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Research Letter

# Twin live births following transfer using eight-cell cleavage stage embryos on Day 4 with developmental arrest

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Most forms of assisted reproduction require maintenance of either or both of the male and female gametes and the resulting embryos *in vitro*. Developmental arrest is one of the mechanisms responsible for increased embryonic demise during the first week of *in vitro* development [1]. In most of the species studied, researchers have observed embryos arrested at specific stages of preimplantation development *in vitro*. The developmental blocks and retardation observed in animals also occur in human embryos between the 4- and 16-cell stages [2], which is the point in time at which embryonic products of transcription and translation become functional [3] and reach the morula stage [4]. Embryos are not blocked in development, however, if they have proceeded to the blastocyst stage.

Research interest has increased in blastocyst development *in vitro* and the benefit of blastocyst transfer has been attributed to facilitating a natural selection of good quality embryos [5,6]. Blastocyst transfer has been reported to generate high implantation rates, whereas lowering the multiple gestation rates following *in vitro* fertilization (IVF) [5,6]. The effort required for blastocyst culture, compounded by concerns about the occasional suboptimal performance of sequential media, has led many clinics to continue to perform Day 2 or 3 embryo transfer (ET).

Recent advances in our understanding of the dynamic physiology of early human embryos have resulted in the development of culture systems now capable of yielding viable blastocysts with greater consistency. At the same time, extended cultures have enabled embryologists to observe human embryo development *in vitro*. Approximately 10–15% of IVF embryos get permanently arrested in mitosis at the two- to four-cell cleavage stage, showing no signs of apoptosis [1]. In our previous report, we suggested that a combined evaluation of the *Z*-score and Day 3 embryo morphology is highly predictive of embryo viability after IVF or intracytoplasmic sperm injection [7]. From our data, all of the zygotes placed in extended culture had a Day 5 embryo survival rate of $56.9 \pm 1.3\%$. These predictions are consistent with empirical data in the literature. The assessment of embryo survival was based on embryo morphology and cleavage speed. Embryos that had the same number of blastomeres at two sequential times (24 hours), together with those zygotes that remained blocked at the pronuclear stage, were considered to have developmental arrest. From our experience, a developmentally arrested embryo *in vitro* always progresses to apoptosis.

Until the present, arrested embryos could not be designated as "alive" and were not appropriate for transfer to the uterus using assisted reproductive techniques. We describe a unique case of twin live births following transfer using three good morphology but developmentally arrested (>24 hours) embryos. We have followed the healthy twins for 2 years as of this writing.

A 35-year-old nulligravida presented with primary infertility for 9 years and polycystic ovarian disease. Her weight was 72 kg and her height was 161 cm with a body mass index of 27.8 kg/m$^2$. She had previously undergone hysterosalpingography, which revealed patent fallopian tubes bilaterally. Before presenting to our center, she had undergone several cycles of clomiphene citrate and one cycle of IVF treatment (with ovarian hyperstimulation syndrome) but failed to conceive. The semen analysis of her husband was within the normal range. She denied any other systemic diseases, except hypertension, for which she had been treated with medication

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for 3 years. The couple presented to our center for IVF. Hysteroscopy was performed to evaluate the endometrial cavity, which revealed multiple submucosal myomas. Thus, depot gonadotropin-releasing hormone agonist was administered for 3 months before the transcervical myomectomies. The protocol for controlled ovarian hyperstimulation followed the downregulation regimen and oocyte retrieval, as we have previously reported [7,8]. A single team of embryologists coordinated all procedures, thereby ensuring that both the culture protocols and the embryo assessment were standardized, as we have described elsewhere [7,9]. All 2PN zygotes were cultured until the day of ET. G1TM medium (Scandinavian IVF Science) was used for culturing embryos on Days 1–3. G2TM medium (Scandinavian IVF Science) was used for culturing embryos from Days 3–5 or Day 6. In our program, we have routinely offered blastocyst transfer to patients with more than 3 eight-cell embryos on Day 3.

