Preventing blastocyst culture failure risk in IVF cycles merits a strategy of cleavage backup combined with blastocyst culture

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Abstract

Background

Blastocyst transfer is associated with a high cycle cancellation rate due to more early embryo arrest. However, cleavage backup combined with blastocyst culture maybe merit prevention blastocyst culture failure.

Methods

We retrospectively analyzed 7026 cycles between October 2017 and August 2020 in our center for reproductive medicine. Two day-3 embryos were transferred or cryopreserved in addition to at least 1 surplus embryo extended the culture for blastocysts. The primary objective was to analyze the factors of influencing blastocyst formation. The secondary objective was to study the fate of sibling cleavage embryos after sparing the embryo culture for blastocysts.

Results

In all included cycles, there was 12.43% occurrence without blastocyst formation (CNB), 5.19% of nonavailable blastocysts (NAB), and 82.38% cycles with at least 1 valuable blastocyst (CHB). The number of day-3 embryos was associated with blastocyst formation outcome (adjusted odds ratio [aOR] 1.71), and older women and short agonist ovary stimulation decreased the odds of blastocyst formation (aOR 0.97 and 0.18, respectively). Female age and numbers of embryos for blastocyst culture were related to blastocyst quality (aOR 0.98 and 1.36, respectively). Some couples underwent fresh cleavage transfer (CNB 453, NAB 203, CHB 2341), and the pregnancy rate was significantly higher with positive culture results (CHB 47.97%, NAB 34.48%, CNB 34.88%, P=0.001). There was a lower chance of being pregnant for advanced aged women (aOR 0.94). Compared with mild ovary stimulation, the long stimulation protocol and modified ultralong protocol were beneficial for pregnancy after day-3 embryo transfer (aOR 1.62 and 2.77, respectively). Cleavage of embryos from cycles with available blastocysts increased the odds of implantation (CHB 35.25%, CNB 23.40%, NAB 24.88%, P<0.001). Pregnancy from cycles of no available blastocyst was associated with higher odds of miscarriage (NAB 19.10%, CNB 9.84%, CHB 10.70%). Outcome of blastocyst formation had no influence on preterm birth rate (CNB 12.03%, NAB 18.21%, CHB 18.45%, respectively, P=0.139), birth defects (CNB 1.05%, NAB 1.09% CHB 0.89% P=0.966), or sex ratio of male proportion (CNB 0.57, NAB 0.55, CHB 0.53, P=0.669).

Conclusions

The pregnancy outcome of sibling cleavage transfer is related to blastocyst formation of spare embryos. For negative blastocyst culture patients, there is a lower but acceptable live birth rate from sibling cleavage transfer. Cleavage backup combined with blastocyst culture may prevent blastocyst formation failure risk.

Introduction

Currently, there are approximately 5 embryos for each couple after routine ovary stimulation (1), and selecting a vigorous embryo is a vital step to obtain a successful assisted reproduction endpoint more rapidly. Morphological assessment is universally used in most centers, but the embryo rating score at the cleavage stage is not closely correlated to embryo euploidy (2, 3), and differences in intra- and inter-embryologist may influence the order of embryo final decisions (4–6). Although arrested embryos occur in any developmental stage, in poor-quality embryos, the arrest phenomenon is more frequently observed with longer culture times (7, 8). On the basis of this concept, blastocyst culture is an option to distinguish a potential embryo, and low-quality cleavage embryos are easily excluded by extending culture. Another intriguing theory of pro-blastocysts is closer to the physiological state. In humans, day 5 embryos arrive at the uterus; day 3 embryos are located in the fallopian tube. A previous study showed that selective single blastocyst transfer can sharply decrease high-order multiple pregnancy sharply without compromising the pregnancy rate (9). Furthermore, compared with cleavage transfer, a higher live birth rate and lower miscarriage rate mean better clinical outcomes (9, 10), which is beneficial for patients to decrease their time to pregnancy (TTP). This probably explains why blastocysts show more potential after a longer screening process, and they are better synchronized with the endometrial environment. However, there is some evidence about concerning newborn risk. Compared with cleavage transfer, an increasing preterm rate (11–13), larger for gestational age (14, 15), and favoring male births (16, 17) are on the list. In addition, unpredictable and elevated cycle cancellation for no suitable blastocyst makes both clinical doctors and patients to select this technique more carefully (18).

A higher cancellation rate reveals some risks in blastocyst culture, and it indicates many discarded embryos bypass transfer procedures. At present, the blastocyst protocol does not apply to those patients who have few early cleavage embryos (19–21). For them, economical and equivalent cleavage transfer is an optimal option. There is not a calculable number of early cleavage embryos to make sure there is an available blastocyst. In terms of the numbers of embryos or bipronuclei oocytes, Fernandez-Shaw suggested that at least 4 zygotes were optional (22). Another study showed that at least 4 good day 3 embryos were a reasonable threshold to obtain a better live birth chance after blastocyst transfer (23). However, currently, there is a lack of proven effectiveness morphological assessment, morphokinetic models, and omics in predicting blastocyst formation (24). In practical clinical treatment, it is common that good prognostic cleavage embryos fail to have a blastocyst (25). In some cycles, a large number of cleavage embryos were cultured, but no blastocysts were harvested (25). While genome loss may be responsible for preventing blastocyst formation (26), many pro-blastocyst culture physicians presumed that cleavage embryos that failed to reach blastocysts in vitro were transferred, and implantation failure was the probability of large events.

