

Article

Improved implantation after preimplantation genetic diagnosis of aneuploidy



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Santiago Munné has been director of PGD at Saint Barnabas Medical Center since 1995. His group there focuses on identifying genetically normal embryos. Originally from Barcelona, Spain, Dr Munné gained his PhD in genetics from the University of Pittsburgh and joined Dr Jacques Cohen at Cornell University Medical College, New York in 1991. There he developed the first PGD test to detect embryonic numerical chromosome abnormalities. His work has been recognized by several prizes: in 1994, 1995 and 1998 from the Society for Assisted Reproductive Technology, and in 1996 from the American Society for Reproductive Medicine. Recently the PGD team has shown higher pregnancy rates in women of advanced age undergoing PGD. This team has performed more than 250 PGD cycles for translocations and over 1600 PGD cycles for chromosome abnormalities related to advanced maternal age. Dr Munné has more than 100 publications to his name, and is a frequent lecturer, both nationally and internationally, on his team's work and the field of preimplantation genetics.

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Abstract

The objective of this study was to assess the improvement in implantation rates after preimplantation genetic diagnosis (PGD) of numerical abnormalities for the sole indication of advanced maternal age when compared with a control group. Each PGD patient was matched to a control patient according to several parameters prior to obtaining pregnancy results. The diagnosis was based on the analysis of chromosomes X, Y, 13, 15, 16, 18, 21 and 22 plus a ninth probe (1, 7, 14 or 17) on a single cell per embryo. The results were also analysed in relation to the previous number of IVF cycles and the number of dipronucleated zygotes obtained, when replacing presumptively chromosomally normal embryos on day 4 of development. It was found that women of advanced reproductive age (average age 40 years) had a higher implantation rate (18%) than their matched controls treated with standard IVF (11%) ($P < 0.05$). This increase was not observed in patients with two or more previous IVF cycles or patients with fewer than eight zygotes. Patients with eight or more 2PN zygotes and one or no previous cycles showed the greatest improvement in implantation rate, from 8.8% in controls to 19.2% in the PGD group (average age 40 years) ($P < 0.025$).

Keywords: aneuploidy, mosaicism, PGD, recurrent miscarriage, repeated IVF failure, trisomy 21

Introduction

Preimplantation genetic diagnosis (PGD) consists of testing individual cells biopsied from a cohort of embryos before their replacement, with the intention of selecting against chromosomally or genetically abnormal embryos. With the use of fluorescence in-situ hybridization (FISH), current PGD protocols can detect a substantial proportion of numerical chromosome abnormalities often present in cleavage-stage embryos. These include aneuploidies for the chromosomes studied, polyploidy, haploidy and the fraction of mosaic embryos detectable with the probes used.

PGD testing for numerical chromosome abnormalities has four potential benefits: (i) to prevent trisomic offspring by analysing chromosomes X, Y, 13, 18 and 21 (Kuliev *et al.*, 2002); (ii) to reduce spontaneous abortions (Warburton *et al.*, 1986; Munné *et al.*, 1998a, 1999; Sarosi *et al.*, 1998; Vidal *et*

al., 1998; Pellicer *et al.*, 1999; Rubio *et al.*, 2003); (iii) to reduce multiple pregnancy by minimizing the number of embryos necessary for replacement and successful pregnancy (Munné, 2002); and (iv) to improve implantation (International Working Group on Preimplantation Genetics, 2001).

The last objective, to improve implantation, is based on evidence indicating that the decrease in implantation in older women is predominantly an oocyte rather than uterine factor (Navot *et al.*, 1994). Also, aneuploidy increases with maternal age (Warburton *et al.*, 1980, 1986; Hassold *et al.*, 1980) but rates found in first trimester conceptions are much lower than those found in oocytes and cleavage stage embryos (Munné *et al.*, 1995; Dailey *et al.*, 1996; Verlinsky and Kuliev 1996; Márquez *et al.*, 2000). This suggests that a sizeable proportion of chromosomally abnormal embryos are eliminated before clinical recognition. This embryo loss could account for the

decline in implantation with maternal age (Magnuson *et al.*, 1985; Sandalinas *et al.*, 2001). The correlation between age-related aneuploidy and implantation also suggests that removal of chromosomally abnormal embryos could reverse this trend (Munné *et al.*, 1993).