During the first ultra-long downregulation and controlled ovarian hyperstimulation regimen, the estradiol level was 2447 pg/mL on the human chorionic gonadotropin day and 20 oocytes were retrieved, but complete fertilization failure was noted the next day. Two months later, another treatment regimen was initiated following a standard downregulation. On the 12$^{th}$ day of the cycle, transvaginal ultrasound-guided oocyte retrieval was performed and 11 oocytes were obtained. All of the collected oocytes were injected via intracytoplasmic sperm injection, and 10 zygotes (6 Z1 and 4 Z2) were obtained on Day 1. On Day 3, nine embryos developed to the eight-cell cleavage stage (six embryos with eight cells and blastomeres of equal size and no cytoplasmic fragments; three embryos with eight cells blastomeres of equal size and less than 20% cytoplasmic fragments). On Day 4, all nine embryos had developmental arrest at the eight-cell cleavage stage. We chose three eight-cell embryos (Z1 8E1; Grade I) to transfer to the uterus on Day 4 (Fig. 1A). The other three arrested embryos (Z1 8E1; Grade I) were frozen. A positive urine test for human chorionic gonadotropin was noted 2 weeks after ET. An initial ultrasound at the fourth week of gestation revealed two gestational sacs with positive cardiac motion and an appropriate size yolk sac.

All prenatal examinations were unremarkable, except poor control of blood pressure (160/110 mmHg) with proteinuria beginning at 30 weeks gestation. At $32 + 3$ weeks gestation, the patient presented to our emergency department with vaginal bleeding and lower abdominal pain, a blood pressure of 140/90 mmHg, and bilateral lower limb edema. Preterm labor with chronic hypertension was diagnosed, and she was admitted to our antepartum unit for treatment with $MgSO_4$ and antihypertension medications.

At 34 weeks gestation, persistent poorly controlled blood pressure (170/110 mmHg in average) and decreased fetal heart rate variability was noted. Suspecting placental hypoperfusion and imminent fetal distress, a cesarean section was performed

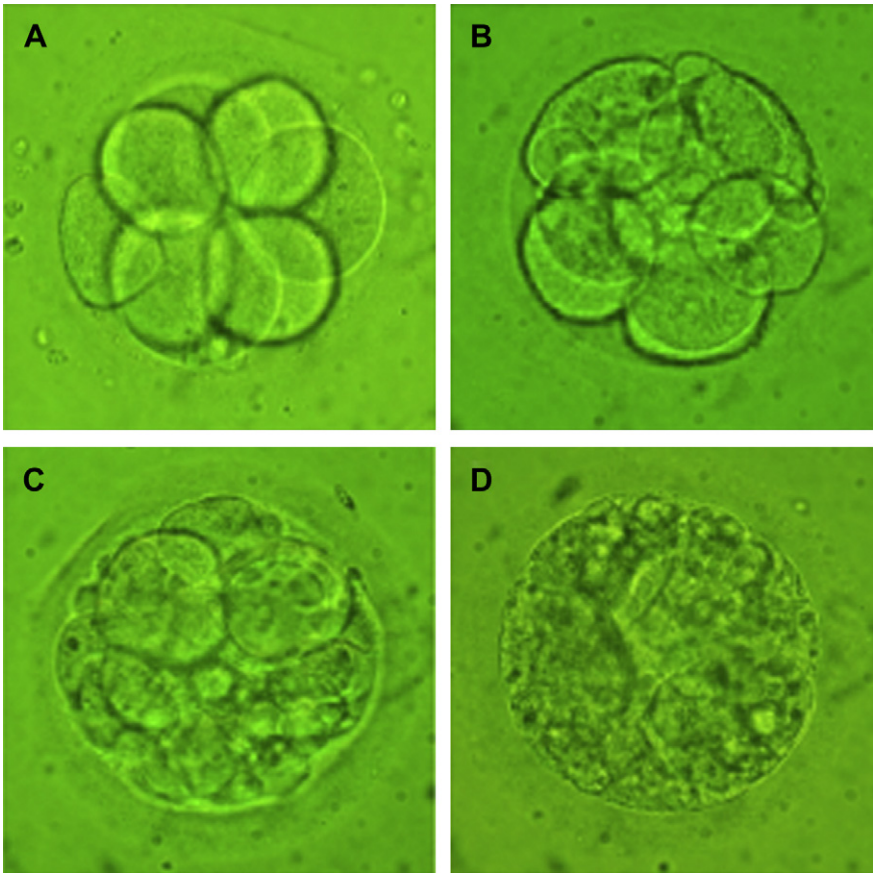


Fig. 1. (A) The embryo had developmental arrest in less than 24 hours at the eight-cell cleavage stage. (B) On the thawed day, embryos were at Day 3 cleavage stage and (C and D) displayed morphological signs of apoptosis on Day 4 and Day 5.

immediately, with delivery of a 1420 g female and a 1740 g male with Apgar scores of 7 and 8, and 5 and 7 at 1 minute and 5 minutes, respectively. The patient was discharged 1 week postoperatively without complications. She was followed in the Cardiovascular Department for blood pressure control. The twins were discharged 1 month later. At 1 year of age, the twins had no clinically significant sequelae. We thawed the three vitrified embryos per our protocol [10], and all embryos underwent apoptosis *in vitro* (Fig. 1B−D).