In fact, we are still far from aware of in vivo conditions, and there are many distinct conditions in the reproductive tract in vivo. A lower oxygen concentration is different in the oviduct and in utero compared with the popular 5% O_2 setpoint in incubator (27). The monic environment is obviously different from the stable status in vitro. In clinical data, the cumulative pregnancy rate was not superior in day 5 transfer

compared with day 3 transfer (28), and some researchers were prone to cleavage transfer to improve cumulative live births per one oocyte retrieval cycle (29). For those couples who had available cleavage embryos but failed to reach the blastocyst stage, it is important to discover whether they benefit from extending culture, which is important for them in subsequent cycles. In our center, in all of OPU cycles, two priority cleavage embryos were transferred or frozen, and the remaining embryos were extended to culture.

In this study, we retrospectively analyzed those cycles in which at least one day-3 embryo(s) was cultured in addition to two useful cleavage embryos for transfer or freeze in fresh cycles, but ultimately, no blastocysts were obtained. At the same time, those cycles with developed blastocysts were designated as control group. The purpose of this study was to discover the fate of last cleavage embryos in blastocyst failure cycles, and demonstrate confusing reasons and provide some information for prolonging culture.

Materials And Methods

Study design

In this study, we retrospectively analyzed all cycles between October 2017 and August 2020 in our center for reproductive medicine. According to our standard operation protocol, patients were informed and chose to freeze/transfer two top priority day 3 embryos, and supernumerary embryos were cultured to blastocyst for cryopreservation until day 7.

The development and pregnancy outcomes were compared according to blastocyst formation results. The patient data analysis was approved by our hospital ethics commission.

Data collection

All following cycles were included: (i) two day 3 embryos were kept for fresh transfer or cryopreservation); (ii) at least 1 prolonged culture embryo for 5–7 days. The exclusion criterions were as following: (i) abnormal chromosome in males or females and (ii) repeated implantation failure.

Conventional ovary stimulation

GnRH agonist or antagonist, or luteal phase stimulation protocols were used. Serial serum estradiol, luteinizing hormone and progesterone were measured in latter-stage stimulation, and follicular diameter was monitored by TVS. When there was a follicular diameter ≥ 17mm, serum estradiol, LH, and P levels were assessed to determine ovulation trigger.

Fertilization method and embryo culture

Oocyte pick-up was applied at 36 hours after HCG administration. ICSI or IVF were up to previous pregnancy history, routine sperm parameters, and experiencing fertilization history. Insemination was limited at 2–4 hours after oocyte collection. For those patients with normal sperm motility and primary

pregnancy history, fertilization status was estimated according to second polar body extrusion in 6 hours after insemination. If less than 1/4 mature oocytes failed to observe two polar bodies, rescue ICSI was used. For ICSI patients, cumulus cells were removed with hyaluronidase digestion. Experienced technicians were approved of the manipulation of ICSI.

Fertilization oocytes were assessed at 16–18 hours on day 1, and fertilized oocytes were transferred into pre-equilibrium cleavage medium. Multinucleation was assessed on day 2, and day 3 embryo scoring was recorded according to the Istanbul consensus, including cell number, grade, and reason for the grade. Two embryos with the highest score were selected for fresh transfer or cryopreservation if not suitable for transfer in fresh cycles. The above embryo quality evaluation was accomplished by two experienced embryologists. Later on, the supernumerary embryos were transferred into blastocyst medium. The embryo development stage was observed from day 5 until day 7, and blastocyst grade was measured on basis of blastocyst stage, inner cell mass and trophoblast cells. The discarded blastocyst was both inner cell mass and trophoblast cells were assessed as C.

Pregnancy outcome measures

Serum hCG levels were determined 14 days after embryo transfer. For pregnant patients, continuous progesterone and E_2 were given, and ultrasound examination was conducted at 1 and 2 months respectively. The gestational sac is considered as implantation, and clinical pregnancy means a gestational sac with fetal heart activity at 1 month after transfer. All newborn data were collected and confirmed by telephone communication.

Statistical analysis

Quantitative data were analyzed on the basis of normality tests and tests of homogeneity of variance. For homogeneous variance, the values are expressed by mean ± SD. A *t*-test or one way ANOVA was used to detect significant differences, and the Mann-Whitney or Kruskal-Wallis test was used for heterogeneous variance. Qualitative data are presented as n and percentages, and were compared with the chi-squared test or Fisher's exact test. To identify a possible independent correlation between blastocyst outcome, and each possible confounding factor, such as couples' ages, fertilization methods, the number of oocytes and cleavage embryos, etc. Binary logistic regression was conducted for blastocyst failure and pregnancy outcome in the group without blastocysts, and the forwards conditional method was entered into the equation. The software tool SPSS 16.0 was used to analysis difference.

Results

In this study, all selected cycles met the requirement of 2 cleavage embryos for fresh transfer or freezing. Additionally, there was at least 1 extended culture embryo(s) for blastocysts. Finally, a total of 7026 OPU cycles with at least 1 spare embryo were performed in blastocyst culture in addition to two top cleavages transfers or cryopreservation. In these cycles, adverse blastocyst culture results contributed to 17.62%.

