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ASSISTED REPRODUCTION TECHNOLOGIES

Comparison of clinical outcomes between single and double vitrified-warmed blastocyst embryo transfer according to the day of vitrification

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Abstract
Purpose To compare the efficacy of single vitrified-warmed blastocyst embryo transfer (SVBT) versus double vitrified-warmed blastocyst embryo transfer (DVBT) according to the day of vitrification.
Methods This retrospective study included a total of 1,051 cycles in women less than 37 years of age with their autologous SVBT cryopreserved on day 5 (5d-SVBT, n=737) or day 6 (6d-SVBT, n=154) and DVBT on day 5 (5d-DVBT, n=129) or day 6 (6d-DVBT, n=31) from January 2009 to December 2011.
Results The clinical pregnancy rate (41.8 % vs. 48.1 %, p=0.184) and ongoing pregnancy rate (36.6 % vs. 45.0 %, p=0.072) were not significantly different between the 5d-SVBT group and the 5d-DVBT group. However, the clinical pregnancy (29.9 % vs. 58.1 %, p=0.003) and ongoing pregnancy rates (23.4 % vs. 51.6 %, p=0.001) were significantly lower in the 6d-SVBT group compared with those in the 6d-DVBT group. The implantation rate (42.2 % vs. 34.5 %, p=0.03) of the 5d-SVBT group was significantly higher than that of the 5d-DVBT group, while the implantation rate (29.9 % vs. 37.1 %, p=0.303) of the 6d-SVBT group was not statistically different compared with that in the 6d-DVBT group. The multiple pregnancy rates (1.0 % in the 5d-SVBT group vs. 38.7 % in the 5d-DVBT group, p<0.001 and 0 % in the 6d-SVBT group vs. 22.2 % in the 6d-DVBT group, p=0.001) were statistically significantly lower in the SVBT group compared with those in the DVBT group regardless of the day of vitrification.
Conclusions This study showed that the 5d-SVBT resulted in comparable clinical outcomes compared to the 5d-DVBT while the 6d-SVBT yielded significantly lower clinical outcomes compared to the 6d-DVBT.

Keywords Single embryo transfer · Single vitrified-warmed blastocyst embryo transfer · Double vitrified-warmed blastocyst embryo transfer · Vitrification · Multiple pregnancies

Capsule Transferring single vitrified-warmed blastocyst developed on day 5 could result in acceptable pregnancy rates while reducing the risk of multiple pregnancies.

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Introduction

Embryo freezing technology has been markedly progressing since the first human pregnancy from human 8cell-stage frozen-thawed embryo transfer (FET) in 1983 [27]. Now, it is one of the indispensable core technologies in human in vitro fertilization-embryo transfer (IVF-ET) programs. Cryopreservation of embryos was mainly performed on pronucleus-stage or cleavage-stage embryos in the past. However, recently, a great amount of research on blastocyst-stage freezing has been performed by increasing the incidence of blastocyst formation through improvement of the culture medium and culture conditions. It has been known that blastocyst-stage freezing has a higher viability than early cleavage-stage embryo freezing and that frozen-thawed blastocyst-stage embryo transfer could improve implantation and pregnancy rates because it is physiologically suited to the environment of the uterus [24, 32].

After the introduction of elective single embryo transfer (eSET), many countries have performed eSET in order to reduce the risk of multiple pregnancies regarded as a complication of IVF-ET. Although elective single cleavage-stage embryo transfer (eSCET) causes significantly lower pregnancy and delivery rates than double cleavage-stage embryo transfer (DCET), eSCET combined with single frozen-thawed embryo transfer shows similar cumulative pregnancy and delivery rates with those of DCET while maintaining a significantly lower multiple pregnancy rate [26]. On the other hand, the clinical outcomes of single blastocyst embryo transfer (SBET) were similar to those of double blastocyst embryo transfer (DBET) in fresh cycles [19]. If single frozen-thawed blastocyst transfer is combined with SBET, it will be able to produce a significantly higher cumulative clinical outcome than DBET. The above studies indicate that FET cycle would be important in the eSET program in such a way that patients could escape from anxiety about single embryo transfer due to the existence of frozen embryos and reduce the economic burden of controlled ovarian hyperstimulation.

