Original Article
Clinical value of transferring blastocysts derived from 0PN/1PN or grade III embryos

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Abstract: The aim of this study was to characterize blastocyst formation from Day 1 abnormal pronuclear (0PN/1PN) embryos or Day 3 poor-quality (2PN grade III) embryos and establish the clinical outcomes following transfer of these embryos. We retrospectively analyzed blastocyst formation from Day 3 cleaved embryos derived from 0PN (group A), 1PN (group B), 2PN grade III (group C) and 2PN grade I/II (group D) following ART (assisted reproductive technology) cycles. To establish embryo transfer outcomes, clinical data associated with the transfer of frozen-thawed useable blastocysts from the four groups were analyzed. Results showed that blastocyst formation rate, useable blastocyst formation rate and high-quality blastocyst formation rate were significantly lower in group A, B and C compared to group D (*P* < 0.001). Useable blastocyst formation rate and high-quality blastocyst formation rate were significantly higher in group A relative to group C (*P* = 0.001). Differences in clinical pregnancy rate, embryo implantation rate, ongoing pregnancy rate and abortion rate among the four groups were not significantly different (*P* > 0.05). No defects, such as malformations were observed in the newborns. In conclusion, even though 0PN/1PN or grade III embryos have significantly lower development potential, near-normal clinical outcomes may be achieved when they are cultured to the useable blastocyst stage, improving chances of success.

Keywords: Blastocyst formation, abnormal pronuclear, poor-quality embryo, clinical pregnancy

Introduction

Since the birth of the first *in vitro* fertilization and embryo-transfer (IVF-ET) baby in 1978 [1], assisted reproductive technology (ART) has helped millions of couples conceive. However, many ART-associated factors require further research. For example, the first day after insemination (day 1) may result in various types of abnormal embryos, including non-pronuclear (0PN), mono-pronuclear (1PN), and multi-pronuclear (MPN) embryo. By day 3, poor-quality embryos with high fragmentation, few and/or asymmetry blastomeres may occur.

Abnormal pronuclear or poor-quality embryos are associated with low development potential, high chromosome abnormality, and high abortion rates [2-8]. According to the European society of human reproduction and embryology (ESHRE) guidelines on good practice in IVF lab-oratories, abnormal embryos are unsuitable for clinical use [9]. Thus, many reproductive centers discard abnormal or poor-quality embryos [10-13]. However, at some centers, the transfer of non-pronuclear [14, 15], mono-pronuclear [16, 17] or poor-quality [18] embryos has resulted in healthy infants. Additionally, sometimes all embryos produced by IVF-ET may be abnormal (non-2PN) or in poor-quality. How to handle such scenarios is a significant challenge for embryologists and clinicians.

Here, we retrospectively evaluated blastocyst formation and the clinical outcomes of transferring 0PN, 1PN or grade III (2PN) embryos during frozen-thawed cycles. The study assessed patients who underwent ART at the reproductive medical center of the second affiliated hospital of Zhejiang University between April 2017 and March 2020.

Materials and methods

Study design

This retrospective study evaluated 578 cases that underwent conventional *in vitro* fertilization (cIVF) and blastocyst culture at the reproductive medical center of the Second Affiliated Hospital of Zhejiang University between April 2017 and March 2020. In these cases, one or two grade I/II cleaved embryos were frozen or transferred first on day 3 to ensure cycle oocyte utilization. With the patients' consent, the remaining cleaved 0PN, 1PN or 2PN embryos were cultured to the blastocyst stage. Embryos of useable blastocyst standards were then frozen and transferred. On day 3, any non-cleaved or MPN embryos were excluded from further blastocyst culture. Cases resulting non-viable blastocysts after thawing, and abnormal uterine cavity factors such as uterine malformations, uterine fibroids and adenomyosis or endometriosis, were excluded for blastocyst transplantation. Cleaved embryos or high-grade blastocysts derived from 2PN zygotes were transferred first, and any useable blastocysts derived from 0PN or 1PN zygotes were subsequently transferred until there were no embryos available for transfer due to safety concerns. Ethical approval for this study was approved by the ethics committee of the Second Affiliated Hospital of Zhejiang University (reference no. 20170209). All participants provided written informed consent.

