



OPEN Biphasic oxygen tension promotes the formation of transferable blastocysts in patients without euploid embryos in previous monophasic oxygen cycles

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This study evaluated whether the concentration of biphasic O₂ (5–2%) promotes the formation of qualified blastocysts (QBs) and euploid blastocysts and the probability of cycles with transferable blastocysts. The paired experimental design included a total 90 patients (180 cycles) without euploid blastocysts in previous monophasic O₂ (5%) cycles were enrolled for an additional cycle of biphasic O₂ (5–2%). In the biphasic O₂ (5–2%) group, the QB rate (35.8%, 225/628) was significantly higher than that in the monophasic O₂ (5%) group (23.5%, 137/582; $p < 0.001$). In addition, the euploid blastocyst number (0.5 ± 0.8) and the percentage of cycles with transferable blastocysts were significantly higher in the biphasic O₂ (5–2%) group (57.8%, 52/90) than those in the monophasic O₂ (5%) group (0 and 35.6%, 32/90, respectively; $p < 0.01$). Multivariable regression analysis also indicated that the QB rate and the probability of cycles with transferable blastocysts correlated with O₂ tension (OR 1.535, 95% CI 1.325–1.777, and OR 3.191, 95% CI 1.638–5.679, respectively; $p < 0.001$). Biphasic O₂ culture can be used as an alternative strategy to increase the euploid QBs and the probability of cycles with transferable blastocysts in patients with a poor prognosis.

Recent evidence indicates that the preimplantation embryo of most mammals develops in an increasingly hypoxic and dynamic O₂ tension environment (2–8%) in vivo^{1–3}. Although the majority of in vitro fertilization (IVF) centers have recognized the superiority of monophasic O₂ (5%) tension in in vitro blastocyst development and intracellular redox balance, the biphasic O₂ (5–2%) concentration in blastocyst culture systems remains more similar to the increasingly hypoxic and dynamic O₂ tension environment in vivo^{4–7}. According to previous research, human embryos in vivo usually cross the utero-tubal junction on day 3⁸; thus, the O₂ tension is reduced from 5 to 7% in the oviduct to 1–5% in the uterus^{2,8–10}. Therefore, for blastocyst development in vitro after day 3 of culture, O₂ tension may need to be reduced from 5 to 2% to mimic the physiologic O₂ tension dynamics in vivo⁶. According to a recent study, biphasic O₂ (5–2%) has beneficial effects on pre- and peri-implantation embryonic development in mice¹¹; biphasic O₂ may be appropriate for human blastocyst formation⁶. In addition, biphasic O₂ (5–2%) tension culture may be advantageous for patients with poor embryonic development by increasing the number of available high-quality blastocysts¹².

According to previous studies, reducing O₂ tension in the culture environment may reduce the levels of reactive oxygen species (ROS) and apoptosis, thereby promoting the in vitro development of embryos^{5,6}. In addition to its effect on the quality of embryos, the O₂ concentration may be linked to the mitochondrial structure¹³ and chromosomal errors^{14,15}. ROS-induced damage increases the incidence of chromosome aneuploidy in mouse

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embryos obtained through IVF, and this damage is primarily mediated by chromosome missegregation¹⁶. The embryo culture system is another factor associated with ploidy during embryonic development, and the rate of embryonic aneuploidy may be related to the culture conditions of oocyte donor cycles in IVF laboratories¹⁷. Numerous factors, including culture medium (pH and constituents), light, temperature, and gas phase, may affect the quality of in vitro cultures, resulting in a substantial increase in the concentration of ROS¹⁸. Thus, during in vitro cultures, oxidative stress induced DNA damage is inevitable, disrupting the development of embryonic cells^{19,20}. Aneuploidy is the leading cause of embryonic development arrest²¹. However, compared with high O₂ (20%) tension conditions, low O₂ (5%) conditions generate a larger number of high-quality embryos and euploid blastocysts available for embryo transfer²².