PGD of numerical chromosome abnormalities has been applied in more than 2000 cycles (Munné *et al.*, 1993, 1995, 1998a,b, 1999; Verlinsky *et al.*, 1995, 1998; Manor *et al.*, 1996; Verlinsky and Kuliev, 1996; Gianaroli *et al.*, 1999a; Kahraman *et al.*, 2000; Magli *et al.*, 2001; Pehlivan *et al.*, 2002). The results indicate increased implantation and/or live births and a reduction in spontaneous abortions when biopsy and analysis was performed on day 3 of development and compared with untested controls (Gianaroli *et al.*, 1999a; Munné *et al.*, 1999).

To date, PGD is offered in only a few IVF centres, mostly because the costs restrict widespread application. An alternative to in-house PGD has recently been to ship processed (e.g. fixed blastomeres) to existing laboratories for analysis by qualified staff with up-to-date equipment. However, communication and shipping may delay embryo replacement until day 4. Several studies (Grifo *et al.*, 1998; Gianaroli *et al.*, 1999a) have indicated that day 4 replacement of biopsied embryos produces results similar to day 3 replacement.

Not all IVF patients of advanced maternal age may benefit from PGD. Preliminary indications are that patients with repeated IVF failure do not show an improvement in implantation after PGD (Gianaroli *et al.*, 1999a,b; Kahraman *et al.*, 2000; ESHRE PGD Consortium Steering Committee, 2002). Similarly, if the improvement in implantation is caused by the selection of the best embryos out of a large cohort of embryos, but a patient produces only as many embryos as would be replaced, there is no possible improvement in implantation.

The main objective of this study was to identify female patients of advanced reproductive age (≥ 35 years) that would benefit the most from PGD. A second aim of the study was to determine if previously reported beneficial effects on implantation rates of PGD when replacement was performed on day 3 (Gianaroli *et al.*, 1999a), were also found when replacement was performed on day 4.

Materials and methods

Patients undergoing PGD of aneuploidy and their controls

Patients undergoing PGD of aneuploidy were all 35 years and older. Of the 138 patients, 55 were younger than 39; of those 17 had recurrent miscarriages, 14 had a history of repeated IVF failure (RIF), and five had previous trisomic conceptions. Most patients 39 and older ($n = 83$) had a combination of RIF, trisomic offspring and/or spontaneous abortions, as is usual for that age. Some but not all patients with very few embryos at the day of biopsy had their PGD procedure cancelled.

To determine if PGD for aneuploidy in patients with advanced maternal age improves implantation, patients were matched

one-to-one with patients undergoing IVF in the same centre but who declined PGD. Thus, this is a retrospective non-randomized study with regard to when the test was offered, but prospective non-randomized with regard to pregnancy results.

The matching occurred prior to confirmation of pregnancy. A one-to-one matching was chosen over several controls per test patient because some test patients over 40 years with several previous IVF attempts were difficult to match with more than one patient; so multiples in the control group would have created a bias towards a total number of controls with better prognosis than the total test.

PGD cases were matched using the following variables listed in order of matching priority: maternal age (± 1 year); number of 2PN embryos (± 2 embryos), number of previous IVF cycles, date of retrieval, and oestradiol serum concentration on day +1 after human chorionic gonadotrophin (HGC), in pg/ml. There was a $\pm 20\%$ difference between control and test). The rationale for matching patients according to these factors, as well as maternal age, was to take into account the number of embryos available for selection, to minimize changes in laboratory performance, and to control for ovarian reserve (Scott and Hofmann, 1995). The number of previous IVF cycles was taken into account for two reasons. First, it was an indirect measure of RIF because in this centre, most patients repeated cycles because of previous failure. Second, in this centre, implantation rate decreases significantly between the first, second and third IVF attempts, a phenomenon not always observed in other centres (Meldrum *et al.*, 1998). Thus, patients undergoing PGD because of repeated IVF failure were not matched to controls according to the number of previously failed cycles but to the total number of IVF cycles. This may underestimate the utility of PGD for RIF; but was necessary for the non-RIF patients for the second reason mentioned above.

Implantation was considered to be the presence of a fetal heartbeat at 3 weeks of pregnancy.

None of the patients included in the study was chromosomally abnormal, but the patients were not matched according to other indications, such as previous repeated spontaneous abortions.