Patient conditions (age, embryo quality, endometrial thickness, etc.) have been established for single embryo transfer in fresh cycles. However, conditions have not been yet established in frozen-thawed embryo transfer. According to the current status of assisted reproductive technology in Korea, 2008, two to four frozen-thawed embryos are usually transferred [2]. The results showed a twin delivery rate of 31.2 % and a triplet delivery rate of 0.6 %. In order to reduce multiple birth rates after FET, reducing the number of embryos transferred will be the most effective approach. When the clinical outcomes of the SBET and DBET groups were compared with respect to the FET cycle, although the pregnancy and delivery rates were significantly lower after SBET as compared with DBET, they reported that single frozen-thawed blastocyst transfer resulted in acceptable results [3]. However, their study was limited in that embryo freezing was conducted on day 5 or 6 but the results were not separated by the day of cryopreservation. Of course, there was a previous study which reported that live birth rates were similar between two groups regardless of the day of cryopreservation when transferring high-grade blastocysts frozen on day 5 or day 6 [6]. On the other hand, it has been reported that the pregnancy rate of blastocysts transferred on day 6 was nearly two times lower than that of blastocysts transferred on day 5 in the fresh cycle [23].

The aim of this retrospective study was therefore to compare the efficacy of single vitrified-warmed blastocyst embryo transfer (SVBT) versus double vitrified-warmed blastocyst embryo transfer (DVBT) according to the day of vitrification in women less than 37 years of age who received a good quality vitrified-warmed blastocyst.

Materials and methods

Patients studied

This study was a retrospective analysis and approved by the Maria Fertility Hospital Institutional Review Board. All patients gave written informed consent for vitrified-warmed blasotycst embryo transfer. A total of 1,051 cycles with autologous SVBT cryopreserved on day 5 (5d-SVBT, n=737) or day 6 (6d-SVBT, n=154) and DVBT on day 5 (5d-DVBT, n=129) or day 6 (6d-DVBT, n=31) at Daegu maria fertility clinic from January 2009 to December 2011 were included in the study. All the participants were less than 37 years old at the time of cryopreservation and were transferred a good quality vitrified-warmed blastocyst.

Ovarian stimulation and blastocyst culture

Ovarian stimulation was performed using the gonadotropin-releasing hormone (GnRH) agonist long protocol or GnRH antagonist protocol and recombinant FSH (Gonal-F, Merck Serono, Germany). 10,000 IU of hCG (IVF-C, LG Life Science, Daejon, Korea) was injected when more than two follicles over 17–18 mm in diameter were visible on ultrasonography. Oocyte retrieval was undertaken by transvaginal ultrasound-guided aspiration after 36 h of hCG administration. In vitro fertilization was induced using conventional insemination or intracytoplasmic sperm injection (ICSI). Embryos having two pronuclei were co-cultured with autologous cumulus cells (ACC) in 20 μl of MRC#D16 medium (YS medium [33], Biosupply Co., Korea) containing autologous follicular fluid (AFF). 10 % AFF was added to the medium during the first 48 h, and then 20 % AFF was added until day 5 or 6. Culture medium was exchanged for pre-equilibrated fresh medium every morning.

Protocol for vitrification and warming of blastocyst

After embryo transfer, regardless of the embryo transfer date, surplus embryos were co-cultured to day 5 or 6 and the embryos that reached the blastocyst-stage were cryopreserved. The method of blastocyst vitrification was the same as described in a previous study [24]. Briefly, embryos were artificially shrunken for dehydration of blastocoele using two 29-gauge needles. After complete shrinkage of the blastocoel, the shrunken blastocysts were incubated for 5 min in MRC#CBS (Biosupply Co., Korea), and then vitrification of the blastocysts was carried out after exposure to equilibration solution for 1.5 min. The equilibration solution was MRC#CBS containing 20 % (v/v) ethylene glycol (EG), while the vitrification solution was MRC#CBS supplemented with 40 % (v/v) EG, 18 % (w/v) ficoll, and 0.3 M sucrose. The equilibrated

embryos were transferred into the vitrification solution, loaded onto an EM-grid, and directly plunged in liquid nitrogen within 30 s. Finally, the EM grid was moved in a cryovial previously submerged under liquid nitrogen. The cryovial was stored in liquid nitrogen.

