Effect of Laser-Assisted Hatching on the Clinical Outcomes of Human Vitrified-Thawed Embryo Transfer Cycles with Different Post-Thaw Culture Duration

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Research

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Abstract

Background: Zona pellucida(ZP)hardening caused by prolonged in vitro culture and exacerbated by the freeze–thaw process making ZP hatching difficult; In theory, assisted hatching may facilitate the hatching process and have the potential to increase implantation and/or pregnancy rates in frozen embryo transfer (FET) cycles. However, a number of studies have shown controversial results on the clinical benefit of laser-assisted hatching (LAH) in FET cycles. This study firstly investigated the efficacy and safety of LAH using vitrified-thawed embryos with different post-thaw culture duration in FET cycles.

Methods: Data from the center's IVF database were retrospectively analyzed, only embryos thawed for the first FET cycle of each ovarian pick-up were eligible for this study, and only cycles in which at least one embryo was available for transfer were included in the present study. Finally, a total of 1225 infertile couples who underwent 1225 FET cycles between July 2013 and March 2015 were analyzed in this study. According to the duration of post-thaw culture in FET cycles, these patients were allocated to three subgroups: the short culture (4-5 h) group (LAH, n=205; control, n=201), overnight culture (20-24 h) group (LAH, n=197; control, n=203), and blastocyst culture (44-48 h) group (LAH, n=210; control, n=209), respectively.

Results: In the short culture(4-5 h) subgroup, no statistically significant differences were found related to the implantation, clinical pregnancy and live birth rates between the two groups (28.0% versus 27.8%, 38.0% versus 36.8%, and 30.7% versus 30.3%, respectively, P□0.05). When the perinatal outcomes of two groups were compared, there was no significant difference in the gestational weeks (37.96±2.23 versus 37.59±2.35, P□0.05), birth weight (2.70±0.56 versus 2.82±0.62, P□0.05), as well as the preterm birth (15.4% versus 17.6%, P□0.05), ectopic pregnancy (2.6% versus 1.4%, P□0.05), and miscarriage rates (16.7% versus 16.2%, P□0.05).

In the overnight culture(20-24 h) subgroup, no statistically significant differences were found regarding the implantation, clinical pregnancy and live birth rates (29.5% versus 29.1%, 40.1%versus 37.4%, 33.0%versus 30.5%, respectively, P□0.05).As to the perinatal outcomes, there was no significant difference in the gestational weeks (36.86±2.28 versus 35.69±2.95, P□0.05), birth weight (2.73±0.76 versus2.62±0.52, P□0.05), as well as the preterm birth (15.2% versus 17.1%, P□0.05), ectopic pregnancy (3.8% versus 2.6%, P□0.05), and miscarriage rates (13.9% versus 15.8%, P□0.05).

In the blastocyst culture(44-48 h) subgroup, the two groups did not differ significantly in the implantation, clinical pregnancy and live birth rates (56.3% versus 59.3%, 68.6% versus 66.5%, and 55.7% versus56.5%, respectively, P□0.05).Furthermore, there were also no significant difference in the gestational weeks (38.68±2.76 versus 36.95±2.59, P□0.05), birth weight (2.78±0.74 versus 2.72±0.59, P□0.05), as well as the preterm birth (8.3% versus 11.5%, P□0.05), ectopic pregnancy (2.8% versus 2.2%, P□0.05), and miscarriage rates (16.0% versus 12.9%, P□0.05).

Conclusions: Our results suggested that LAH does not improve the clinical outcomes in FET cycles, irrespective of the duration of post-thaw culture. Though the risk of perinatal period did not increase, it is

still necessary to conduct further investigations to validate the safety of LAH.

Introduction

Embryo implantation is crucial to the success of assisted reproductive technology (ART) program, which is a complex process, occurs in a limited time period known as the window of implantation [1]. Zona pellucida (ZP) hatching is a crucial step in the physiological process of embryo implantation. Under physiological conditions, ZP is a special layer of glycoprotein complex that wraps around the membrane of the oocyte, under physiological conditions, and becomes thinner gradually as the embryo cleavage develops. The hatching of embryo takes place around the 6th day of embryonic development, when the embryo in the blastocyst state is freed from ZP, and comes out [1–2].