Specifically, 12.43% (n = 873) of all cycles showed no blastocyst (CNB), and 5.19% (n = 365) of the included cycles yielded blastocysts but were not available (NAB). In addition, 5788 cycles (CHB, 82.38%) had two cleavage embryos and at least 1 valuable blastocyst (Fig. 1).

The number of day 3 embryos was obviously related to blastocyst culture outcome. As the number of cultured embryos was elevated, the possibility of blastocyst formation failure gradually decreased. The blastocyst failure rate reached a plateau when the number of cultured embryos was more than 6. In these cycles with a large amount of spare cleavage embryos, there were about approximately 3% cycles without blastocysts (Fig. 2). To investigate possible factors of blastocyst formation, binary logistic regression analysis was operated. As Table 1 shows, crude and adjusted for confounding factors (BMI, basal reproductive endocrine, doses of gonadotropin), advanced aged women had more difficulty obtaining blastocysts (aOR = 0.97, [95% CI 0.94–0.99] P < 0.05), for every year of growth, the odds of blastocyst formation decreased by 3%. The short agonist stimulation protocol showed a higher chance of blastocyst failure (aOR = 0.18, [95% CI 0.04–0.88] P < 0.05), but the number of embryos for blastocyst culture was a positive factor (aOR = 1.71, [95% CI 1.63–1.78] P < 0.01). These were independent variables for blastocyst failure. Furthermore, basal LH instead of the ovary stimulation protocol had a positive effect on blastocyst quality (aOR = 1.05, [95% CI 1.00-1.10] P < 0.05). Female age (aOR = 0.98, [95% CI 0.92–0.99] P < 0.05) and the numbers of embryos for blastocyst culture (aOR = 1.36, [95% CI 1.29–1.44] P < 0.01) were still related to the results of whether there were available blastocysts, while both female age and numbers of extending embryos had a weaker effect on blastocyst quality than blastocyst formation (Table 2).

Table 1
Association between blastocyst formation and patient's characteristic

Characteristic	OR (95% CI)	aOR (95% CI)
Female age	0.92(0.91, 0.93)	0.97(0.94, 0.99)
Male age	0.95(0.93, 0.96)	0.99(0.96, 1.03)
Infertility duration	0.93(0.92, 1.08)	0.93(0.81, 1.01)
Infertility type(ref: primary infertility)	0.88(0.66, 1.18)	1.04(0.88, 1.25)
Tubal factor	1.01(0.96, 1.06)	0.94(0.78, 1.14)
PCOS factor	0.39(0.27, 0.56)	0.71(0.47, 1.08)
Male factor	1.19(1.02, 1.41)	1.02(0.86, 1.23)
BMI	0.90(0.96, 1.04)	0.89(0.84, 1.02)
Basal FSH	0.94(0.93, 1.02)	0.92(0.91, 1.05)
Basal E2	1.01(0.99, 1.02)	0.90(0.69, 2.14)
Basal LH	1.04(0.81, 1.32)	0.82(0.74, 1.10)
Dose of gonadotrophin	0.80(0.70, 1.02)	0.97(0.92, 1.00)
Stimulation protocol (ref: mild stimulation)		
Long protocol	2.86(2.08, 3.95)	1.01(0.70, 1.45)
Short protocol	0.09(0.02, 0.36)	0.18(0.04, 0.88)
Ultra-long protocol	3.19(1.84, 5.56)	1.63(0.90, 2.97)
Modified ultra-long protocol	1.98(1.28, 3.07)	0.81(0.49, 1.32)
GnRHA protocol	1.84(1.31, 2.60)	1.06(0.73, 1.55)
Luteal phase	1.58(1.09, 2.29)	0.87(0.58, 1.32)
Insemination method (ref: IVF)	1.30(1.10, 1.54)	1.22(0.65, 1.49)
Number of cultured embryos	1.73(1.66, 1.81)	1.71(1.63, 1.78)

Note: aOR = adjusted odds ratio; CI = confidence interval; OR = odds ratio, PCOS = polycystic ovary syndrome; BMI = body mass index; E2 = estradiol; FSH = follicle stimulating hormone; GnRHA = gonadotropin releasing hormone antagonist; IVF = fertilization in vitro; ref = reference

Binary logistic regression considered reporting patient and treatment characteristics. Final models included female and male age (years), infertility duration (years), infertility type (primary infertility yes, no), infertility factors (tubal, PCOS, male yes, no), BMI, basal endocrine (FSH, E2, LH), dose of

gonadotrophin, ovary stimulation protocol (reference = mild stimulation), insemination method (IVF yes, no), and number of cultured embryos.