Ovarian stimulation

The antagonist protocol, long agonist protocol or short agonist protocol was used for ovary stimulation depending on the patient's age, anti-Müllerian hormone (AMH) level, basal follicle number and other indicators [19]. After at least three follicles attained > 18 mm in diameter, ovulation was induced by administering 6500~8000 IU of hCG (human chorionic gonadotrophin) (Merck Serono), and oocytes were retrieved by an ultrasound-guided method at 36-37 h after hCG administration.

Gamete or embryo manipulation in vitro

Oocytes were retrieved and cultured at 37°C, 6% CO_2, in a humidified incubator for 3-4 hours. Spermatozoa were collected by density gradient centrifugation or swim-up technique. Approximately 200,000 motile sperm cells/ml were used for conventional IVF. The day of insemination was recorded as Day 0. Pronuclear morphology and number were evaluated under an inverted microscope at 17±1 h [20] after insemination and embryos classified as 2PN, 1PN, 0PN, and MPN. MPN embryos were discarded. 2PN, 0PN, and 1PN embryos were separately collected in individual 30 μL droplets of cleavage-stage culture medium (G-1) (Vitrolife) on 60 mm Petri dishes (Falcon). The embryos were cultured at 37°C, 6% CO_2, under humidified conditions. Embryo grading was done on Day 3. After the transfer or freezing of one or two grade I/II embryos, the remaining cleaved embryos were transferred into individual 30 μL microdrops of blastocyst-stage culture medium (G-2) (Vitrolife). They were then cultured at 5% O_2, 6% CO_2 and 89% N_2 in a hypoxic incubator. Blastocyst grading was done on Day 5/6, and those meeting useable standards were shrunk by laser and frozen using the Cryotop methodology [21].

Cleavage-stage embryos and blastocysts grading

Cleavage-stage embryo grading was done according to universal standardization guidelines of the society for assisted reproductive technology (SART) [22]. Embryos with 7-9 blastomeres, perfect symmetry and 0-10% fragmentation were categorized as grade I (good). Embryos with more than 5 blastomeres, moderate symmetry, 11-25% fragmentation were categorized as grade II (fair). Embryos with < 6 blastomeres, asymmetry and > 25% fragmentation were categorized as grade III (poor). Blastocysts were evaluated as described previously [23]. Briefly, expansion and hatching status was graded from stage 1 to 6, while the inner cell mass (ICM) quality and the trophectoderm (TE) quality were both graded as A, B and C. Embryos that reached the stage 2 were counted as blastocyst formation. Blastocysts that reached 3BC/3CB or higher quality were defined as useable blastocysts, and 3BB or better were defined as high-quality blastocyst.