ZP hardening caused by prolonged in vitro culture and/or exacerbated by the freeze–thaw process in ART, has been observed the inducing alteration in glycoprotein matrix, and might make it difficult for ZP to hatch. It is believed that embryo hatching failure may be one of the major factors limiting the success of ART program [3–4]. Therefore, assisted hatching (AH) has been proposed as a laboratory procedure aimed to improve the capacity of implantation following in vitro fertilization (IVF) [5]. The AH method involves the artificial thinning or breaching of an embryo's ZP by laser, mechanical, or chemical means. Especially, laser-assisted hatching (LAH) is commonly used for in vitro fertilization of embryos to facilitate the hatching process, which has been shown to be faster and easier than other methods [6–8].

Theoretically, AH may facilitate the hatching process and may increase the implantation and/or pregnancy rates in frozen embryo transfer (FET) cycles. However, a number of studies have shown controversial results on the clinical benefit of LAH in FET cycles. So far, there were reports that LAH significantly improved the implantation rate and/or the clinical pregnancy rate of FET compared with the control group (non-LAH) [9–11]. In contrast, some studies reported no improvement in the FET outcome using LAH [12–13].

In general, the efficacy of LAH is still debated. Moreover, only a few studies concern the safety of LAH, so, more studies are needed to evaluate the effect of LAH on assisted reproduction outcomes. Considering the causes mentioned above, included prolonged in vitro culture and the freeze–thaw process that make ZP hardening in ART. We have been committed to the application of assisted reproductive technology. In the present study, we investigated the efficacy and safety of LAH using vitrified-thawed embryos with different post-thaw culture duration in FET cycles, which, as far as is known, has not been previously investigated.

Materials And Methods

Study population

This was a retrospective cohort study to evaluate the effect of laser-assisted hatching (LAH) on the clinical outcome in FET cycles with different post-thaw culture duration. This study was conducted at the

reproductive medicine center of the Third Affiliated Hospital, Guangzhou Medical University. Our reproductive center is a large-scale center with nearly 9,000 oocyte retrieval cycles per year, which under the supervision and guidance of the National Health and Family Planning Commission. Informed consent was obtained from all couples, and the study was approved by the Institutional Review Board of the Ethical Committee of the Third Affiliated Hospital of Guangzhou Medical University. Data from the center's IVF database were retrospectively analyzed, only embryos thawed for the first FET cycle of each ovarian pick-up were eligible for this study, and only cycles in which at least one embryo was available for transfer were included in the present study.

Ovarian Stimulation And Oocyte Retrieval

Ovarian stimulation was performed using recombinant FSH or hMG. The LH surge was inhibited using either GnRH antagonists or GnRH-a. In all cycles, hCG (5,000 or 10,000 IU) was administered as soon as three follicles reached a mean diameter of 17 mm. Ultrasound-guided transvaginal oocyte retrieval was performed 34–36 hours later.

Vitrification And Thawing

The procedures of vitrification were performed at room temperature (22–25 °C) with Kitazato vitrification kit (BioPharma, Shizuoka, Japan). Briefly, embryos were equilibrated in the equilibration solution contained 7.5% (v/v) ethylene glycol and 7.5% (v/v) dimethyl sulfoxide for 5 min, then transferred into the vitrification solution that was composed of 15% (v/v) ethylene glycol, 15% (v/v) dimethyl sulfoxide and 0.5 M sucrose and loaded onto the cryotop (Kitazato, Fujinomiya, Japan) within 1 min. Finally, the straw was immediately plunged into the protective plastic cover inside liquid nitrogen for cryopreservation.

For thawing, the plastic cover was removed in liquid nitrogen and the tips of the cryotop with embryos were quickly submerged in 1 ml 1.0 M sucrose warmed to 37 °C for 1 min. Then the embryos were transferred to a room temperature solution containing 0.5 M sucrose for 3 min and 0.25 M sucrose for another 3 min. After two subsequent washing in basic solutions without sucrose for 6 min altogether at room temperature, embryos were transferred into G2 medium (Vitrolife, Gothenburg, Sweden) and incubated until AH procedure. Embryos were considered to have survived if ≥ 50% of the blastomeres were intact.