Blastocyst warming was conducted by two-step dilution with sucrose on day 4 after ovulation. The EM-grid having a blastocyst was removed from the cryovial and transferred to a 100 ul drop of 0.5 mol/l sucrose for 5 min. Thereafter, the blastocysts were transferred sequentially to 100 ul drops in MRC#CBS supplemented with 0.25 mol/l and 0 mol/l of sucrose each for 5 min at room temperature. The warmed blastocysts were rinsed several times in culture medium and then cultured with MRC#D46 medium (Biosupply Co.) until embryo transfer.

Vitrified-warmed blastocyst-stage embryo transfer

The blastocysts that had re-expanded 18~20 h after warming were judged to have survived. The quality of the warmed blastocysts was assessed before embryo transfer according to the criteria of Gardner and Schoolcraft [8]. A "good" quality blastocyst at the time of FET was defined as having a well defined expanded blastocoele cavity (≥grade 4), a well defined inner cell mass and trophectoderm (≥BB). One or two surviving vitrified-warmed blastocysts were transferred into the uterine cavity on day 5 after ovulation in spontaneous cycles for patients with regular ovulatory cycles or in ovulation induction cycles (clomiphene citrate, 50~150 mg daily for 5 days) for patients with irregular menstrual cycles.

Cycle outcome measures

Serum β-hCG concentration was measured 9 days after embryo transfer to verify pregnancy. Clinical pregnancy was judged by observation of the gestational sac (G-sac) on vaginal ultrasonography after 6–7 weeks of gestation. On-going pregnancy was judged by fetal cardiac activity after 12 weeks. Monozygotic twins were considered as two gestation sacs, and ectopic pregnancy was not counted as implantation or clinical pregnancy.

Statistical analysis

Statistical analysis was performed with SPSS 14.0 (SPSS Inc., Chicago, IL, USA) program, and the average value was expressed as mean ± standard deviation. For comparison of continuous variables, Student's *t*-test was used, and for comparison of non-continuous variables, the Chi-square test was used. *p*-values less than 0.05 were considered statistically significant.

Results

Of 1051 vitrified-warmed blastocyst embryo transfer cycles, SVBT was 891 cycles (737 cycles in the 5d-SVBT group and 154 cycles in the 6d-SVBT group) and DVBT was 160 cycles (129 cycles in the 5d-DVBT group and 31 cycles in the 6d-DVBT group). Maternal age (31.9±2.8 year vs. 31.7±2.8 year) and cause of infertility were not different between the SVBT group and the DVBT group. The percentage of cycles using ICSI to induce fertilization was 38.2 % in the SVBT group and 33.8 % in the DVBT group (p=0.289). The percentage of cycles vitrified on day 5 was similar in the two groups (82.7 % in the SVBT group and 80.6 % in the DVBT group). The survival rate was 96.6 % in the SVBT group and 97.8 % in the DVBT group (p=0.279). However, duration of cryopreservation (12.6±15.4 months vs. 17.1±18.6 months, p<0.001) was significantly shorter in the SVBT group than that in the DVBT group (Table 1).

The clinical outcomes between the two groups according to the day of vitrification are shown in Table 2. The clinical pregnancy rate (41.8 % vs. 48.1 %, p=0.184) and ongoing pregnancy rate (36.6 % vs. 45.0 %, p=0.072) of the 5d-SVBT group were lower than those of the 5d-DVBT group, but the differences were not statistically significant. However, the clinical pregnancy rate (29.9 % vs. 58.1 %, p=0.003) and ongoing pregnancy rate (23.4 % vs. 51.6 %, p=0.001) were significantly lower in the 6d-SVBT group than those in the 6d-DVBT group. The implantation rate (42.2 % vs. 34.5 %, p=0.03) of the 5d-SVBT group was significantly higher than that of the 5d-DVBT group, while the implantation rate (29.9 % vs. 37.1 %, p=0.303) was slightly lower in the 6d-SVBT group than that in the 6d-DVBT group, without reaching statistical significance. Multiple pregnancy rates (1.0 % in the 5d-SVBT group vs. 38.7 % in the 5d-dDVBT group, p<0.001 and 0 % in the 6d-SVBT group vs. 22.2 % in the 6d-DVBT group, p=0.001) were significantly lower in the SVBT group than those in the DVBT group regardless of the day of vitrification. The ectopic pregnancy rates were similar between the SVBT and DVBT groups regardless of the day of vitrification. The miscarriage rate was slightly higher in the SVBT group than that in the DVBT group, without reaching statistical significance.