Frozen-thawed blastocyst transfer

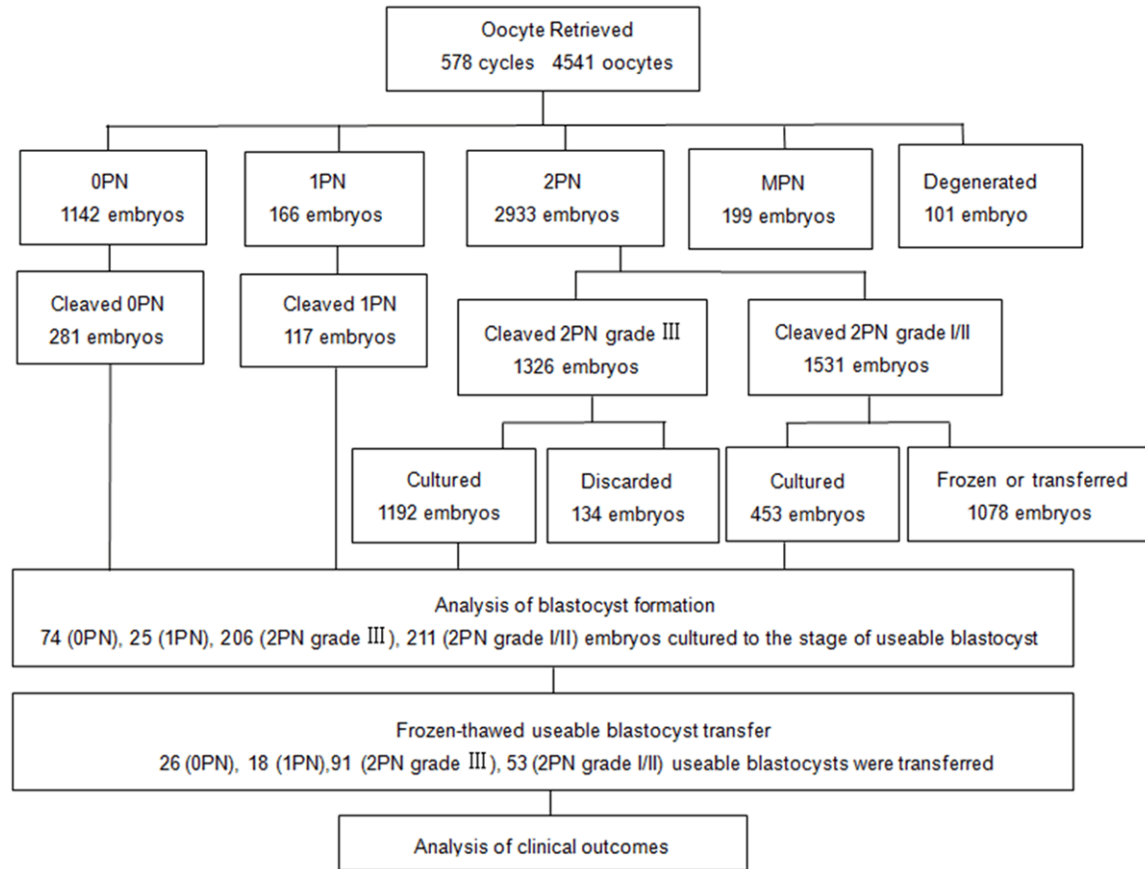Identical endometrial preparation protocols were used during artificial hormone replace-

Figure 1. Flow chart of embryo development in this study.

ment cycles for frozen-thawed blastocyst transfer [24]. After the blastocysts were trawled [21], they were laser-assisted hatched and incubated *in vitro* for 3-4 h before they were transferred [20]. Before transfer of blastocysts derived from 0PN and 1PN, patients were fully informed of the risks, and advised to undergo follow-up amniotic fluid chromosome inspection or non-invasive prenatal DNA testing (NIPT). All participants gave written informed consent. Routine luteal support was given after transplantation. Clinical pregnancy was established 28 days after embryo transfer by ultrasound visualization of the gestational sac. Ongoing pregnancy was defined as the establishment of a viable pregnancy after the 12th gestational week. Abortion was defined as spontaneous pregnancy termination before 12 gestational weeks. Implantation rate was established by dividing the number of gestational sacs by the number of transferred embryos. Live birth rate was calculated by dividing the total of live births by the total number of cycles.

Statistical analysis

Statistical analyses were performed using SPSS 22.0 (SPSS Inc.). Data are presented as means ± SD. Continuous variables were compared using student's t-test. Categorical variables were evaluated using chi-square test or the Fisher exact test. $P < 0.05$ was considered statistically significant.

Results

A flowchart of this study is shown in **Figure 1**. 578 cycles and 4541 oocytes from conventional IVF done between April 2017 and March 2020 were analyzed. These cases included at least one cleaved day 3 embryos (0PN, 1PN or 2PN) that underwent further blastocyst culture. The incidence rates of 0PN, 1PN, and 2PN were 25.1% (1142/4541), 3.7% (166/4541), and 64.6% (2933/4541), respectively. The cleavage rate of 0PN was 24.6% (281/1142). These rates are consistent with previous studies [25, 26].

Table 1. Comparison of blastocyst formation among four groups [% (n/N)]

Parameter	group A	group B	group C	group D	p-value
	0PN	1PN	2PN	2PN	
	n = 281	n = 117	grade III n = 1192	grade/II n = 453	
blastocyst formation (%)	37.4% (105/281)[a]	32.5% (38/117)	34.9% (416/1192)[a]	63.8% (289/453)	< 0.001
useable blastocyst formation (%)	26.3% (74/281)[a,b]	21.4% (25/117)[a]	17.3% (206/1192)[a]	46.6% (211/453)	< 0.001
high-quality blastocyst formation (%)	14.2% (40/281)[a,b]	9.4% (11/117)[a]	7.7% (92/1192)[a]	27.2% (123/453)	< 0.001

Note: 0PN: cleaved embryos derived from 0PN, 1PN: cleaved embryos derived from 1PN, 2PN grade III: cleaved embryos derived from 2PN on Day 3 with grading III, 2PN gradeI/II: cleaved embryos derived from 2PN on Day 3 with grading I/II. Blastocyst formation = embryos reach to the stage 2 of blastocyst [23], useable blastocyst = blastocysts reach to 3BC/3CB or better quality, high-quality blastocyst = blastocysts reach to 3BB or better quality. [a]means $P < 0.001$ compared with group D, [b]means $P = 0.001$ compared with group C.