Laser-assisted Hatching Procedure

Laser-assisted hatching was performed on the thawed cleavage stage embryos with a laser treatment (Zilos-tk Laser, Hamilton Thorne Research, USA) after the warming procedure as described previously [14]. This equipment uses a 1.48-μm infrared diode laser beam and does not have any adverse effect on the development of human embryos and the subsequent perinatal outcomes [11]. Briefly, the embryo was

positioned and the point in the circle of the laser beam was focused where the perivitelline space was widest. The laser beam was then activated at pulse duration of 500 μs and formed an opening (about 40 μm) in the zona pellucida from outward to inward by several laser shots. After LAH, embryos were washed several times and cultured at 37 °C in a humidified atmosphere of 5% O_2, 6% CO_2, and 89% N_2 until the day of embryo transfer.

Frozen-thawed Embryos Transfer And Assessment Of Pregnancy

Depending on the post-thawed culture period, FET cycles were divided into three study groups. In the short culture group, the frozen-thawed embryos were evaluated for survival, blastomere number, degree of fragmentation and blastomere asymmetry and transferred 4–5 h after thawing. In the overnight culture group, embryos were cultured for 20–24 h and all transferred embryos were confirmed to have further cleavage during the culture period. In the blastocyst culture group,2–5 embryos were thawed and cultured for 44–48 h and at least one blastocyst was available for transfer in this study.

The post-thaw embryos were transferred in either natural cycles or hormone replacement treatment cycles. One to two embryos were transferred, depending on the patient's age and embryo availability and quality. Luteal phase support was continued with progesterone for at least two weeks after embryo transfer. Serum β-hCG was measured two weeks later, and a clinical pregnancy was confirmed by the presence of a gestational sac with fetal heartbeat via ultrasonography examination four weeks after embryo transfer. All pregnant women were followed up after parturition. All delivered infants were evaluated for complications during pregnancy or at delivery, including gestational age, birth weight, defects and so on.

Statistical analysis

The results were compared by using Student’s t-test, chi-squared analysis and Fisher's exact test. A P value of < 0.05 was considered significant. All data analysis was performed using the Statistical Package for Social Sciences (SPSS) version 19.0 (SPSS Inc., USA).

Results

Finally, a total of 1225 infertile couples who underwent 1225 FET cycles were performed between July 2013 and March 2015 were analyzed in this study. Additionally, according to the duration of post-thaw culture in FET cycles, these patients were allocated to three groups: the short culture(4–5 h) group (LAH group, n = 205; control group, n = 201), overnight culture(20–24 h) group (LAH group, n = 197; control group, n = 203), and blastocyst culture(44–48 h) group (LAH group, n = 210; control, n = 209 group), respectively.

1. Effect of LAH on clinical and perinatal outcomes of FET in the short culture group

During the study period, 406 patients who met the inclusion criteria were enrolled into the short culture group, included 205 patients with LAH treatment (LAH group), 201 patients without LAH treatment (Control group), respectively. As shown in Table 1, the female age at thawing, body mass index (BMI), duration of infertility, type of infertility(primary), indications for infertility and the endometrial thickness on embryo transfer (ET)day were all similar between the two groups. In addition, the number of embryos transferred were also similar (1.83 versus1.79, P>0.05).Consequently, no significant differences were found related to the implantation, clinical pregnancy and live birth rates between the two groups (28.0% versus 27.8%, 38.0% versus 36.8%, and 30.7% versus 30.3%, respectively, P>0.05). Moreover, when the perinatal outcomes of two groups were compared, there was no significant difference in the gestational weeks (37.96 ± 2.23 versus 37.59 ± 2.35, P>0.05), birth weight (2.70 ± 0.56 versus 2.82 ± 0.62, P>0.05), as well as the preterm birth (15.4% versus 17.6%, P>0.05), ectopic pregnancy (2.6% versus 1.4%, P>0.05), and miscarriage rates (16.7% versus 16.2%, P>0.05).

Table 1
Comparison of the characteristics of patients and clinical outcomes (short culture) between the LAH and control groups.