In humans, the effect of biphasic O₂ (5–2%) on embryonic ploidy remains unknown. Similarly, whether biphasic O₂ (5–2%) conditions are useful for embryonic ploidy remains unclear. In preimplantation genetic testing for aneuploidy (PGT-A) cycles, selecting euploid embryos for transfer may eventually increase the probability of embryonic implantation^{23,24}. However, the success rate of cycles with PGT-A remains limited by the high incidence of cycles with unsuccessful PGT-A or cycles without transferable euploid embryos²⁵. Previous studies have reported the no-euploid-embryo rate of 25% for transfer²⁶. However, the factors associated with the formation of embryos with an euploidy in humans vary greatly^{27,28}. Even though oocytes are collected from young patients, such as oocyte donors²⁹ or young patients (22–25 years) within fertility³⁰, the aneuploidy rate in some IVF cycles seems to be high. Therefore, this study enrolled patients without euploid blastocysts for transfer in previous monophasic O₂ (5%) cycles for determining whether the use of biphasic O₂ (5–2%) in the next culture cycle can promote the formation of qualified blastocysts (QBs) and euploid blastocysts. In addition, improvements in cycles with at least one euploid blastocyst or with at least one transferable blastocyst (non-aneuploidy) were also compared between the monophasic O₂ (5%) and the biphasic O₂ (5–2%) groups. Our primary goal was to examine the formation of euploid blastocyst in two O₂ tension groups. Our secondary goal was to determine the rate of QBs formation, transferable blastocysts and the probability of cycles with transferable blastocysts in the two O₂ tension groups.

Results

Differences in baseline characteristics between biphasic O₂ (5–2%) and monophasic O₂ (5%) groups. Table 1 lists the baseline characteristics of the monophasic O₂ (5%) group ($n=90$) and biphasic O₂ (5–2%) group ($n=90$). The average age of the women included in this study was 39.5 ± 3.7 (27–45) years, and their average anti-Müllerian hormone (AMH) level and body mass index (BMI) were 2.3 ± 1.8 (0.5–13.7) ng/mL and 22.9 ± 3.8 (17.6–36.1) kg/m², respectively. Paired comparisons revealed no significant differences in the type of ovarian stimulation protocol, the dosage of FSH, the level of E₂ on the day of hCG injection, the level of P₄ on the day of hCG injection, the numbers of MII oocytes, and the numbers of 2PN between the groups. However, the level of luteinizing hormone (LH) on the day of human chorionic gonadotropin (hCG) injection was significantly higher in the monophasic O₂ (5%) group (2.6 ± 2.5 IU/L) than in the biphasic O₂ (5–2%) group (1.7 ± 1.7 IU/L) ($p=0.001$). In addition, the average number of oocytes was significantly smaller in the monophasic O₂ (5%) group (10.7 ± 4.7) than in the biphasic O₂ (5–2%) group (11.5 ± 5.9) ($p=0.047$).

Differences in embryological and ploidy outcomes between biphasic O₂ (5–2%) and monophasic O₂ (5%) groups. Paired comparisons revealed no significant differences in the total number of blastocysts between the groups (2.9 ± 1.7 vs 3.8 ± 2.4 , Table 2). The number of QBs in the biphasic O₂ (5–2%) group (2.5 ± 1.6) was significantly higher than that in the monophasic O₂ (5%) group (1.5 ± 1.2) ($p<0.001$). However, the formation rates of blastocysts, QBs, and day-5 QBs were significantly higher in the biphasic O₂ (5–2%) group (54.8%, 35.8%, and 17.5%, respectively) than in the monophasic O₂ (5%) group (45.4%, 23.5%, and 10.5%, respectively) ($p<0.05$). Nine cycles (10%, 9/90) in the monophasic O₂ (5%) group produced no QBs for biopsy. However, all cycles in the biphasic O₂ (5–2%) group produced at least one QB for biopsy. A total of