Table 2
Association between blastocyst quality and patient's characteristic

Characteristic	OR (95% CI)	aOR (95% CI)
Female age	0.94(0.92, 0.96)	0.98(0.92, 0.99)
Male age	0.96(0.94, 0.97)	0.97(0.97, 1.03)
Infertility duration	0.88(0.73, 1.13)	1.01(0.96, 1.05)
Infertility type(ref: primary infertility)	1.02(0.79, 1.18)	0.84(0.66, 1.08)
Tubal factor	0.69(0.44, 1.37)	0.88(0.68, 1.14)
PCOS factor	0.36(0.21, 0.64)	0.57(0.31, 1.03)
Male factor	0.93(0.74, 1.17)	0.80(0.63, 1.01)
BMI	0.65(0.54, 1.17)	0.99(0.96, 1.02)
Basal FSH	0.68(0.94, 1.01)	1.04(0.98, 1.09)
Basal E2	0.92(0.70, 1.32)	0.83(0.69, 1.21)
Basal LH	1.13(1.02, 1.25)	1.05(1.00, 1.10)
Dose of Gonadotrophin	0.76(0.65, 1.01)	0.91(0.80, 1.17)
Stimulation protocol (ref: mild stimulation)		
Long protocol	2.53(1.58, 4.09)	1.29(0.72, 2.05)
Short protocol	0.63(0.27, 3.38)	0.31(0.02, 4.22)
Ultra-long protocol	1.35(0.70, 2.61)	0.93(0.46, 1.85)
Modified ultra-long protocol	2.97(1.45, 6.11)	1.71(0.81, 3.62)
GnRHA protocol	2.40(1.41, 4.07)	1.58(0.91, 2.74)
Luteral phase	2.07(1.17, 3.69)	1.32(0.73, 2.40)
Insemination method (ref: IVF)	0.84(0.68, 2.14)	1.04(0.79, 1.38)
Number of cultured embryos	1.37(1.31, 1.43)	1.36(1.29, 1.44)

Note: aOR = adjusted odds ratio; CI = confidence interval; OR = odds ratio, PCOS = polycystic ovary syndrome; BMI = body mass index; E2 = estradiol; FSH = follicle stimulating hormone; GnRHA = gonadotropin releasing hormone antagonist; IVF = fertilization in vitro; ref = reference

Binary logistic regression considered reporting patient and treatment characteristics. Final models included female and male age (years), infertility duration (years), infertility type (primary infertility yes,

no), infertility factors (tubal, PCOS, male; yes, no), BMI, basal endocrine (FSH, E2, LH), dose of gonadotrophin, ovary stimulation protocol (reference = mild stimulation), insemination method(IVF yes, no), and number of cultured embryos.

There were 2997 cycles that underwent cleavage-stage fresh embryo transfer; 453 cycles without any blastocysts, 203 cycles without available blastocysts, and 2341 cycles with at least one blastocyst. Compared with the number of cycles of frozen blastocysts, the pregnancy rate was significantly decreased in the CNB (aOR = 0.57; 95% CI 0.46–0.70; P < 0.001, reference = the NHB group) and NAB groups (aOR = 0.64; 95% CI 0.47–0.86; P < 0.001). Female age was a risk factor for clinical pregnancy (aOR = 0.94; 95% CI 0.93–0.96; P < 0.001). In the study, the female age distribution was different in the three cycles (Table 3). Patients in the CNB groups age were older than those in the other two groups. The protocols of stimulation resulted in differences in the pregnancy rate. The long protocol (aOR = 1.62; 95% CI 1.05–2.49; P < 0.05) and modified ultralong protocol (aOR = 2.77; 95% CI 1.63–4.71; P < 0.001) were beneficial for improving clinical pregnancy in fresh cleavage-stage transfer (Table 4).

Table 3
A comparison of the baseline characteristic parameters in three group of blastocyst outcome

Characteristic	CNB group[a]	NAB group[b]	CHB group[c]	
n	453	203	2341	
Age (mean ± SD)	32.64 ± 5.52	31.76 ± 5.58	31.01 ± 4.67	P < 0.01
Infertility diagnosis				
Primary infertility (n, %)	221(48.79%)	100(49.26%)	1163(49.68%)	NS
Tubal factor (n, %)	171(37.75%)	78(38.42%)	928(39.64%)	NS
Endometriosis (n, %)	92(20.31%)	48(23.65%)	597(25.50%)	NS
Uterine factor (n, %)	20(4.42%)	10(4.93%)	110(4.70%)	NS
Ovulation dysfunction (n, %)	67(14.79%)	27(13.30%)	338(14.44%)	NS
Male factor (n, %)	188(41.50%)	78(38.42%)	958(40.92%)	NS
Unexplained (n, %)	49(10.82%)	25(12.32%)	261(11.15%)	NS
Duration (y) (mean ± SD)	3.77 ± 3.18	3.40 ± 2.96	3.25 ± 2.55	P < 0.01
BMI (kg/m2) (mean ± SD)	23.64 ± 4.74	23.47 ± 4.29	22.84 ± 4.41	NS
FSH (mIU/ml) (mean ± SD)	6.72 ± 3.75	7.04 ± 3.47	6.74 ± 3.81	NS
E2 (pg/ml) (mean ± SD)	44.75 ± 22.12	43.18 ± 30.8	41.51 ± 29.59	NS
LH (mIU/ml) (mean ± SD)	4.31 ± 2.43	4.02 ± 3.11	3.98 ± 3.24	NS
Oocytes collected (n)	12.82 ± 7.26	12.29 ± 6.91	13.05 ± 7.35	NS
Embryos (mean ± SD)	8.47 ± 5.25	8.63 ± 5.82	9.11 ± 6.79	NS
Good embryo rate of transferred (n, %)	698(77.04%)	318(78.33%)	3756(80.22%)	NS

Note: BMI = body mass index; E2 = estradiol; FSH = follicle stimulating hormone; LH = luteinizing hormone

[a] CNB patients included 2 day-3 embryos were frozen or transferred, spare embryos were extended culturing, but there was no blastocyst formation.