Variable	LAH group	Control group	P value
No. of patients(n)	205	201	-
Female age at thawing (y)	33.65 ± 5.01	33.72 ± 4.94	0.797
Body mass index(kg/m2)	21.38 ± 0.15	21.68 ± 0.13	0.765
Duration of infertility (y)	5.70 ± 3.90	5.45 ± 3.59	0.290
Type of infertility (primary) (%)	111/205 (54.1)	117/201 (58.2)	0.752
Indications for infertility			
Ovarian disorders (%)	9(4.4)	8(4.0)	0.837
Endometriosis (%)	6(2.9)	13(6.5)	0.091
Tubal factor (%)	113(55.1)	124(61.7)	0.179
Male factor (%)	38(18.5)	30(14.9)	0.330
Mixed factor (%)	35(17.1)	23(11.4)	0.105
Unexplained (%)	4(2.0)	3(1.5)	1.000
Endometrial thickness on ET day (mm)	8.96 ± 1.90	9.45 ± 2.14	0.556
No. of embryos transferred	1.83 ± 0.36	1.79 ± 0.43	0.297
Implantation rate (%)	105/375 (28.0)	100/360(27.8)	0.853
Clinical pregnancy rate (%)	78/205(38.0)	74/201(36.8)	0.880
Live birth rate (%)	63/205(30.7)	61/201(30.3)	0.929
Gestational weeks(w)	37.96 ± 2.23	37.59 ± 2.35	0.838
Birth weight (Kg)	2.70 ± 0.56	2.82 ± 0.62	0.209
Preterm birth rate (<37 weeks)	12/78(15.4)	13/74(17.6)	0.953
Ectopic pregnancy rate (%)	2/78(2.6)	1/74(1.4)	0.527
Miscarriage rate (%)	13/78(16.7)	12/74(16.2)	0.619

Values are shown as n/total (%) or means ± standard deviations (SD). LAH = laser-assisted hatching; ET = embryo transfer. No statistically differences were found between the two groups.

2. Effect of LAH on clinical and perinatal outcomes of FET in the overnight culture group

The infertile couples' characteristics and clinical outcomes of the overnight culture group are summarized in Table 2. The LAH group and control group did not differ significantly in the female age at thawing, BMI,

duration of infertility, type of infertility, indications for infertility and the endometrial thickness on ET day. Similarly, no statistically differences were found regarding the number of embryos transferred, the implantation, clinical pregnancy and live birth rates (1.93 versus 1.84, 29.5% versus 29.1%, 40.1%versus 37.4%, 33.0%versus 30.5%, respectively, P0.05).

As to the perinatal outcomes, there was no significant difference in the gestational weeks (36.86 ± 2.28 versus 35.69 ± 2.95, P0.05), birth weight (2.73 ± 0.76 versus2.62 ± 0.52, P0.05), as well as the preterm birth (15.2% versus 17.1%, P0.05), ectopic pregnancy (3.8% versus 2.6%, P0.05), and miscarriage rates (13.9% versus 15.8%, P0.05).

Table 2
Comparison of the characteristics of patients and clinical outcomes (overnight culture) between LAH and control groups.

Variable	LAH group	Control group	*P* value
No. of patients(n)	197	203	-
Female age at thawing (y)	32.11 ± 4.61	32.37 ± 4.61	0.627
Body mass index(kg/m2)	21.18 ± 0.15	22.23 ± 0.21	0.762
Duration of infertility (y)	4.85 ± 3.08	4.79 ± 3.27	0.200
Type of infertility (primary) (%)	114/197(57.9)	107/203(52.7)	0.300
Indications			
Ovarian disorders (%)	10(5.1)	4(2.0)	0.091
Endometriosis (%)	8(4.1)	5(2.5)	0.368
Tubal factor (%)	103(52.3)	123(60.6)	0.094
Male factor (%)	38(19.3)	28(13.8)	0.139
Mixed factor (%)	36(18.3)	40(19.7)	0.715
Unexplained (%)	2(1.0)	3(1.5)	1.000
Endometrial thickness on ET day (mm)	9.65 ± 2.16	9.95 ± 1.94	0.685
No. of embryos transferred	1.93 ± 0.26	1.84 ± 0.40	0.480
Implantation rate (%)	112/380(29.5)	109/374(29.1)	0.713
Clinical pregnancy rate (%)	79/197(40.1)	76/203(37.4)	0.637
Live birth rate (%)	65/197(33.0)	62/203(30.5)	0.290
Gestational weeks(w)	36.86 ± 2.28	35.69 ± 2.95	0.868
Birth weight (Kg)	2.73 ± 0.76	2.62 ± 0.52	0.409
Preterm birth (%)	12/79(15.2)	13/76(17.1)	0.856
Ectopic pregnancy rate (%)	3/79(3.8)	2/76(2.6)	0.687
Miscarriage rate (%)	11/79(13.9)	12/76 (15.8)	0.538

Values are shown as n/total (%) or means ± standard deviations (SD). LAH = laser-assisted hatching; ET = embryo transfer. No statistically differences were found between the two groups.