All control embryos were cultured until their replacement on day 3 in human tubal fluid (HTF) with 5% HSA or maternal serum (Alikani *et al.*, 1999).

Biopsy, fixation, FISH procedure and embryo classification

IVF with PGD was performed at the Institute for Reproductive Medicine and Science of Saint Barnabas and written consent was obtained from patients in accordance with the Institutional Review Board (IRB) protocol. Inclusion of patients followed these criteria: maternal age was equal or greater than 35 years; and all replaced embryos were classified as chromosomally normal. Cycles where all embryos were found to be abnormal and the patients chose not to have any replacement were also included in the study.

On day 3 of development, each embryo had a single cell biopsied (Munné *et al.*, 2003), unless the nucleus could not be

found after fixation, in which case a second cell was biopsied. Fixation was performed as described previously. Normally developing embryos classified as presumptively chromosomally normal by PGD were replaced in the patient on day 4 unless they had arrested development or were in excess of the appropriate number of replaceable embryos. Some non-replaced embryos were reanalysed with all or most of their cells fixed individually, as described previously (Munné *et al.*, 1996). Not all non-replaced embryos were re-analysed, either because of time constraints or because the embryo degenerated in culture. Qualified non-replaced embryos were disaggregated and all cells fixed individually (Velilla *et al.*, 2002).

For both the PGD analysis and the re-analysis, cells were analysed at least with DNA probes for chromosomes X, Y, 13, 15, 16, 18, 21 and 22 (Vysis, Downer's Grove, IL, USA). Some were also analysed for chromosome 1 and the rest for chromosome 14, 7 or 17 using previously published FISH protocols (Munné *et al.*, 1998a; Bahçe *et al.*, 2000). Analyses for chromosomes 1, 14, 7 were later discontinued because its aneuploid forms are rarely found in embryos with better chances of replacement. Embryos that were not replaced or degenerated were reanalysed with the same set of probes that were analysed during the PGD process.

Not all embryos classified by PGD as chromosomally abnormal could be fully reanalysed, due to time constraints or the lack of patient consent; so embryos were classified based on single cell analysis (without re-analysis) as follows: 1) when the cell had two copies of each chromosome analysed, the embryo was classified as presumptively normal; 2) when the cell had three or more copies of each chromosome, the embryo was classified as polyploid; 3) when the cell had one or fewer copies of each chromosome, the embryo was classified as haploid; 4) when the cell had one or two chromosomes with an abnormal number of copies, the embryo was classified as aneuploid; and 5) when the cell had three or more chromosomes with an abnormal number of copies but the cell was not haploid or polyploid, the embryo was classified as complex abnormal (mosaic). The latter criteria are based on the observation that triple and higher multiple aneuploidies are rare, even in cleavage-stage embryos and that full analysis of embryos with three or more abnormal chromosomes shows they are usually mosaic (Munné *et al.*, 1995; Márquez *et al.*, 2000).

When re-analysis of embryos not replaced or degenerate was performed, the classification criteria for normal, aneuploid, mosaic, polyploid or haploid derived from FISH results from most or all of the cells of each embryo were as previously described (Munné *et al.*, 1998c).

Culture to day 4 after PGD

PGD embryos were cultured from day 1 to day 3 in HTF or P1 with 5% HSA. The biopsy was performed in HEPES-buffered HTF with 5% HSA supplemented with 0.05 mol/l sucrose and the media was free of calcium and magnesium. After biopsy, the embryos were washed and cultured in CCM (Co-Culture Media, Scandinavia IVF) until replacement on day 4 of development.

Statistical analysis

Since the variables of interest consisted of proportions (implantations, pregnancy), generalized linear model (GLM) methods were used to carry out the analyses. The dependent variable consisted of the logistic transform of the proportions, and various possible explanatory variables were included in the model and tested. Of particular interest was the factor derived by grouping the patients as either control or PGD, as defined above. The number of embryos replaced per cycle was also a potentially important factor, as was the reason for employing PGD. The stratification brought about by the matching process defined above, which was likely to improve the precision of the investigation, was also included as a factor. Although for technical reasons the analyses were conducted on the logistic scale, the summaries will be on the original scale of proportions, these back-transformations having been generated within the package employed (GENSTAT, 1993). Thus the logistic transform is given by $\varphi = \ln[p/(1-p)]$ where p is the proportion, and the corresponding inverse transformation is given by $P = e^{\varphi}/[1 + e^{\varphi}]$.