[b] NAB patients included 2 day-3 embryos were frozen or transferred, spare embryos were extended culturing, but there was no available blastocyst formation.

[c] CHB patients included 2 day-3 embryos were frozen or transferred, spare embryos were extended culturing, but there was at least 1 available blastocyst formation.

Table 4
Analysis of influencing factors of clinical pregnancy in cleavage stage transfer cycles

Characteristic	OR (95% CI)	aOR (95% CI)
Female age	0.94 (0.93, 0.96)	0.94 (0.93, 0.96)
Male age	0.96 (0.95, 0.97)	1.00 (0.99, 1.01)
Infertility duration	0.95 (0.93, 0.98)	0.98 (0.95, 1.01)
Infertility type(ref: primary infertility)	1.04 (0.90, 1.20)	0.87 (0.74, 1.02)
Tubal factor	0.95 (0.82, 1.10)	1.15 (0.97, 1.36)
PCOS factor	0.77 (0.64, 0.92)	0.78 (0.65, 0.95)
Male factor	0.80 (0.42, 1.53)	0.93 (0.48, 1.80)
BMI	0.97 (0.94, 0.99)	0.98 (0.96, 1.01)
Basal FSH	1.03 (0.99, 1.07)	1.03 (0.99, 1.07)
Basal E2	1.00 (0.99, 1.00)	0.91 (0.83, 1.04)
Basal LH	1.00 (0.96, 1.04)	0.99 (0.95, 1.03)
Stimulation protocol (ref: mild stimulation)		
Long protocol	2.19 (1.45, 3.32)	1.62 (1.05, 2.49)
Short protocol	0.90 (0.16, 5.17)	0.98 (0.16, 6.10)
Ultra-long protocol	2.17 (1.27, 3.71)	1.59 (0.92, 2.76)
Modified ultra-long protocol	3.77 (2.25, 6.30)	2.77 (1.63, 4.71)
GnRHA protocol	1.34 (0.84, 2.14)	1.16 (0.71, 1.89)
Insemination method (ref: IVF)	0.95 (0.81, 1.12)	0.81 (0.68, 1.26)
Blastcyst formation result (ref: CHB[a] group)		

Note: aOR = adjusted odds ratio; CI = confidence interval; OR = odds ratio, PCOS = polycystic ovary syndrome; BMI = body mass index; E2 = estradiol; FSH = follicle stimulating hormone; GnRHA = gonadotropin releasing hormone antagonist; IVF = fertilization in vitro; ref = reference

[a] CNB patients included 2 day-3 embryos were frozen or transferred, spare embryos were extended culturing, but there was no blastocyst formation.

[b] NAB patients included 2 day-3 embryos were frozen or transferred, spare embryos were extended culturing, but there was no available blastocyst formation.

[c] CHB patients included 2 day-3 embryos were frozen or transferred, spare embryos were extended culturing, but there was at least 1 available blastocyst formation.

Characteristic	OR (95% CI)	aOR (95% CI)
CNB[b] group	0.51 (0.42, 0.63)	0.57 (0.46, 0.70)
NAB[c] group	0.59 (0.44, 0.78)	0.64 (0.47, 0.86)
Note: aOR = adjusted odds ratio; CI = confidence interval; OR = odds ratio, PCOS = polycystic ovary syndrome; BMI = body mass index; E2 = estradiol; FSH = follicle stimulating hormone; GnRHA = gonadotropin releasing hormone antagonist; IVF = fertilization in vitro; ref = reference		
[a] CNB patients included 2 day-3 embryos were frozen or transferred, spare embryos were extended culturing, but there was no blastocyst formation.		
[b] NAB patients included 2 day-3 embryos were frozen or transferred, spare embryos were extended culturing, but there was no available blastocyst formation.		
[c] CHB patients included 2 day-3 embryos were frozen or transferred, spare embryos were extended culturing, but there was at least 1 available blastocyst formation.		

Binary logistic regression considered reporting patient and treatment characteristics. Final models included female and male age (years), infertility duration (years), infertility type (primary infertility yes, no), infertility factors (tubal, PCOS, male; yes, no), BMI, basal endocrine (FSH, E2,LH), ovary stimulation protocol (reference = mild stimulation), insemination method(IVF yes,no), and blastocyst formation result (reference: CHB group).

Available blastocysts were an indicator of sibling cleavage-stage embryo viability. The implantation rate was correlated with blastocyst culture outcome (Table 5). This rate was obviously higher in the CHB group (35.25%); only 23.40% of embryos were implanted in the CNB group, and 24.88% of embryo successfully implanted in the NAB group ($P < 0.001$). Women without available blastocysts were more likely to have a higher pregnancy loss rate (19.10%) than women in the other two groups (9.84% in the CNB group, and 10.70% in the CHB group, $P < 0.05$). A similar tendency appeared in the live birth rate (CHB 47.97%, NAB 34.48%, CNB 34.88%, $P < 0.001$). Regardless of whether there was a blastocyst until day 7 in the remaining embryo culture, there was no impact of blastocyst culture results on preterm birth (CNB 12.03%, NAB 18.21%, CHB 18.45%, respectively, $P = 0.139$). The incidence of very preterm birth before 32 weeks was significantly higher in only the poor-quality blastocyst group (NAB 7.04%, CNB 1.27%, CHB 1.77%, respectively, $P < 0.01$). Birth defects showed a similar occurrence rate in the three groups, 1.05% in the CNB group, 1.09% in the NAB group, and 0.89% in the CHB group ($P = 0.966$). Those cycles of transferring two top day 3 embryos had no difference in the sex ratio of the male proportion (CNB 0.57, NAB 0.55, CHB 0.53, respectively, $P = 0.669$).