Characteristics	Monophasic (5%) O ₂	Biphasic (5–2%) O ₂	<i>p</i> value
OPU cycles (patients)	90	90	–
<i>Ovarian stimulation</i>			
GnRH agonist (%)	15.6 (14/90)	22.4 (20/90)	0.245
GnRH antagonist (%)	85.0 (76/90)	77.6 (70/90)	0.203
Total dose of FSH (IU, mean \pm SD)	3015 \pm 556 (2100–4575)	3117 \pm 641 (1200–5775)	0.126
E ₂ levels (pg/mL, mean \pm SD)	1562 \pm 1180 (141–8763)	1423 \pm 862 (104–4510)	0.619
P ₄ levels (ng/mL, mean \pm SD)	0.84 \pm 0.48 (0.13–2.66)	0.78 \pm 0.55 (0.15–3.89)	0.340
LH levels (IU/L, mean \pm SD)	2.6 \pm 2.5 (0.2–17.3)	1.7 \pm 1.7 (0.2–10.3)	0.001
Numbers of retrieved oocytes (mean \pm SD)	10.7 \pm 4.7 (2–24)	11.5 \pm 5.9 (3–33)	0.047
Numbers of mature oocytes (MII) (mean \pm SD)	8.4 \pm 3.8 (2–19)	8.8 \pm 4.7 (2–29)	0.285
Numbers of 2PN (mean \pm SD)	6.5 \pm 3.0 (2–15)	7.0 \pm 3.5 (2–20)	0.173

Table 1. Baseline characteristics of biphasic O₂ (5–2%) and monophasic O₂ (5%) concentration groups. The chi-square (χ^2) test and Wilcoxon signed rank test were performed to analyze statistical significance. Values in the parentheses were presented as minimal-maximal values or ratio.

Parameters	Monophasic (5%) O ₂	Biphasic (5–2%) O ₂	p value
Cycles (patients)	90	90	–
Numbers of blastocysts (mean ± SD)	2.9 ± 1.7 (0–8)	3.8 ± 2.4 (1–14)	0.095
Numbers of qualified blastocysts (QBs) (mean ± SD)	1.5 ± 1.2 (0–6)	2.5 ± 1.6 (1–9)	<0.001
Blastocyst rates (%)	45.4 (264/582)	54.8 (344/628)	0.001
QB rates (%)	23.5 (137/582)	35.8 (225/628)	<0.001
Rates of day 5 QBs (%)	10.5 (61/582)	17.5 (110/628)	<0.001
Rates of day 6 QBs (%)	13.1 (76/582)	18.2 (114/628)	0.015
Cycles without QBs (%)	10% (9/90)	0 (0/90)	0.002
<i>Embryo ploidy</i>			
Numbers of euploid blastocysts (mean ± SD)	0 (0–0)	0.5 ± 0.8 (0–3)	<0.001
Numbers of aneuploid blastocysts (mean ± SD)	1.0 ± 1.0 (0–5)	1.4 ± 1.2 (0–5)	0.020
Numbers of mosaic blastocysts (mean ± SD)	0.5 ± 0.8 (0–3)	0.7 ± 1.0 (0–5)	0.209
Numbers of transferable blastocysts (mean ± SD)	0.5 ± 0.8 (0–3)	1.1 ± 1.4 (0–5)	<0.001
Euploidy rate	0 (0/137)	19.6% (44/225)	<0.001
Rate of transferable blastocyst (%)	32.8% (45/137)	45.8% (103/225)	0.015
Percentage of cycles with at least one euploid blastocyst (%)	0 (0/90)	33.3 (30/90)	<0.001
Percentage of cycles with at least one transferable blastocyst (%)	35.6 (32/90)	57.8 (52/90)	0.003

Table 2. Embryological outcomes in biphasic O₂ (5–2%) and monophasic O₂ (5%) concentration groups. The chi-square (χ^2) test and Wilcoxon signed rank test were performed to analyze statistical significance. Values in the parentheses were presented as minimal-maximal values or ratio. QB: qualified blastocyst; BR: blastocyst rate per 2PN.