3. Effect of LAH on clinical and perinatal outcome of FET in blastocyst culture group.

As displayed in Table 3, the characteristics of patients and clinical outcomes (blastocyst culture) between LAH and control groups were compared, no significant differences were observed between the two groups

in female age at thawing, BMI, duration of infertility, type of infertility, indications for infertility and the endometrial thickness on ET day. Consequently, the two groups did not differ significantly in the number of embryos transferred, the implantation, clinical pregnancy and live birth rates between the two groups (1.75 ± 0.42 versus 1.69 ± 0.51, 56.3% versus 59.3%, 68.6% versus 66.5%, and55.7% versus56.5%, respectively, P 0.05).Furthermore, there were also no significant difference in the gestational weeks (38.68 ± 2.76 versus 36.95 ± 2.59, P 0.05), birth weight (2.78 ± 0.74 versus 2.72 ± 0.59, P 0.05), as well as the preterm birth (8.3% versus 11.5%, P 0.05), ectopic pregnancy (2.8% versus 2.2%, P 0.05), and miscarriage rates (16.0% versus 12.9%, P 0.05)

Table 3
Comparison of the characteristics of patients and clinical outcomes (blastocyst culture) between LAH and control groups.

Variable	AH group	Control group	P value
No. of patients(n)	210	209	-
Female age at thawing (y)	31.80 ± 3.79	30.56 ± 3.81	0.524
Body mass index BMI(kg/m2)	20.11 ± 0.25	21.23 ± 0.29	0.769
Duration of infertility (y)	4.32 ± 3.14	4.28 ± 2.60	0.223
Type of infertility (primary) (%)	100(47.6)	116(55.5)	0.106
Indications			
Ovarian disorders (%)	18(8.6)	24(11.5)	0.321
Endometriosis (%)	7(3.3)	2(1.0)	0.175
Tubal factor (%)	133(63.3)	130(62.2)	0.811
Male factor (%)	25(11.9)	32(15.3)	0.309
Mixed factor (%)	24(11.4)	16(7.7)	0.189
Unexplained (%)	3(1.4)	5(2.4)	0.503
Endometrial thickness on ET day (mm)	10.45 ± 2.26	10.78 ± 2.32	0.475
No. of embryos transferred	1.75 ± 0.42	1.69 ± 0.51	0.166
Implantation rate (%)	207/368(56.3)	210/354(59.3)	0.701
Clinical pregnancy rate (%)	144/210 (68.6)	139/209 (66.5)	0.652
Live birth rate (%)	117/210(55.7)	118/209(56.5)	0.385
Gestational weeks(w)	38.68 ± 2.76	36.95 ± 2.59	0.764
Birth weight (Kg)	2.78 ± 0.74	2.72 ± 0.59	0.379
Preterm birth rate (%)	12/144(8.3)	16/139(11.5)	0.856
Ectopic pregnancy rate (%)	4/144 (2.8)	3/139 (2.2)	0.468
Miscarriage rate (%)	23/144(16.0)	18/139(12.9)	0.967

Values are shown as n/total (%) or means ± standard deviations (SD). LAH = laser-assisted hatching; ET = embryo transfer. No statistically differences were found between the two groups.

Discussion

Failure to hatch may be the main factor limiting the efficiency of ART program. In particular, prolonged in vitro culture and/or the process of freezing and thawing embryos might make ZP hardening and difficult to hatch [15]. Therefore, the assisted hatching of zona pellucida may contribute to the hatching process, thus facilitating embryo implantation. Among these different methods, LAH appears to be easier and more popular than the other methods [16–17]. Most previous studies employed the strategy that LAH was performed on the post-thaw cleavage or blastocyst stage embryos before ET, then compared the clinical outcomes between the LAH group and control group (non-LAH group) [18].