Table 5
Reproductive outcomes after day-3 fresh embryo transfer in different blastocyst formation groups

	CNB group[a]	NAB group[b]	CHB group[c]	P value
transfer cylces	453	203	2341	
implantation rate (n, %)	212(23.40)	101(24.88)	1650(35.24)	P < 0.001
clinical pregnancy rate (n, %)	183(40.40)	89(43.84)	1337(57.11)	P < 0.001
miscarriage rate (n, %)	18(9.84)	17(19.10)	143(10.70)	P < 0.05
live birth rate (n, %)	158 (34.88)	70(34.48)	1123(47.97)	P < 0.001
preterm rate (n, %)	21(12.03)	18(18.31)	229(18.45)	NS
birth defect rate (n, %)	2(1.05)	1(1.09)	6(0.89)	NS
sex ratio of male proportion	0.57	0.55	0.53	NS

[a] CNB patients included 2 day-3 embryos were frozen or transferred, spare embryos were extended culturing, but there was no blastocyst formation.

[b] NAB patients included 2 day-3 embryos were frozen or transferred, spare embryos were extended culturing, but there was no available blastocyst formation.

[c] CHB patients included 2 day-3 embryos were frozen or transferred, spare embryos were extended culturing, but there was at least 1 available blastocyst formation.

Discussion

Today, cumulative knowledge of culture systems makes people be eager to design a media to obtain more robust blastocysts. Compared with cleavage-stage embryo transfer, blastocyst transfer to the uterus is closer to the physiological state, an easier and more reliable protocol in single births (30), and a more popular regime in the current PGT procedure. Most likely, decreased adverse obstetric and better perinatal outcomes encouraged clinicians to adopt blastocyst transfer (31). This retrospective research focuses on the risk of blastocyst culture failure. There is still a low failure possibility in cycles of more than 6 surplus embryos to extend culture, even in cycles without blastocyst formation, and selected cleavage embryos from sibling oocytes can result in a live birth in fresh transfer. As previous articles have reported, the number of prolonged culture embryos was significantly related to blastocyst yield outcome, and age and stimulation protocol were also involved.

Oocytes are derived from primordial follicles; primordial follicle oocytes continue to decrease in number after birth (32), and finite oocytes are constantly enduring adverse effects, such as free radical attack (33). The deficiency of excessive DNA damage can account for a higher proportion of aneuploidy in aged oocytes (34). For infertile couples, implantation failure or spontaneous abortion often occurs in women more than 35 years old, especially in women over 40 years old (35), while oocyte donor cycle data clearly

indicate oocyte quality instead of uterine factors which are responsible for adverse pregnancy outcomes (36). Maternal age is an apparent negative factor in obtaining a blastocyst, and elevated age is related to lower quality blastocysts. In addition to compromised oocytes of DNA damage, a lack of a vigorous repair system makes aged oocytes more sensitive to the culture environment (37–39). The paternal effects, including age, and subfertility factors, were weakened to blastocyst formation by oocyte repair ability (40, 41).

To improve the success rate in one oocyte-pick-up cycle, ovary stimulation is universally adopted in clinics. A GnRH agonist or antagonist is applied to prevent premature luteal surge. Compounded with specific gonadotropins, different individualized controlled ovary stimulation protocols can be realized. In addition, with the nonspecific adjuvant drugs letrozole and clomiphene citrate, soft or mild stimulation is recommended in poor ovary reservation patients. To date, ovary function, medication safety, minimized risk, and number of oocytes are under consideration to design an individualized protocol (42), so it is unrealistic to promote the best stimulation protocol to fit all the patients. Compared with the GnRH agonist protocol, the GnRH antagonist protocol is more recommended for reducing the risk of OHSS, especially for high or normal responders (43, 44), but there is no evidence of a difference of in the pregnancy rate and live birth rate in different stimulation protocols (44). Short stimulation without excessive pituitary suppression fits women with insufficient ovary function, while it may have a deleterious effect on blastocyst formation (45, 46). Luteal phase ovary stimulation is a flexible, economic, and friendly choice for IVF patients. The basal LH concentration was correlated with blastocyst quality after adjusting for confounding effects of PCOS. This finding suggests that attention should be given to the LH effect on early follicular development. LH plays an important role in accelerating the synthesis of follicular steroids, which influence bidirectional communication between follicles and oocytes by targeting theca and granulosa cells (47). Presumably, LH is correlated with oocyte maturation and later embryo development.