Discussion

The most efficient way to reduce the risk of multiple pregnancies and to increase the birth of healthy babies is to transfer a single embryo with the highest implantation potential. Transferring blastocyst-stage embryos in fresh or frozen-thawed cycles would improve the likelihood of pregnancy because embryos are selected during extended culture

Table 1 Patient demographic characteristics of SVBT and DVBT groups

	SVBT (n=891)	DVBT (n=160)	p value
Maternal age (yrs)	31.9±2.8	31.7±2.8	0.507
Duration of cryopreservation (months)	12.6±15.4	17.1±18.6	<0.001
Etiology of infertility			
Female factor	491 (55.1)	88 (55.0)	0.980
Male factor	157 (17.6)	26 (16.3)	0.674
Mixed	44 (4.9)	8 (5.0)	0.974
Unknown	199 (22.3)	38 (23.8)	0.693
No. of ICSI attempts	340 (38.2)	54 (33.8)	0.289
No. of cycles cryopreserved on day 5	737 (82.7)	129 (80.6)	0.523
No. of blastocysts survived	1050/1087 (96.6)	347/355 (97.8)	0.279

Values are presented as mean ± SD or number (%)

SVBT single vitrified-warmed blastocyst embryo transfer; *DVBT* double vitrified-warmed blastocyst embryo transfer; *ICSI* intracytoplasmic sperm injection

[7, 28]. Also, unlike eSCET results, some studies have shown that SBET tends to show a significantly lower risk of multiple pregnancies without reducing the overall pregnancy rate compared to DBET [19, 31]. On the other hand, Berin et al. [3] reported that when the clinical outcomes of SBET and DBET in frozen-thawed cycle were compared, the clinical pregnancy rate and live birth rate of SBET were significantly lower than those of DBET. However, transferring two frozen-thawed blastocysts resulted in increased risk of twin pregnancy. Although our retrospective study has a disadvantage of including a small study population in the 6d-DVBT group, the results showed that patients who were <37 years of age had a lower clinical pregnancy rate (41.8 % vs. 48.1 %, p=0.184) and a lower ongoing pregnancy rate (36.6 % vs. 45.0 %, p=0.072) in the 5d-SVBT group than those in the 5d-DVBT group, without reaching statistical significance. However, the clinical pregnancy rate (29.9 % vs. 58.1 %, p=0.003) and ongoing pregnancy rate (23.4 % vs. 51.6 %, p=0.001) of the 6d-SVBT group were significantly lower than those in the 6d-DVBT group (Table 2). Transferring two vitrified-warmed blastocysts resulted in a high multiple pregnancy rate in women with good prognosis (age, blastocyst quality) regardless of the day of vitrification in our study.

In the present study, a significantly shorter duration of cryopreservation was observed in the SVBT group compared with the DVBT group. The reason is due to the enforcement policy of eSET in our clinic. Patients less than 37 years of age undergoing their first or a second IVF-ET cycle have been routinely receiving an elective single embryo regardless of the embryo transfer date since August 2008. Similarly, we also follow the rule of transferring a single blastocyst to patients less than 37 years of age in the frozen-thawed cycle. However, blastocysts are cryopreserved two units per ampoule before eSET policy. After thawing, if these patients wanted to have two blastocysts transferred, they receive two. There were cycles that cryopreserved before August 2008. Those were 33 of 129 cycles in the 5d-DVBT group and 16 of 31 cycles in the 6d-DVBT group. The clinical pregnancy rate was 48.5 % (16/33) in the 5d-DVBT group, and 50.0 % (8/16) in the 6d-DVBT group. These results indicated that the results of the present study were not affected by the duration of cryopreservation.

