports there appear to be three main factors that could affect the viability of the embryos after manipulation (Giménez et al., 1994):

- Removal of the zona pellucida
- Disaggregation of the blastomeres
- Reduction of the cell number

Removal of the zona pellucida

The zona pellucida has been reputed as an egg protective covering that:

Preserves the embryo from the maternal immunological system. It has been suggested that even a slight damage to the zona pellucida during manipulation procedures (assisted hatching or preimplantation genetic diagnosis) could lower the gestational rate after transfer in humans (Cohen et al., 1990); therefore an immunodepressing protocol has been proposed to increase the implantation rate when such embryos are going to be transferred. Our own data in a mouse

model (Velilla et al., 1999) confirm this assertion using zona-free embryos which present a higher developmental capacity than intact ones after this kind of treatment.

Avoids ectopic implantation, preserving the embryo inside a coat while passing through the oviduct. This would not represent any problem in human artificial twinning because embryo transfer is normally performed at the uterine level, where implantation takes place.

Keeps the blastomeres in contact to allow compaction.

Disaggregation of the blastomeres

As previously mentioned, removing the zona pellucida would affect the compaction process. Compaction is an event that takes place in late preimplantation mammalian embryos and implies a tight adhesion between blastomeres. The physical contact between cells during preimplantation development seems to unavoidably end up in compaction; otherwise disaggregation of the embryo would occur. An alternative could be the reintroduction of the isolated blastomeres into foster empty zonae, as performed in mice by Tsunoda and McLaren (1983).

Reduction of the cell number

The reduction in the cell number seems to be the major handicap to be overcome by the embryo to survive. Moreover, not only the reduction in the cell number but also the stage at which the isolation is performed has an influence in the final developmental capacity of the embryo.

In mice, Giménez et al. (1994) obtained about 10% liveborn after transferring 1/2 or 2/4 embryos at the blastocyst stage (postcompacted embryos), while Lawitts and Graves (1988) reported that 2/4 and 4/8 embryos had higher developmental capacity than 1/2 embryos.

In humans no data are available; however different authors (Tarín et al., 1992; Hardy et al., 1990) suggested, during the analysis of the feasibility of preimplantation genetic diagnosis (PGD) procedures, that the reduction in the cell numbers of the inner cell mass (from which the body of the fetus is derived), would be the main cause of the reduced embryo viability after removing several cells in cleaving stages.

So, how many cells are the minimum necessary to develop a whole normal human being? This is an important question because it implies the following one: How many copies of the same embryo are we going to be able to obtain using this procedure?

The animal models mentioned above could be non informative for the human situation, especially because there are important differences in the time of acquisition of the genetic control by the embryonic genome among species, i.e. the time that the embryonic cells remain totipotent.

On the other hand, no research has been done in this field in human embryos. However, accidental situations offer the possibility to have an idea about this question. For instance, it has been described (Veiga et al. 1987) that a single intact cell remaining from a 4-cell frozen-thawed human embryo was able to develop a normal healthy baby after transfer. No data are available from 8-cell human embryos, but it seems plausible to assume that one single cell could develop into a normal human being. Nevertheless, viability of the embryo is severely affected when diminishing the cell number (as mentioned before), so 1/8 embryos probably would have extremely low chances to arrive to term. According to that, it seems that artificial twinning would not produce more than four (exceptionally eight) copies of the same individual.

Clinical indications of artificial twinning

According to what we have just mentioned, what would be the advantages and clinical indications, if any, of artificial twinning in humans?

Two main applications have been proposed for artificial twinning in humans:

- Increasing available embryos for transfer in assisted reproduction procedures.
- Increasing the number of normal embryos in PGD programs.

Increasing available embryos

The number of embryos available for transfer in assisted reproductive techniques could be a limiting factor in the success of such procedures. This is particularly true in those cases where the woman is a bad responder to the hormonal stimulation, so that few oocytes will be available for fertilization. Normally the number of spermatozoa never becomes a problem, specially after microassisted fertilization procedures have been developed, namely ICSI (Intracytoplasmic sperm injection).

It seems clear that the more embryos good enough to transfer, the higher the chances to obtain a pregnancy. In fact a linear positive correlation between the number of embryos transferred (Table I) and the pregnancy rate has been established (Berkley et al., 1991; Hecht, 1993) and, although a sort of «cooperation in implantation» between embryos after transfer had been suggested, this extreme could not be confirmed (Speirs et al., 1996).