A capable blastocyst is a first crucial step to reach successful implantation. According to the ESHRE published document about the key performance index of the embryo lab, only 60% of zygotes developed to blastocyst (48), which means that 40% of fertilized oocytes showed developmental arrest of preblastocysts. Even though there was a similar cumulative pregnancy rate between the cleavage and blastocyst methods, until now, there was no compelling evidence to support the concept that cleavage embryos that failed to form blastocysts in vitro would do so if they were transferred into the uterus. Some authors found that a higher cumulative pregnancy rate in the cleavage stage gave a possible speculation of inferiority in vitro (29).

ROS are an observational index to reveal culture conditions in vitro. Higher oxygen stress is significantly related to a high incidence of cleavage arrest (7). For complicated two-faced characteristics of oxygenic radicals, exogenous antioxidants such as glutathione do not work all the time (49, 50). Interestingly, media from different brands result in different gene expression profiles regarding ROS, as proven by nested PCR, and neither of them can mimic female reproductive tract conditions to decrease the adverse effects of ROS (51). Similarly, on the basis of animal observational or experimental data, different

compositions, pH and osmotic pressure merchandised media were developed. All of these media cannot simulate the environment in vivo, which is a well-orchestrated autocrine and paracrine network that provides a dynamic ideal milieu including pH and osmotic pressure. Previous studies have shown that a lack of a complex growth factors derived from follicles, oviducts and endometrium (52), and suboptimal in vitro conditions are prone to aberrant gene expression. GLUT1, as a stress-related protein, may be upregulated compared with in vivo, and cell cycle arrest proteins such as BUB3, NOLC1, PCAF, GABPA and CNOT4 were significantly differentially expressed in another study (53). OCT4 expression also showed differences in different culture media (54). Epigenetics research found that compared to in vivo-derived embryos at an early stage, commercial media cause the genomic imprinting problem of methylation loss (55). It is reasonable to speculate that a suboptimal culture environment compromises potential embryo.

Lack of embryo quality comparison maybe influence bias of result. Meta-analysis supported that the number of retrieval oocytes was correlated with embryo quality(56). Meanwhile, we try to enlarge the sample of size to minimize the bias of embryo quality. Another major limitation of the study is that we do not survey gene defect about embryo development. An abnormal cleavage pattern may be the main reason for arrest in early embryos, and the aberrant divisions result in chromosome uneven distribution to daughter cells (26). Some maternal gene defects, such as TUBB8 (57), TRIP13 (58), NOP2 (59) etc. interfering with cyclic checkpoints or chromosome assembly, resulting in early embryo arrest. Apparently, intrinsic contraction cannot be changed by an additional setting in vitro. A possible reason for lower implantation capacity maybe the gene defects if sibling cleavage embryos were transferred.

A good oocyte is necessary for later development. Patients' age, stimulation protocol, and environment in vitro are involved in blastocyst formation, and cycles with available blastocysts point to better pregnancy outcomes. In cycles with adverse blastocyst culture outcomes, cleavage embryos from sibling oocytes still have the potential to have a birth.

Abbreviations

ART
Assisted reproductive technology
IVF
In vitro fertilization
ICSI
Intracytoplasmic sperm injection
ROS
Rective Oxygen Species
NAB
Cycles without any blastocyst formation
CAB
Cycles with available blastocyst formation
CNB

Cycles with nonavailable blastocyst formation
PCOS
Polycystic ovary syndrome
PGT
Preimplantation genetics test
aOR
Adjusted odds ratio
CI
Confidence interval
OPU
Oocyte pick-up
TTP
Time to pregnancy

Declarations

Ethics approval and consent to participate

The study plan was approved by the Ethics Committee of the First Affiliated Hospital of USTC. All methods were carried out in accordance with the guidelines of the Declaration of Helsinki.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

J.R.T and T.X.H contributed to the conception of the study; J.R.T and Z.N.R performed the data analyses and wrote the manuscript; J.X.H helped perform the analysis with constructive discussion; X.B, L.F.T collected and analyzed the data; L.L.H and L.H.B interpreted the patient data regarding the ovary stimulation protocol, age and embryo development. All authors read and approved the final manuscript.