Table 2. Comparison of characteristics of patients who underwent transferring blastocyst among four groups ($\overline{x} \pm sd$)

	group A	group B	group C	group D	p-value
	0PN	1PN	2PN grade I	2PN grade/II	
Total no. of cycles	23	17	69	37	
Age (year)	30.4±2.9	28.8±2.3	30.6±4.1	31.3±3.6	0.141
BMI (kg/m^2)	21.8±3.1	20.8±1.7	21.0±2.5	21.4±2.3	0.482
serum AMH level (ng/ml)	2.9±1.2	2.9±1.1	3.2±1.3	3.1±1.1	0.672
duration of infertility (year)	3.3±2.6	3.4±2.3	3.1±2.7	3.5±2.1	0.878
thickness of endometrium (mm)	9.5±1.8	10.6±2.1	10.2±1.9	10.1±1.6	0.276
the number of blastocysts transferred (n)	1.1±0.7	1.1±0.2	1.3±0.5	1.4±0.5	0.077

Note: 0PN: cleaved embryos derived from 0PN, 1PN: cleaved embryos derived from 1PN, 2PN grade III: cleaved embryos derived from 2PN on Day 3 with grading III, 2PN gradeI/II: cleaved embryos derived from 2PN on Day 3 with grading I/II, BMI: Body Mass Index, AMH: Anti-Müllerian Hormone.

On day 3, cleaved 2PN embryos were divided into 2 groups, grade III (group C) and grade I/II (group D). Blastocyst formation rate, useable blastocyst formation rate and high-quality blastocyst formation rate for 0PN (group A), 1PN (group B), and 2PN grade III (group C) were significantly lower than those of 2PN grade I/II (group D) ($P < 0.001$, **Table 1**). No significant differences in blastocyst formation rate were observed between group A (37.4%), B (32.5%) and C (34.9%) ($P > 0.05$). However, in group A, the useable blastocyst formation and high-quality blastocyst formation rates (26.3% and 14.2%, respectively) were significantly higher than those of group C (17.3% and 7.7%) ($P = 0.001$).

Comparison of clinical features of patients who underwent 0PN, 1PN, 2PN grade III and 2PN grade I/II blastocyst transfer revealed no significant differences across the 4 groups in terms of age ($P = 0.141$), BMI ($P = 0.482$), serum AMH level ($P = 0.672$), duration of infertility ($P = 0.878$), endometrial thickness ($P = 0.276$) and average number of transferred blastocysts ($P = 0.077$, **Table 2**).

A total of 188 useable blastocysts from 146 cycles were used for transplantation after thawing. However, 0PN or 1PN derived blastocysts were transferred only when 2PN derived embryos were lacking. The proportion of high-quality blastocysts transferred, as well as clinical outcomes, including clinical pregnancy rate ($P = 0.928$), embryo implantation rate ($P = 0.94$), ongoing pregnancy rate ($P = 0.617$) and abortion rate ($P = 0.353$) were not significantly different across the 4 groups (**Table 3**). By the end of March 2020, a total of 46 newborns (6 from 0PN, 5 from 1PN, 19 from 2PN grade III, and 16 from 2PN gradeI/II) were delivered without any defects, such as malformations found. 28 cycles (5 from 0PN, 3 from 1PN, 13 from 2PN grade III and 7 from 2PN gradeI/II) were at > 12 gestational weeks. The 5 0PN and 3 1PN cycles in gestation were subjected to amniotic fluid chromosome inspection or non-invasive prenatal DNA testing (NIPT). For 7 cases (one missing), the risk of chromosomal abnormalities was minimal. Two abortions were recorded in the 0PN group, one abortion each in the 1PN group and 2PN grade I/II, and eight abortions in 2PN grade III