137 and 225 QBs from the monophasic (5%) and biphasic O₂ (5–2%) groups, respectively, were biopsied and subjected to PGT-A. The rate of euploidy in the biphasic O₂ (5–2%) group (19.6%, 44/225) was significantly higher than that in the monophasic O₂ (5%) group (0%, 0/137) ($p < 0.001$). The number of euploid blastocysts in the biphasic O₂ (5–2%) group (0.5 ± 0.8) was significantly higher than that in the monophasic O₂ (5%) group (0) ($p < 0.001$). The number and formation rate of transferable blastocysts in the biphasic O₂ (5–2%) group (1.1 ± 1.4, 45.8%, 103/225) were significantly higher than those in the monophasic O₂ (5%) group (0.5 ± 0.8, 32.8%, 45/137) ($p < 0.001$ and $p = 0.015$, respectively). After incubation under biphasic O₂ (5–2%) conditions, the incidence of cycles with at least one euploid blastocyst increased from 0 to 33.3% (30/90). In addition, the incidence of cycles with at least one transferable blastocyst increased from 35.6% (32/90) to 57.8% (52/90).

Correlation between O₂ tension and the QB formation rate. Univariate regression analysis was performed to identify the individual variables were associated with the formation rate of QBs (Supplementary Table 1). The results indicated that the formation rate of QBs was significantly correlated with the LH level on the day of hCG injection (odds ratio [OR] 0.950, 95% confidence interval [CI] 0.907–0.995; $p = 0.031$), AMH level (OR: 0.923, 95% CI 0.856–0.996; $p = 0.040$), number of oocytes (OR: 0.973, 95% CI 0.953–0.992; $p = 0.007$), number of MII oocytes (OR: 0.965, 95% CI 0.941–0.990; $p = 0.007$), and O₂ tension (OR: 1.516, 95% CI 1.295–1.775; $p < 0.001$). Considering the LH level on the day of hCG injection and the number of oocytes, the multivariable regression analysis indicated that the biphasic O₂ (5–2%) condition was positively correlated with the QB formation rate, as compared with the monophasic O₂ (5%) condition (adjusted OR: 1.535, 95% CI 1.325–1.777; $p < 0.001$) (Table 3).

Correlation between O₂ tension and the probability of cycles with transferable blastocysts. Univariate regression analysis was performed to identify potential variables correlated with cycles with transferable blastocysts (Supplementary Table 2). The results indicated that cycles with transferable blastocysts

Parameters	B	p value	OR	95% CI
LH levels (IU/L)	–0.036	0.074	0.964	0.926–1.004
Numbers of retrieved oocytes	–0.034	<0.001	0.966	0.949–0.984
Biphasic O ₂ (5–2%) culture	0.428	<0.001	1.535	1.325–1.777
Monophasic O ₂ (5%) culture	0		1	

Table 3. The multivariate regression analysis to determine the correlations between variables and the rate of QBs. GEE regression was performed to analyze statistical significance. B: B-coefficient, OR: Odds Ratio, 95% CI: 95% confidence interval.

were significantly correlated with the age of the women (OR: 0.781, 95% CI 0.700–0.871; $p < 0.001$), level of E_2 (OR: 1.001, 95% CI 1.000–1.001; $p = 0.003$), number of 2PN (OR: 1.136, 95% CI 1.038–1.243; $p = 0.006$), number of QBs (OR: 2.336, 95% CI 1.740–3.136; $p < 0.001$), and O_2 tension (OR: 2.480, 95% CI 1.478–4.161; $p = 0.001$). Therefore, on the basis of these results, age, E_2 level on the day of hCG injection, and number of 2PN were selected as confounders for adjustment. As shown in Table 4, multivariable regression analysis revealed that the biphasic O_2 (5–2%) condition was positively correlated with the probability of cycles with transferable blastocysts, as compared with the monophasic O_2 (5%) condition (adjusted OR: 3.191, 95% CI 1.638–5.679; $p < 0.001$). In addition, patients were further divided into subgroups with age ≤ 38 years and age > 38 years (Supplementary Table 3). The consistent effects of biphasic O_2 tension strategy were revealed to significantly improve QB rates (37.4% vs. 26.4% and 35.6% vs. 22.4%), euploid rates (25% vs. 0% and 16.8% vs. 0%), and percentages with cycles with at least one transferable blastocyst (46.2% vs. 0% and 28.1% vs. 0%) in both age groups (Supplementary Table 3).