Since no multiple comparison procedures were employed in the summaries displayed in **Table 1**, the P -values should be regarded simply as a measure of the magnitudes of the effects in question.

Results

Chromosome abnormalities

Each patient underwent only a single cycle of PGD. The 138 patients undergoing PGD with their IVF cycle produced 1231 bipronucleate (2PN) embryos in total, of which 1120 (91%) had four or more cells and were biopsied. Only embryos with four or more cells were biopsied, while embryos with fewer than four cells (III) were considered morphologically and developmentally unsuitable for replacement and were not biopsied.

Of the 1120 biopsied embryos, 49 had no results after PGD, due either to unsuitable fixation or to FISH problems ($n = 14$) or lost nucleus ($n = 35$). The remaining 1071 embryos were analysable and after PGD, 318 (29.7%) were classified as normal, 426 (39.8%) were aneuploid, 267 (24.9%) were complex mosaic, 31 (2.9%) were polyploid and 29 (2.7%) were haploid.

Of the presumptively normal embryos, 272 were replaced in 119 cycles, while the others were either frozen, or discarded because they were developmentally and morphologically abnormal. The other 19 patients did not have a replacement for lack of chromosomally normal embryos.

Of the embryos not replaced, 258 were reanalysed, of which 32 were confirmed as not being abnormal after re-analysis (12% false positive rate).

Correcting the PGD data with the re-analysis data, which is a more accurate diagnosis, since it involved the analysis of more cells, the chromosome abnormalities in this study were as follows. Thirty-five still had no results, and of the remaining 1085 embryos, 347 (32.0%) were normal; 419 (38.6%) were

Table 1. Comparison of implantation rates in control and PGD patients.

	Cycles (n)	Average maternal age (years)	Average no. 2PN	Average oestradiol (pg/ml)	Average no. Embryos cycles (n)	Average no. Embryos transferred (n)	Implantation rate (%) (FHB /embryos replaced)	P-value
<i>All cases</i>								
All control	138	39.4	8.7	2290	1.5	508 (3.7) ^a	10.6 (54/508)	
All PGD	138	39.8	8.9	2344	1.7	272 (2.0) ^b	17.6 (48/272)	<0.05
<i>Less than two previously failed cycles</i>								
All control	84	39.1	7.9	2305	0.6	300 (3.6)	10.0 (30/300)	
All PGD	84	39.6	8.6	2223	0.4	174 (2.1)	19.5 (34/174)	<0.01
<i>Two or more previously failed cycles</i>								
All control	54	39.9	10.0	2273	2.8	209 (3.9)	11.5 (24/209)	
All PGD	54	40.1	9.4	2518	3.6	98 (1.8)	14.3 (14/98)	NS
<i>Eight or more zygotes</i>								
All control	81	39.9	10.6	2649	1.4	311 (3.8)	9.3 (29/311)	
All PGD	81	39.9	11.6	2734	1.7	196 (2.4)	16.8 (33/196)	<0.025
<i>Less than eight zygotes</i>								
All control	57	38.9	6.0	1758	1.6	197 (3.5)	12.7 (25/197)	
All PGD	57	39.6	5.1	1762	1.6	76 (1.3)	19.7 (15/76)	NS
<i>Less than two previously failed cycles and 8 or more zygotes</i>								
All control	46	40.0	10.0	2765	0.5	171 (3.7)	8.8 (15/197)	
All PGD	46	40.0	11.7	2704	0.3	120 (2.6)	19.2 (23/120)	<0.025

^a versus ^b: $P < 0.001$.

NS = not significant.

aneuploid (79 were also mosaics, and two polyploid); 247 (22.8%) were mosaics or complex abnormal; 41 (3.8%) were polyploid and 31 (2.9%) were haploid.

Overall implantation

A total of 138 patients undergoing PGD for aneuploidy because of advanced maternal age were matched to 138 controls according to maternal age, number of 2PN zygotes, number of previous IVF cycles, day of retrieval and serum concentrations of oestradiol. All the patients that underwent PGD did so only for one cycle. As shown in **Table 1** (total results), the parameters for which the controls and PGD cycles were matched had similar values.