To the best of our knowledge, this is the first study to evaluate the impact of LAH on

vitrified-thawed embryos with different post-thaw culture duration in FET cycles. This study found that there was no improvement on pregnancy outcomes when applying LAH and transferred the D3, overnight, or blastocysts embryo cultured from vitrified-thawed cleavage embryos. Our results were in agreement with some of the previous findings. Ng et al found that LAH and non-LAH groups had similar rates of implantation (26.2% vs 27.3%), clinical pregnancy (29.1% vs 30.3%), clinical pregnancy loss (24.0% vs 17.8%), and live birth (19.9% vs 20.5%),which suggested that LAH prior to transfer of vitrified-thawed blastocysts was not associated with improved pregnancy outcomes [19].Similarly, Nakasuji et al. found no improvement in the clinical pregnancy and live birth rate in the FET patients who underwent AH in a retrospective study [20].

On the other hand, Wan et al. found a significant increase in the implantation rate (34.2% vs 23.6%) and clinical pregnancy rate (51.0% vs 35.3%) with AH, but not live birth rate (40.6% vs 28.4%). They suggested that LAH improve the clinical outcomes of vitrified-thawed blastocysts, especially of the day 6 vitrified blastocysts developed from low-grade cleavage-stage embryos [10].Another retrospective study which analyzed 415 cleavage-stage embryos transfer cycles with D3 cryopreserved embryo transfer that carried out by Lu et al., demonstrated that the implantation rate (31.2% versus 24.6%) and clinical pregnancy rate(49.3% versus 38.9%) in the LAH group were significantly higher than the control group ($P < 0.05$). The rates of live birth (44.8% versus 35.8%), preterm birth (22.8% versus 17.1%), miscarriage (7.2% versus 5.4%), and ectopic rates (1.9% versus 0%) did not differ significantly between the two groups ($P > 0.05$). Their study showed that LAH significantly improves the clinical outcomes, especially the clinical pregnancy and implantation rates, associated with FET cycles among patients with previous repeated failure [21].

These conflicting results may be attributed to several reasons, such as variations in study population, embryo cryopreservation technique, artificial shrinkage and so on. It should be noted that artificial shrinkage, a procedure similar to LAH that creates a small hole at the cellular junction of the trophectoderm, which is a routine procedure in the IVF center before blastocyst vitrification. Hence, the most published results conducted on blastocysts might involving this issue inevitably. Generally, we employed vitrified-thawed day 3 embryo, exposed to prolonged culture to blastocyst, which eliminated laser shrinkage before routine blastocyst vitrification, which could evaluate the impact of LAH more truthfully.

To date, despite the longtime use of LAH, few clinical studies have evaluated its impact on perinatal and neonatal outcomes [11, 19]. In the present study, there were no differences between the LAH group and control group in the gestational weeks, birthweight, as well as the preterm birth, ectopic pregnancy and miscarriage rates. And the results are similar in spite of the different post-thaw culture period. Take the D3 embryo as an example, the preterm birth rate between the

LAH group and control group were 15.4% and 17.6% ($P > 0.05$). With respect to the ectopic pregnancy and miscarriage rates, there were also no difference between two groups (2.6% versus1.4%,16.7% versus16.2%, respectively, $P > 0.05$). Liu reported that the incidence of monozygotic twinning had no significant difference in FET cycles between the LAH group and non-LAH group [22].Considering the previous results that overall risks for major congenital anomalies were not significantly different after AH treatment[11, 23], indicating that LAH is a safe embryological intervention in ART. However, safety is a top priority when an extra manipulation in human embryos. Considering the increasing trend of LAH use, LAH is not recommended as a routine treatment strategy for FET patients.

In conclusion, we firstly evaluated the efficacy and safety of LAH on vitrified-thawed embryos with different post-thaw culture duration in FET cycles. Our results suggested that LAH does not improve the clinical outcomes in FET cycles, irrespective of the duration of post-thaw culture. Though the risk of perinatal period did not increase, it is still necessary to conduct further investigations with larger sample sizes and long-term follow-up of babies to validate the safety of LAH.

Declarations

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Authors' contributions

YL and YS designed the study, performed the statistical analysis and drafting of the manuscript. FP contributed to the statistical analysis. LL edited the manuscript. All authors reviewed and approved the final version of this article.

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Availability of data and materials

The data used and analyzed during this study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

Ethical approval was obtained from the Ethics Committee of Third Affiliated Hospital of Guangzhou Medical University, Guangzhou, China.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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