Discussion

In this study, we evaluated the effects of biphasic O_2 (5–2%) cultures on embryonic ploidy and development in women without euploid embryos in a previous cycle with monophasic O_2 (5%). We discovered that biphasic O_2 (5–2%) is one of the major factors that improves the development of blastocysts. The results of the biphasic O_2 (5–2%) culture revealed that (1) the number and proportion of QBs considerably increased, (2) the number of euploid embryos and the number of aneuploid embryos increased and decreased, respectively, and (3) the number of transferable QBs and the probability of cycles with transferable QBs increased. Multivariable regression analysis also revealed that the biphasic O_2 (5–2%) culture was strongly correlated with an increase in the QB formation rate and the probability of cycles with transferable blastocysts.

Although the consumption of O_2 substantially increases at compaction³¹, during the phase of blastomere differentiation into TE and ICM, low O_2 tension promotes the metabolic preference of glucose being the primary energy substrate^{31,32}, thereby protecting against excess oxidative stress, which is associated with high metabolic activity³³. Relatively low metabolic levels are strongly linked to embryonic development^{34,35}, and surviving embryos usually exhibit reduced oxidative phosphorylation activity and O_2 consumption³⁶. Compared with less metabolically active embryos, embryos with higher active metabolism exhibit increased ROS levels³⁷, excessive DNA damage³⁸, and poor embryonic development³⁷. In addition to endogenous ROS, in embryonic cultures in vitro, high levels of exogenous ROS are formed from the environment during embryonic culture or manipulation³⁹. Previous studies have reported that reducing O_2 tension under culture conditions may decrease the ROS level and cell apoptosis, thereby improving embryonic development in vitro^{5,6}. However, hypoxia promotes the formation of blastocysts in cattle, especially during the post compaction phase⁴⁰. These beneficial effects of the low O_2 concentration during the development of blastocysts in vitro may be stage-dependent⁶. Previous animal studies have reported that biphasic O_2 (7–2% or 5–2%) tension promotes the formation and quality of preimplantation blastocysts^{9,41,42}. Therefore, we hypothesized that adjusting the biphasic O_2 concentration for different embryonic stages, depending on the requirements of stage-specific metabolism during cleavage and blastocyst formation, has a positive effect on the in vitro cultivation of human embryos.

Despite the effects of hypoxia on metabolism, studies have indicated that the O_2 concentration affects the expression of genes in preimplantation embryos⁴³. In hypoxic environments, O_2 tension promotes the development of mouse blastocysts and reduces apoptosis by regulating HIF-2 α , thereby increasing the expression of antioxidant enzymes, such as MnSOD, PRDX5, and vascular endothelial growth factor (VEGF)⁴⁴. In bovine and mouse preimplantation embryos, biphasic O_2 (7–2% or 5–2%) changes the expression of genes involved in embryonic metabolism^{9,45,46}. Compared with embryos cultured under monophasic O_2 (20% or 7%) tension, blastocysts cultured under biphasic O_2 (7–2%) tension have higher ICM and glucose transporter 1 (GLUT-1), glucose transporter 3 (GLUT-3), and VEGF expression⁴⁵. Unlike monophasic O_2 (5% or 20%) tension, biphasic O_2 (5–2%) tension increases the developmental capacity and the activation of the HIF-1 α transcription factor and related genes of embryonic development, including H2az, Cdx2, Oct-41, and 16 s rRNA⁹. According to Kaser et al., biphasic O_2 (5–2%) cultures produce an increased total yield of blastocysts and a large number of usable blastocysts, thereby recapitulating the physiologic O_2 environment in vivo⁶. Brouillet's study (2021) has also highlighted the clinical outcomes of biphasic O_2 (5–2%) culture⁴⁷. Generally, biphasic O_2 (5–2%) cultures are associated with considerably increased cumulative live birth and total blastocyst and usable blastocyst formation rates⁴⁷. Under biphasic O_2 conditions, changing the relative abundance of anabolic amino acids and metabolites