Implantation rate was measured as fetal heartbeats (FHB) per embryo replaced. PGD cycles and controls had overall implantation rates of 17.6% (48/272) and 10.6% (54/508), respectively. The same difference was observed when implantation was calculated as number of sacs per embryos replaced, with 20.6% (56/272) implantation on the PGD group and 13% (66/508) in the control group. Further analysis of the data was performed, considering implantation as FHB per embryo replaced.

When the statistics (FHB/embryo replaced) were back-transformed onto the scale of proportions, the average implantation rate for the PGD group was significantly higher

(0.180 ± 0.029) than for the control group (0.108 ± 0.016) ($P < 0.05$).

Implantation according to number of previous IVF cycles

PGD patients were subdivided into two groups according to the number of previous IVF cycles; one of women with less than two previous IVF cycles (i.e. none or one previous); the other of women with two or more previous IVF cycles.

As shown in **Table 1**, the implantation rate of the PGD group with fewer than two previous cycles (19.5% or 34/174) was significantly higher ($P < 0.01$) than their respective controls (10% or 30/300). However, the group with ≥ 2 previous cycles showed an implantation rate (14.3%, 14/98) similar to their respective controls (11.5% or 24/209) and with the PGD group having less fetal hearts than the control group. Thus, patients with many previous cycles, usually RIF patients, did not benefit from PGD of aneuploidy.

The proportion of presumptively chromosomally normal embryos in the PGD subgroup with ≥ 2 previous cycles was 31%, similar to the 33.4% found in PGD patients with less than two previous cycles, thus indicating that the poor implantation rate in the first PGD group was not caused by an excess of chromosomally abnormal embryos.

Implantation according to number of embryos available

While implantation probably depends on indication, pregnancy also depends on the number of embryos replaced, which is related to the number of embryos available. **Table 1** shows the implantation and pregnancy rates of all PGD cases, and their respective controls, according to the number of 2PN zygotes obtained.

As shown in **Table 1**, implantation was significantly higher ($P < 0.025$) in the PGD group, with eight or more zygotes (16.8% or 33/196), than in its respective control groups (9.3% or 29/311). In contrast, in the group with fewer than eight zygotes, the differences in implantation between the PGD patients (19.7% or 15/76) and controls (12.7% or 25/197) were not statistically significant; but more importantly, there were fewer FHB in the PGD group than in the control group, indicating that this group of patients may not benefit from PGD.

Thus, the patients that most benefited from PGD seem to be those with less than two previous IVF cycles and with eight or more zygotes. These patients showed an implantation rate of 19.2% (or 23/120) compared with 8.8% or (15/171) in their respective controls ($P < 0.025$) (**Table 1**).

Discussion

Two previous studies with controls have evaluated implantation after PGD of aneuploidy (Gianaroli *et al.*, 1999a; Munné *et al.*, 1999). Both reviewed embryo replacement on day 3 of development. Munné *et al.* (1999) used only five to six probes (XY, 13, 18, 21 with or without 16) and found no increase in implantation; however, they reported a significant increase in take-home baby rates. Gianaroli *et al.* (1999a) targeted eight chromosome pairs (XY, 13, 14, 15, 16, 18, 21, 22) and detected a significant increase in implantation after PGD.

The main goal of this study was to identify sub-groups of infertile couples, where the female partner is 35 years and older, that may benefit from PGD. The present results indicate that not all patients benefit from PGD of aneuploidy. As indicated in **Table 1**, patients with ≥ 2 previous cycles, which are mostly RIF patients at this centre, did not benefit from PGD, and their poor results were not influenced by a lack of chromosomally normal embryos for replacement. Two other groups have performed PGD on RIF patients. In one of these studies, Gianaroli *et al.* (1999a) found high rates of chromosome abnormalities and no increase in implantation when compared with a control group. The other study (Kahraman *et al.*, 2000) compared a group of young patients undergoing PGD for RIF (average age 30.3) to another group of PGD patients with the indication of advanced maternal age (average age 37.9), and while they found slightly higher rates of chromosome abnormalities in the younger group than in the older, the groups had similar implantation rates. Thus, these two previous studies appear to agree with the present results regarding the lack of a clear beneficial effect of PGD on RIF patients. There may be several reasons for this lack of success. Firstly, the chromosomes studied may not be the appropriate ones. A recent article by Wilton *et al.* (2003) compares the