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Figures

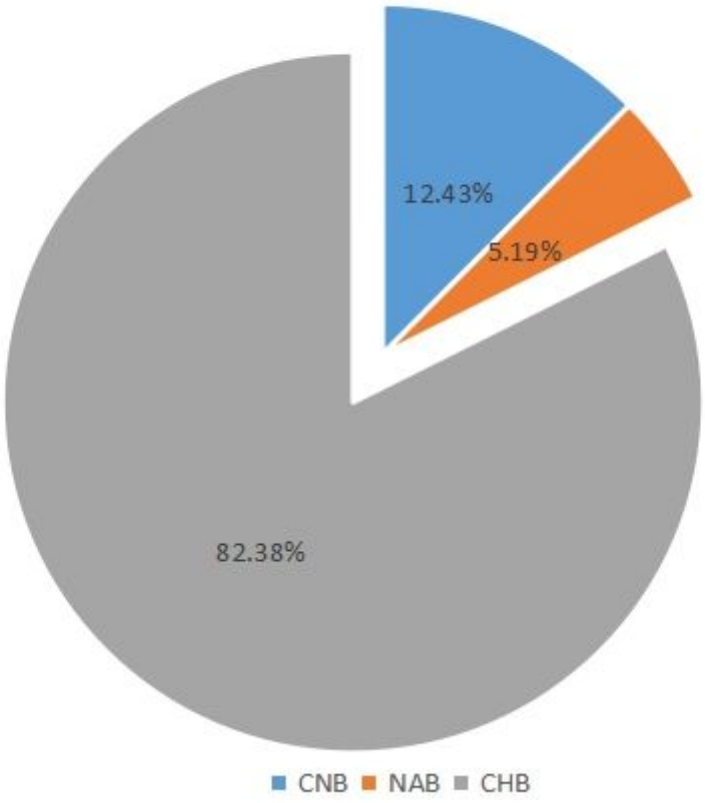

Figure 1

Distribution of blastocyst culture outcome with two day-3 embryos unenrolled. Two of the top-scoring embryos were cryopreserved or transferred, and the surplus day-3 embryos were extended and cultured for blastocyst. The culture outcome was divided into three groups; there was no blastocyst formation (CNB); only poor blastocysts were got (NAB); and CHB was defined as cycles in which there was at least 1 blastocyst kept.

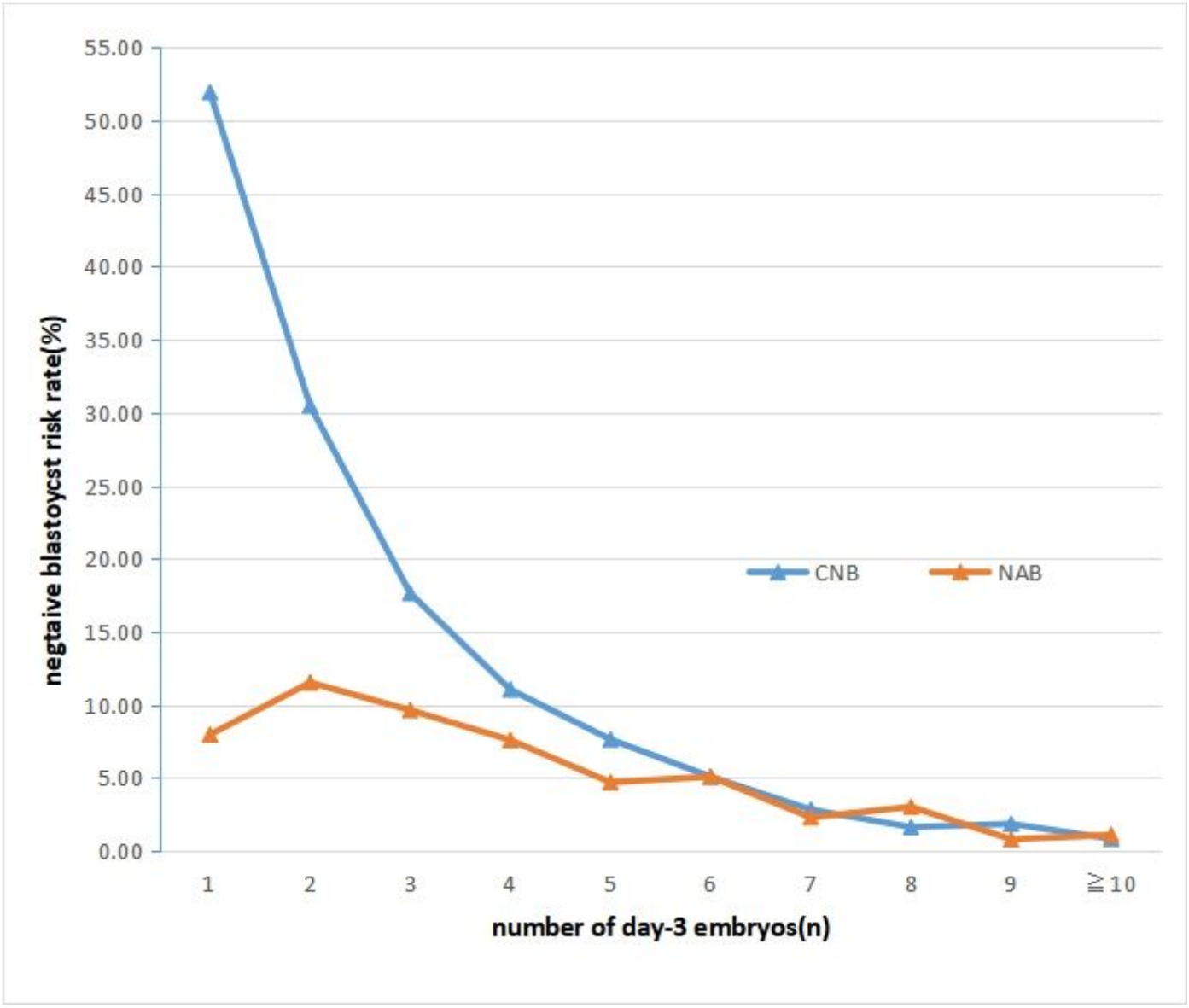

Figure 2

Adverse blastocyst formation outcomes based on the number of day-3 embryos. Two of the top- scoring embryos were cryopreserved or transferred, and then surplus day-3 embryos were extended and cultured for blastocyst. The negative blastocyst formation outcome included two groups, there was no blastocyst formation (CNB); only poor blastocysts were obtained (NAB).