Parameters	B	<i>p</i> value	OR	95% CI
Women age (years)	– 0.243	< 0.001	0.784	0.684–0.857
E_2 levels (IU/L)	0.000	0.172	1.000	1.000–1.001
Numbers of 2PN	0.101	0.055	1.107	0.998–1.227
Biphasic O_2 (5–2%) culture	1.160	< 0.001	3.191	1.638–5.679
Monophasic O_2 (5%) culture	0		1	

Table 4. The multivariate regression analysis to determine the correlations between variables and the probability of cycles with transferable blastocysts. GEE regression was performed to analyze statistical significance. B: B-coefficient, OR: Odds Ratio, 95% CI: 95% confidence interval.

involved in redox homeostasis and the differential expression level of MUC1 in TE cells may improve the development of human embryos⁶. Moreover, whole transcriptome analysis of blastocysts revealed that 707 RNAs are differentially expressed depending on the strategy of O₂ supplementation⁴⁷. These genes are mainly involved in embryonic development, DNA repair, embryonic stem cell pluripotency, and implantation potential⁴⁷. In this study, we confirmed that in patients with a poor prognosis, the rate of blastocyst formation in biphasic O₂ (5–2%) cultures was higher than that in monophasic O₂ (5%) cultures (Table 2). We also reported that all cycles in biphasic O₂ (5–2%) cultures have at least one QB suitable for embryonic biopsy (Table 2).

To the best of our knowledge, this is the first study to examine the effects of biphasic O₂ (5–2%) tension on blastocyst ploidy. Generally, well-balanced embryonic development is associated with optimal and quiet metabolism³⁵, instead of active metabolism, which negatively affects the quality of embryos by increasing the ROS levels³⁷. In addition, during in vitro cultivation, ROS from endogenous and exogenous sources may result in molecular damage to embryonic chromosome segregation apparatuses and may cause aneuploidy over time⁴⁸. In mouse embryos obtained through IVF, ROS-induced damage seems to increase the incidence of chromosomal aneuploidy¹⁶. During embryonic development, ROS overproduction is associated with negative outcomes, such as impaired mitochondrial function, aging, DNA damage, and impaired chromosome segregation⁴⁹. Therefore, reducing O₂ tension under culture conditions has been suggested to improve the genomic integrity of developing embryos by decreasing the levels of ROS^{5,6}.

Our results revealed that the patients included in this study (with a poor prognosis) had a considerably increased number of euploid blastocysts after biphasic O₂ (5–2%) culture (Table 2). After incubation was performed under a biphasic O₂ (5–2%) condition, the incidence of cycles with at least one euploid blastocyst increased from 0 to 33.3% (30/90) (Table 2). Because the patients had no or a small number of euploid blastocysts suitable for transfer, they were presented with the option of mosaic blastocyst transfer. According to previous studies, mosaic embryos may result in healthy babies^{50,51}. In this study, all euploid and mosaic embryos are defined as transferable blastocysts, the number of transferable blastocysts and the probability of cycles with transferable blastocysts substantially increased under the biphasic O₂ (5–2%) condition compared with under the monophasic O₂ (5%) condition (Table 4). Given the age of the women included in this study, biphasic O₂ (5–2%) tension still increased not only the number of euploid blastocysts but also the rate of euploid blastocyst formation. Studies have indicated that self-correction of aneuploid and mosaic embryos considerably increases after the eight-cell stage⁵², and that approximately 9.7% of day-3 aneuploid embryos undergo complete self-correction to form euploid blastocysts⁵². Therefore, we suggest that an ultralow O₂ (2%) concentration of the biphasic O₂ (5–2%) strategy may further improve self-correction during blastocyst formation by reducing the effects of ROS.