chromosome abnormalities found in RIF embryos when these were analysed either by FISH or by CGH. They found that FISH only detected 69% of the abnormalities that CGH can detect. Secondly, these patients may have problems other than chromosomally abnormal embryos. For instance, limited experience with cytoplasmic transfer for RIF patients showed higher implantation and pregnancy (Brenner *et al.*, 2000). Thirdly, several reports indicate these patients may have high rates of structural chromosome abnormalities (Gekas *et al.*, 2001; Razieli *et al.*, 2002), which will not be detected by PGD of aneuploidy.

The present results suggest similar (at least not higher) chromosome abnormalities in the RIF group when compared with non-RIF patients; while the other studies reported higher chromosomal abnormality rates for RIF patients. The reason for such differences may be related to the poorly defined indication of 'repeated IVF failure'. It should be borne in mind that implantation can differ greatly from one centre to another, so some patients with repeated IVF cycles may have very different reasons for failure. Another reason could be differences in maternal age. The group of RIF patients had an average maternal age of 40 years, compared with 30 in the Gianaroli study (1999a), so the causes of RIF could have been different. Finally, the control group was matched not strictly by previous failed cycles but by total number of previous cycles.

While overall, patients with one or no previous IVF cycles had double the implantation rate compared with controls, the total number of embryos implanted was not statistically different. This is because an average of 2.1 embryos were replaced in the PGD group compared with 3.6 in the controls. In countries and centres with rules allowing the replacement of only two embryos (i.e. Sweden, UK) both implantation and pregnancy could be expected to be much higher in the PGD group than the control group.

A group that did not benefit from PGD were patients with fewer than eight zygotes. A patient with seven or fewer dipronucleated zygotes will produce on average three or four good quality embryos for replacement, and therefore, for an average maternal age of 39 years (control group), about 3.5 embryos would be replaced in this centre. So where all good quality embryos will be selected for replacement in the control group, an equal or smaller number would be replaced in the PGD group, because some of them will be chromosomally abnormal. The result would be similar implantation rates, and because of the damage caused by the biopsy and the error rate of the technique, probably lower total number of embryos implanted.

Patients not falling within the above criteria as candidates for improved implantation through PGD could still benefit by a lower chance of conceiving trisomic offspring. As reported by the ESHRE PGD Consortium (2002), about 65% of patients undergoing PGD of aneuploidy do so not only to increase their chances of conception but also to avoid having to terminate an affected pregnancy.

The other objective of the present study was to evaluate implantation after PGD and day 4 replacements with patients matched to controls prior to pregnancy testing. Delaying the

replacement by 1 day has the advantage of allowing extra time for (i) the possible re-biopsy of another cell from embryos with monosomies, or no results (Gianaroli *et al.*, 1999b); (ii) applying a third hybridization in order to analyse other chromosomes, or to reanalyse one or several of the previously analysed ones with a different probe in order to confirm the chromosome count (Magli *et al.*, 2001); or (iii), simply to allow extra time to perform PGD analyses that require more time (Grifo *et al.*, 1998). Previous reports have shown that pregnancies can be achieved after embryo biopsy and day 4 replacement (Grifo *et al.*, 1998; Gianaroli *et al.*, 1999b; ESHRE Consortium, 2002); and that implantation results were not affected (Gianaroli *et al.*, 1999b). The present results not only confirm that PGD of aneuploidy and replacement on day 4 can achieve pregnancies, but also that it can improve implantation rate.

The false positive error rate in the present study was 12%, higher than in a prior study in which nine probes were also used (Gianaroli *et al.*, 1999a). The reason for such increase is unknown, but an unusually high number of errors were concentrated in a 6-month period, indicating either a machine malfunction or unsuitable reagents. Because several changes were made simultaneously to solve the problem, no clear cause was identified.

In summary, patients that benefited from PGD were those either with less than two previous IVF cycles, or patients with eight or more zygotes. The combination of these two characteristics produced the best results, with more than double implantation rate in the PGD group (19.2%) than the respective controls (8.8%) (Table 1). However, if replacements are limited to two embryos, it should benefit most IVF patients of advanced maternal age.

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