However, increasing the number of transferred embryos beyond four significantly increases the number of multiple pregnancies and their associated risks without an important increase in the pregnancy rate (Table I). Therefore transferring a maximum of three embryos has become a widespread practice in almost all assisted reproduction programs (Hecht, 1993). Moreover, some authors (Staessen et al., 1993; Roest et al., 1997) suggested that transferring two good quality embryos would additionally lower the incidence of multiple pregnancy without significantly lowering the pregnancy rate when compared to that obtained after transferring 3 embryos.

Table I: Pregnancy and multifetal pregnancy rate by number of embryos transferred

No. of embryos transferred	Births/Transfer (%)	Multiple Births (%)
1	6	0
2	11	3
3	12	21
4	18	17
5	17	31
6	18	50

From The American Fertility Society (1989).

Therefore, it seems clear that obtaining more than 3 embryos, probably would not effectively help the couple to increase its chance to get a pregnancy. This would be specially true if low quality (zona-free, manipulated) embryos were to be used, such as those derived from an artificial twinning procedure.

Freezing zona free embryos

An alternative could be freezing the extra embryos to use them in further attempts. Few essays have been done to freeze zona-free embryos (none in human species). In mice, about 60% of 2 cell zona-free embryos survived in culture 48 h after thawing (Grossmann et al., 1994) although a high incidence of blastomere disaggregation was observed among them (Sandalinas et al., 1994). This phenomenon was ascribed to abnormalities in the cytocortex and plasma membrane of the contact regions between blastomeres (Martí et al., 1997). However, this could not represent a handicap if isolated single blastomeres were frozen, since embryos that do not disaggregate immediately after thawing do not disaggregate during *in vitro* culture (Sandalinas et al., 1994).

Obviously, using this approach could induce the appearance of monozygotic twins (clones) of different ages. A solution to avoid this situation could be: first to obtain a DNA finger-print of each embryo to be cloned and second to analyze the DNA of the first born baby, to make sure that other embryos are transferred in future cycles.

Preimplantation genetic diagnosis (PGD)

Finally, artificial twinning has been proposed to increase the number of normal embryos in PGD programs. PGD is based on removing 1 or 2 blastomeres (biopsies) from the embryo to be genetically analyzed, thus transferring only the normal embryos. This is a complex procedure that needs to start with a minimum number of embryos to ensure transfer (Vandervorst et al., 1997). So, sometimes only one embryo is available for transfer. Cloning this single normal embryo would increase the probability of a gestation. The main problem for this approach is

that normally the biopsy is performed at the 8-cell stage when cloning could be specially difficult. An alternative could be to biopsy the embryo at an earlier stage, for instance at the 4-cell stage, although it has been suggested that this may adversely affect the viability of the embryo (Tarín and Handyside, 1993).

Conclusions

In conclusion, it seems that artificial twinning could have a utility in human assisted reproduction techniques. However, more research, either in human or in animal embryos, should be carried on in order to increase our knowledge about the consequences and to solve the many problems that the procedure itself presents.

References

BERKLEY A.S., BEDFORD J.M., ROSENWAKS Z. (1991) «Clinical Aspects of Human *in vitro* Fertilization», in Wassarman P. ed. *Elements of Mammalian Fertilization Vol II. Practical Applications*, pp 34-61. CRC Press Inc. Florida. 1991.

BRAUDE P., BOLTON V., MOORE S. (1988) «Human Gene Expression First Occurs between the Four- and Eight-cell Stages of Preimplantation Development», *Nature*, 332: 459-61.

COHEN L., MALTER H., ELSNER G., KORT H., MASSEY J., MAYER P. (1990) «Immunosupression Supports Implantation of Zona Pellucida Dissected Human Embryos», *Fertility Sterility*, 53: 662-5. 1990.

GIMÉNEZ C., EGOZCUE J., VIDAL F. (1994) «In Vitro and In Vivo Development of Mouse Half Embryos: An Approach to Pre-implantation Diagnosis», *Genetics (Life Sciences Advances)*, 13: 25-34.