In a conference presentation, our study group showed that the multiple pregnancy rate of DBET on day 5 was 52.6 % (30/57) in women less than 37 years of age [16]. According to the results of the present study of women less than 37 years of age, the percentage of multiple pregnancies was 38.7 % in the 5d-DVBT group. When compared fresh DBET and frozen-thawed DBET on day 5, there was no significant difference in multiple pregnancy rates between the 5d-DVBT group and the fresh DBET group. Further efforts are needed to reduce the

Table 2 Clinical outcomes of SVBT and DVBT groups according to the day of vitrification

	Day 5		p value	Day 6		p value
	SVBT (n=737)	DVBT (n=129)		SVBT (n=154)	DVBT (n=31)	
Clinical pregnancies	308 (41.8)	62 (48.1)	0.184	46 (29.9)	18 (58.1)	0.003
Ectopic pregnancies	7 (1.0)	1 (0.8)	0.848	3 (2.0)	0 (0.0)	0.433
Implantation	311 (42.2)	89 (34.5)	0.03	46 (29.9)	23 (37.1)	0.303
Multiple pregnancies	3 (1.0)	24 (38.7)	<0.001	0 (0.0)	4 (22.2)	0.001
Miscarriages	38 (12.3)	4 (6.5)	0.183	10 (21.7)	2 (11.1)	0.327
On-going pregnancies	270 (36.6)	58 (45.0)	0.072	36 (23.4)	16 (51.6)	0.001

The continuous variables are expressed as number (rate)

SVBT single vitrified-warmed blastocyst embryo transfer; *DVBT* double vitrified-warmed blastocyst embryo transfer

number of embryos transferred in order to decrease the incidence of multiple pregnancies in frozen-thawed blastocyst embryo transfer cycles.

Contradictory results have been reported regarding blastocyst-stage embryo transfer leading to increased delivery rate of monozygotic twins compared to cleavage-stage embryo transfer [4, 21]. Kang et al. [14] previously reported a 1.4 % monozygotic twin pregnancy rate in the eSBET group of women less than 37 years of age. In the present study with similar patient status, the percentage of monozygotic twin pregnancies after 5d-SVBT is 1.0 % and is in accordance with our previous study. Also, this rate is similar to the result of Guerif et al. [12] in which they reported it to be 1.6 % of the monozygotic twin rate in the eSCET group. The data presented here demonstrate that the SVBT does not increase the rate of monozygotic twin pregnancies compared to the fresh cleavage-stage or blastocyst-stage embryo transfer.

The miscarriage rate of the SVBT group was slightly higher than that of the DVBT group regardless of the date of vitrification, without reaching statistical significance. This difference could be explained by the fact that 2~3 G-sacs that were reduced to 1~2 were not recorded as abortion in the DVBT group. In the present study, the miscarriage rate of the 5d-SVBT group was 12.3 % and this rate was in accordance with our previous study's miscarriage rate of 13.7 % in the fresh SBET cycle [14]. These results demonstrate that miscarriage was not increased due to the vitrified-warmed process. Nonetheless, the miscarriage rate was higher in 6d-SVBT compared to 5d-SVBT (21.7 % vs. 12.3 %, $p=0.08$), without reaching statistical significance. Although our study has a disadvantage of having younger patients in the 5d-SVBT group than those in the 6d-SVBT group (31.8±2.8 vs. 32.4±2.7, $p=0.023$), the age of the patients aborted was younger in the 6d-SVBT group rather than those in the 5d-SVBT group (30.8±2.9 vs. 32.0±3.5, $p=0.342$), but the differences were not statistically significant. This is thought to be due to delayed blastocyst expansion which may indicate lower embryo quality associated with chromosomal abnormalities. With respect to this issue, it was recently reported that slower developing blastocysts were negatively affected by chromosomal abnormalities compared to faster developing blastocysts [1]. Grifo et al. [10] reported that single thawed euploid blastocyst transfer results in a low miscarriage rate. Therefore, a prospective research should be performed about the relationship between miscarriage and blastocyst developed on day 6.