Table 3. Comparison of clinical outcomes among four groups after transferring blastocysts [% (n/N)]

	group A	group B	group C	group D	p-value
	0PN	1PN	2PN grade III	2PN grade I/II	
No. of transferred cycles (n)	23	17	69	37	
No. of transferred useable blastocysts (n)	26	18	91	53	
proportion of high-quality blastocysts (%)	61.5% (16/26)	55.6 (10/18)	52.7% (48/91)	64.2% (34/53)	0.572
clinical pregnancy rate (%)	56.5% (13/23)	52.9% (9/17)	58.0% (40/69)	62.2% (23/37)	0.928
embryo implantation rate (%)	53.9% (14/26)	50.00% (9/18)	48.4% (44/91)	52.8% (28/53)	0.940
ongoing pregnancy rate (%)	47.8% (11/23)	47.0% (8/17)	46.4% (32/69)	59.5% (22/37)	0.617
abortion rate (%)	8.7% (2/23)	5.9% (1/17)	11.6% (8/69)	2.7% (1/37)	0.353

Note: Useable blastocysts were defined as those with quality reaching 3BC/3CB or higher. High-quality blastocysts were defined as those with quality reaching 3BB or higher. Clinical pregnancy was determined 28 days after embryo transfer by ultrasound visualization of the gestational sac. The embryo implantation rate was determined as the number of gestational sacs divided by the number of embryos transferred. Ongoing pregnancy was defined as viable pregnancy beyond 12 gestational weeks. Abortion was defined as pregnancy terminated spontaneously before 12 weeks of gestation.

group. All spontaneous abortions occurred in the first trimester (within 9 weeks of transplantation) and analysis of the abortion tissue revealed chromosomal abnormalities.

Discussion

Zygotes exhibiting 2 pronuclear and 2 polar bodies 17+1 h post-insemination signified normal fertilization [20]. Abnormal fertilization, including non-pronuclear (0PN), mono-pronuclear (1PN), and multi-pronuclear (MPN) are often unavoidable [27, 28]. Cytogenetic analysis has previously shown that > 90% of MPN embryos are polyploid or chimera and therefore abnormal spindles may cause uncontrollable cell division [25]. Therefore, MPN embryos derived in this study were discarded. The 0PN/1PN embryos are considered to be at high risk of chromosomal abnormalities, which may result in poor implantation or pregnancy rates [13, 29]. 0PN/1PN embryos are often discarded or utilized by limited number of reproductive centers in research programs [10-13]. However, fluorescence in situ hybridization (FISH) studies have shown that 57% of 0PN [30] and 48.7% of 1PN [31] embryos were actually diploid (normal). This may be due to various factors. For instance, while in most cases standardized pronuclear-stage checking is done at 17+1 h post-insemination, some pronuclear may disappear before 16 h post-insemination or occur past 18 h post-insemination [32-33]. Such embryos, without 2 visible pronuclear, were classified as 0PN/1PN. In other cases, the male and female pronuclear may be in a single pronuclear envelope [34] or may appear asynchronously [35], causing

some 2PN embryos to be identified as 1PN. In addition, the limited magnification power of the light microscope may cause 2 stacked pronuclear (2PN) to be mistaken for 1PN embryos. In this study, to offset for such errors, cleaved 0PN/1PN embryos were maintained for longer blastocyst culture to increase the chances of a successful pregnancy. Besides that pronuclear checking time was strictly controlled, an inverted microscope was used to minimize human error.

While SART (society for assisted reproductive technology) provides standardized guidelines as to what constitutes poor-quality embryo [22], there are no guidelines on what to do with such embryos. Poor-quality embryos are often characterized by high chromosomal abnormalities [36], higher abortion rates [37], and lower (< 10%) live birth rates [18, 38]. Therefore, most centers do not use them in ART [10, 11, 39, 40]. However, considering that blastocyst culture may eliminate some chromosomally abnormal embryos and that good-quality blastocysts could develop from poor-quality cleaved embryos [39, 41], cleaved poor-quality embryos were also cultured to the blastocyst stage in this study, with patient's written informed consent.