GROSSMANN M., EGOZCUE J., SANTALÓ J. (1994) «Cryopreservation of Zona-free Mouse Embryos», *Cryoletters*, 15: 103-12.

HARDY K., MARTIN K.L., LEESE H.J., WINSTON R.M.L., HANDYSIDE A.H. (1990) «Human preimplantation Development in Vitro is not Adversely Affected by Biopsy at the 8-cell Stage», *Human Reproduction*, 5: 708-14.

HECHT B.R. (1993) «Iatrogenic Multifetal Pregnancy», *Assisted Reproduction Reviews*, 3:75-87.

LAWITTS J.A., GRAVES C.N. (1988) «Viability of Mouse Half-embryos In Vitro and In Vivo», *Gamete Research*, 20: 421-30.

Martí M., Grossmann M., Santaló J., Egozcue J., Ponsà M. (1997) «Characteristics of Actin Fibers and Ultrastructure of the Contact Regions Involved in the Separation of Blastomeres of Twocell Mouse Embryos, Frozen-thawed without the Zona Pellucida», *Cryobiology*, 34: 94-106.

Medical Research International and the Society of Assisted Reproductive Technology, The American Fertility Society (1989) «In Vitro Fertilization/ Embryo Transfer in the United States: 1987 Results from the National IVF-ET Registry», *Fertility Sterility*, 51.

Roest J., Van Heusden A.M., Verhoeff A. (1997) «A Triplet Pregnancy after In Vitro Fertilisation is a Procedure-related Complication that should be Prevented by Replacement of Two Embryos Only», *Fertility Sterility*, 67: 290-5.

Sandalinas M., Grossmann M., Egozcue J., Santaló J. (1994) «Blastomere Disaggregation of Two-cell Zona-free Mouse Embryos», *Cryoletters*, 15: 343-52.

Santaló J., Grossmann M., Egozcue J. (1996) «Does Ca^2/Mg^2 free Medium have an Effect on the Survival of the Preimplantation Mouse Embryo after Biopsy?», *Human Reproduction Update*, 2: 257-61.

Speirs A.L., Baker H.W.G., Abdullah N. (1995) «Analysis of Factors Affecting Embryo Implantation», Human Reproduction: 11 suppl. 1 Human Conception *In vitro*. Proceedings of the Third Bourn Hall Meeting, 187-91 (1996.)

Staessen C., Janssenswillen C., Van Den Abeel E., Devroey P., Van Steirteghem A.C. (1993). «Avoidance of Triplet Pregnancies by Elective Transfer of Two Good Quality Embryos», *Human Reproduction*, 8: 1650-3.

Tarín J.J., Conaghan J., Winston R.M.L., Handyside A.H. (1992) «Human Embryo Biopsy on the Second Day Post Insemination for Preimplantation Diagnosis: Removal of a Quarter of Embryo Retards Cleavage», *Fertility Sterility*, 58: 970-6.

Tarín J.J., Handyside A.H. (1993) «Embryo Biopsy Strategies for Preimplantation Diagnosis», *Fertility Sterility*, 59: 943-52.

Tsunoda Y., McLaren A. (1983) «Effect of Various Procedures on the Viability of Mouse Embryos Containing Half the Normal Number of Blastomeres», *Journal Reproduction Fertility*, 69: 315-22 .

Vandervorst M., Liebaers I., Van Waesberghe L., Van Steirteghem A., Devroey P. (1997) «Is There a Minimum Number of Cumulus-oocyte-complex Needed for Successful PGD?», *Journal Assisted Reproduction Genetics. Abstracts from Second International Symposium on Preimplantation Genetics*, Chicago: 14: 475

Veiga A., Calderón G., Barri P.N., Coroleu B. (1987) «Pregnancy after Replacement of a Frozen-thawed Embryo with < 50% Intact Blastomeres», *Human Reproduction*, 321-3.

VEIGA A., SANTALÓ J., VIDAL F., CALDERÓN G., GIMÉNEZ C., BOADA M., EGOZCUE J., BARRI P.N. (1994) «Twin Pregnancy after Preimplantation Diagnosis for Sex Selection», *Human Reproduction,* 9: 2156-9.

VELILLA E., GROSSMANN M., EGOZCUE J., SANTALÓ J. (1999) «Effect of 6-beta-methylprednisolone on mouse pregnancy rate». Human Reproduction (14:207-10).