When embryos are cultured *in vitro*, about 50% will arrest during the first week. Human preimplantation embryos exhibit a high rate (∼50%) of cleavage arrest during the four- and eight-cell stage of *in vitro* development [1,7,11,12]. The reasons for this high rate of embryonic loss during early development are unclear but could include chromosomal abnormalities, genetic factors, suboptimal culture conditions, oxidative metabolites, or inadequate oocyte maturation [11,12]. Hardy et al [11] suggested that most arrested embryos (70%) display gross chromosomal anomalies, are already developmentally programmed at the one-cell stage, and that environmental factors merely dominate. From our previous observation about extending to the blastocyst stage *in vitro*, we agree with this hypothesis [7]. In the case presented herein, however, three arrested embryos were transferred and resulted in a successful twin pregnancy with a normal cytogenetic karyotype. So, chromosomal anomalies cannot explain the embryo developmental arrest phenomenon.

It is reasonable to suggest that suboptimal culture conditions or inadequate oocyte maturation led the embryo to development arrest at least 24 hours. In our laboratory, G2TM medium (Scandinavian IVF Science) is routinely used for culturing embryos from Days 3−5 or Day 6. For the case presented herein, G5.2TM medium (Scandinavian IVF Science) instead of G3.2TM medium (Scandinavian IVF Science) was used for the Day 3 cleavage stage embryos. On the other hand, the patient had polycystic ovary disease and a history of fertilization failure, suggesting that the embryos had inadequate oocyte or blastomere maturation. From our experience, those embryos with developmental arrest always have blastomere fragments or retarded cleavage speed or poor grade zygotes. Betts and Madan [1] reported that approximately 10−15% of IVF embryos permanently arrested in mitosis at the two- to four-cell cleavage stage showing no indication of apoptosis. Hardy et al [11] showed that apoptosis does not occur during the early cleavage stages of human preimplantation development, and significant levels are not seen until the morula stage. Our case involved successful implantation with a good eight-cell cleavage stage morphology score without fragments, thus suggesting an embryo with developmental arrest but not programmed to cell death. The reason for this result was perhaps the suboptimal medium or some unknown substance in the uterine environment, which may be essential to embryo development that could not be obtained in *in vitro* medium. In the literature, the time course leading to embryo developmental arrest has not been defined. Hardy et al [11] suggested an approximate correspondence between developmental stages and chronologic time, with day of oocyte retrieval on Day 0. Eight- and 16-cell cleavage embryos usually lie in Day 3−4. Jun et al [13] defined the human cohort embryo phenotype with cleavage arrest as embryos with four of fewer cells on Day 3 *in vitro*. However, it is a broadly accepted concept that pre-embryos in which there is no evidence of cellular division for a minimum of 24 hours are deemed to have undergone cleavage arrest [7,14].

In this case, all previous vitrified embryos underwent demise after thawing, although we had an 89.8% post-thaw survival rate in our report [10]. It was thought that the cryopreservation and thaw were performed on an eight-cell stage *in vitro* before the embryos had reached the blastocyst stage, which exhibits a higher rate in the cleavage stage subjected to damage during the protocol.

Admittedly, the transfers were sometimes mixed, with both early- and late-cleaving embryos being transferred together. This made it difficult to ascertain which of the embryos was actually implanted. However, lowering the number embryos transferred to avoid high-order pregnancies is a worldwide assisted reproductive technique treatment tendency. The embryos with developmental arrest usually were not first priority in transferring, as they were considered poor quality and led to embryo death in our previous report [7]. Even if there is extremely little experience of transferring totally arrested embryos (mostly just one embryo available in poor responders), it always resulted in implantation failure. This is the first report using three arrested embryos with successful live births. Our unique case provides the chance for reviewing the cellular concepts of embryo development. It is of value for clinicians and embryologists to consider an embryo morphology selection transfer strategy if the patient has a lack of choice of appropriate embryos.

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