When blastocyst-stage embryo transfer was performed on day 5 compared to day 6 in fresh cycles, significantly better clinical outcomes could be achieved [15, 23]. However, Hiraoka et al. [13] reported that clinical outcomes of vitrified-warmed blastocyst embryo transfers on day 6 were a similar to that of vitrified-warmed blastocysts embryo transfers on day 5. These differences in clinical outcomes between fresh and frozen-thawed cycles maybe due to the speed of development of blastocysts and/or endometrial receptivity according to the date of embryo transfer. In relation to these issues, Elgindy and Elsedeek [5] reported that blastocysts developed on day 5 had comparable implantation and pregnancy rates whether transferred on day 5 or day 6, whereas the pregnancy rate of expanded blastocysts on day 5 regardless of the time of transfer was significantly higher than that of expanded blastocysts on day 6. They concluded that clinical outcomes were affected by the speed of development of blastocysts rather than endometrial receptivity. However, their study had a disadvantage of a small number of patients who were transferred on day 6. Our study which included more than 100 cycles in 6d-SVBT was consistent with the study by Elgindy and Elsedeek [5]. However, our study also has important disadvantages of having younger patients in the 5d-SVBT group than those in the 6d-SVBT group (31.8±2.8 vs. 32.4±2.7, $p=0.023$). It has been known that women's age is an important determinant of IVF-ET success. Goto et al. [9] reported that clinical outcomes tend to be lower with increasing age when transferred the same quality blastocyst in single frozen-thawed blastocyst embryo transfer cycles. A prospective, randomized study should be performed to determine that embryo quality and/or synchronization between blastocysts and endometrial receptivity is responsible for FET results in blastocysts occurring on day 6.

Cryopreservation of surplus embryos in elective single embryo transfer plays an important role as a useful assisted reproduction technology (ART) that could improve the cumulative clinical outcomes and decrease the costs of ART treatment [29]. However, should surplus embryos be frozen at which developmental stage (cleavage, blastocyst) after embryo transfer? Until now, researchers have reported different results on the freezing stage of surplus embryos [12, 30]. Recently, extended culture to the blastocyst-stage has become more common due to the improvement of culture conditions. Consequently, the necessity of blastocyst-stage cryopreservation has been increasing to a greater extent. Also, some prospective studies have shown that a significantly higher implantation rate in SBET compared to that in eSCET in fresh cycles [12, 20]. However, the biggest drawback of extended culture is related to a higher incidence of embryo transfer cancellation and fewer embryos cryopreserved due to failed blastocysts development [17, 20]. According to a recent prospective study comparing DBET with SBET in women under 36 years of old without top-quality embryos on day 2, the ET cancellation rate of SBET was significantly higher than that of DCET (12 % vs. 0 %, $p<0.001$) [11], but the pregnancy rate and delivery rate per oocyte retrieval of SBET were similar to those of DCET. On the other hand, the multiple delivery rate was significantly lower in SBET compared to that in DCET. Moreover, blastocysts cryopreservation was twice as high in

the SBET group compared with that in the DCET group. These results show that embryos that were not of top quality on day 2 could be developed into the blastocyst-stage and these blastocysts could have a high implantation potential. We have employed the system of culturing all surplus embryos until day 6 and then freezing at the blastocyst-stage after embryo transfer. As a result, in the eSET cycles, the rate of cycle with blastocyst-stage cryopreservation was 89.7 % (582/649) in 2009, 85.0 % (624/734) in 2010, and 89.6 % (740/826) in 2011, respectively.

One of the most important changes in cryopreservation methods of embryos is the use of vitrification instead of slow freezing protocols. Vitrification of human blastocysts has been extensively studied until now since its first human delivery following vitrified-warmed blasotocysts transfer in 2001 [18]. This technique with a simple and fast method compared to slow freezing could be suitable for patients undergoing eSET, because they could have more frozen embryos than patients receiving more than two embryos. Some studies have already suggested that vitrification may improve the embryo survival rate and clinical outcomes [22, 25]. We believe that SVBT combined with eSET may be the best way for the birth of a single healthy baby.

This study showed that transferring a single vitrified-warmed blastocyst developed on day 5 resulted in comparable clinical outcomes compared to DVBT, while transfer of a single vitrified-warmed blastocyst formed on day 6 provided significantly lower clinical outcomes compared to DVBT in patients less than 37 years of age at the time of cryopreservation who received a good quality vitrified-warmed blastocyst. These results suggest that blastocysts cryopreserved on day 5 should transfer one embryo to obtain acceptable pregnancy and to minimize the multiple pregnancy rate. However, it is considered that blastocysts cryopreserved on day 6 should be carefully approached to determine the number of embryo transferred. Further studies are needed to determine the number of cryopreserved day 6 blastocysts transferred.

Conflicts of interest The authors declare that they have no conflict of interest.

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