Here, relative to 2PN grade I/II embryos, cleaved 0PN or 1PN embryos had significantly lower blastocyst formation and useable blastocyst formation rates, which was consistent with previous studies [15-17, 42]. This indicates that abnormal pronuclear embryos with chromosomal abnormalities may have been present but most likely failed to progress to

the blastocyst stage. The blastocyst formation effect of poor-quality (2PN grade III) embryos was also much worse than that of 2PN grade I/II embryos, illustrating that embryo grading helped select embryos with the best development potential. Unlike in previous studies, when the 2PN embryos were divided into poor-quality and good-quality groups, we observed that useable blastocyst formation and high-quality blastocyst formation rates in 0PN/1PN embryos were higher than those in poor-quality (2PN grade III) embryos. This was most apparent in the 0PN vs. the 2PN grade III group (P = 0.001), suggesting that relative to poor-quality normally fertilized embryos, further culturing cleaved 0PN embryos may have better value. Wang [10] compared blastulation status in discarded embryos, and found that compared to the 0PN group, the blastulation rate was significantly higher in 1PN group. However, Yao [11] and Yin [42] reported the opposite finding. In this study, indicators of blastocyst culture in 0PN the group were slightly higher than those in the 1PN group, although the difference was not statistically significant. Therefore, further research is needed to confirm the developmental potential of embryos derived from 0PN/1PN to provide more reliable guidance for clinical applications.

Our comparison of clinical parameters among 0PN, 1PN, 2PN grade III and 2PN grade I/II groups did not show significant differences with regards to clinical pregnancy rate, embryo implantation rate, ongoing pregnancy rate or abortion rate. However, past retrospective studies found that pregnancy rates, embryo implantation rates or live birth rates of 0PN/1PN-derived or poor-quality cleavage-stage embryos were significantly lower than those of healthy 2PN-derived cleavage-stage embryos [15, 18, 29]. This difference in observed clinical results may result from the transfer of day 3 cleavage-stage embryos in earlier studies, as opposed to the transfer of day 5/6 blastocyst-stage embryos in our study. We observed that only useable blastocysts could be transferred, although the useable blastocyst formation rates in groups 0PN, 1PN, and 2PN gradeIII were much lower than those in group 2PN grade I/II (P < 0.001). Through blastocyst culture, 73.7% of the 0PN embryos, 78.6% of the 1PN embryos and 82.7% of the 2PN grade III embryos were excluded. The exclusion rate of 2PN grade I/II embryos was only 53.4%. After eliminating a large number of embryos with low developmental potential from the 0PN, 1PN, and 2PN grade III groups, blastocysts derived from abnormal pronuclear (0PN/1PN) or poor-quality (2PN grade III) embryos attained similar clinical outcomes relative to good-quality (2PN grade I/II) embryos. Through the comparative genome hybridization array (aCGH), Yin [42] showed that 0PN-derived blastocysts and 2PN-derived blastocysts had similar normal chromosomal rates (64.71% vs. 69.39%). Bradley [17] used CGH or next-generation sequencing (NGS) to show that adjusted abnormality rates of 1PN-derived blastocysts were comparable to 2PN-derived blastocysts (39.7% vs. 41.1%) in cIVF cycles. Liao [43] reported that the diploid rate of 1PN-derived blastocysts was significantly higher than that of cleavage-stage 1PN embryos (74.6% vs. 31.6%). These reports support the observation that blastocyst culture may be an efficient method of selecting "healthy" embryos for transfer. This might also explain why the abortion rates of 0PN/1PN-derived blastocysts were not significantly higher than those in 2PN-derived blastocysts, and why clinical pregnancy rates were not significantly lower in this study.

Here, no apparent defects were observed in the 46 delivered newborns and amniotic fluid chromosome inspection or NIPT testing results during ongoing pregnancy cycles were normal. Nonetheless, blastocysts derived from abnormal pronuclear or poor-quality embryos may harbor inherent pathological defects. Follow-up of these newborns' long-term health data and discussing related epigenetics issues are needed. In this study, we recorded 12 abortions. Currently, the most accurate method for selecting euploid embryos is preimplantation genetic diagnosis (PGD). We speculate that if molecular genetics technology was used widely, abortion could be avoided. PGD analysis requires highly experienced laboratory embryologists and is currently done in a limited number of qualified reproductive centers in China. We hope that through multilateral cooperation, more reproductive centers will get the opportunity to utilize pre-implantation molecular genetics technology, and an increasing number of patients will benefit.

Disclosure of conflict of interest

None.

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