In addition, women's age was also related to ploidy status in this study. The spearman's correlation (Supplementary Table 4) analysis showed that women age significantly negatively correlation with euploidy ($r: -0.189, p < 0.05$) and positively correlation with aneuploidy ($r: 0.437, p < 0.01$) rates. The multivariable regression analysis revealed that the women age was negatively correlated with the probability of cycles with transferable blastocysts (adjusted OR: 0.784, 95% CI 0.684–0.857; $p < 0.001$; Table 4) after adjustment of O₂ conditions. This study demonstrated that even in the biphasic O₂ (5–2%) culture condition, women age remained an important factor associated with embryo ploidy. The increase of euploid rate (from 0 to 25%) in the younger patient group was higher than that (from 0% to 16.8%) in the older patient group (Supplementary Table 3). Previous studies have reported that increased ROS and elevated vulnerability of oocytes to ROS lead to reduce the fertilization and developmental competency in aged patients^{53,54}. Therefore, the beneficial effects of biphasic O₂ (5–2%) culture might be influenced by the insufficient antioxidant capability in older patients⁵⁵.

Because the oocytes derived from one stimulation cycle could not be divided into different groups in some of the target patients, we compared the effects of biphasic O₂ (5–2%) and monophasic O₂ (5%) cultures by using a design of self-paired comparison from two continuous cycles of individual patients. However, one limitation must be acknowledged; that is, we discovered that a few baseline characteristics differed between cycles (i.e., number of retrieved oocytes) (Table 1). Nevertheless, the numbers of mature oocytes and 2PN were similar between the two groups, and Spearman's correlation analysis revealed that the baseline characteristics were not correlated with the primary and secondary outcomes (Table 1 and Supplementary Table 4). Therefore, by considering confounders, we further confirmed the beneficial effects of biphasic O₂ (5–2%) cultures on the rate of QBs formation and the probability of cycles with transferable blastocysts. In general, embryos cultured at reduced O₂ levels from 20 to 2% require a nitrogen gas system and quality control procedures for the O₂ sensors. Compared with the atmospheric or monophasic O₂ (5%) culture conditions adopted by the majority of embryological laboratories, biphasic O₂ (5–2%) cultures may result in increased laboratory equipment costs. Moreover, given the nature of retrospective studies, which lack randomization and may involve selection bias, randomized controlled trials are suggested to robustly assess these interesting results.

In this study, we included women who had a small number of QBs and lacked euploid blastocyst formation under monophasic O₂ (5%) culture conditions. The results revealed that biphasic O₂ (5–2%) culture not only improved the development of QBs but also promoted the formation of euploid blastocysts, thereby overcoming the challenges of embryonic development. This technique also considerably increased the probability of cycles with transferable blastocysts. In conclusion, biphasic O₂ (5–2%) culture can be used as an alternative strategy to increase the incidence of transferable blastocysts and to reduce the cancellation rate of blastocyst transfer in patients with a poor prognosis.

Materials and methods

Patient selection. This was a retrospective cohort study and a single-center clinical trial that aimed to evaluate the effects of O₂ tension, including monophasic O₂ (5%) and biphasic O₂ (5–2%) culture systems, on blastocyst formation and ploidy in PGT-A cycles. The study cohort included 90 women with 180 cycles who were

referred to Lee Women's Hospital, Taiwan, and treated with PGT-A from March 2018 to March 2021. Patients who had no euploid blastocysts in monophasic O₂ (5%) cultures in previous cycles and underwent biphasic O₂ (5–2%) cultures in the following cycles were included, whereas oocyte and sperm donors and patients with male factors, such as sperms obtained from testicular or epididymal sperm aspiration or extraction, were excluded. PGT-A cycles were divided into two subgroups of embryo culture, namely monophasic O₂ (5%) and biphasic O₂ (5–2%). The treatment histories and clinical outcomes of all patients were recorded from the database of Lee Women's Hospital. This retrospective data analysis was approved by the Institutional Review Board of Chung Shan Medical University, Taichung, Taiwan (CS1-21156). Our study methods and analysis conform to the guidelines and regulations set by the agreement with the Institutional Review Board of Chung Shan Medical University IRB. The need to obtain informed consent in this study was waived by the Institutional Review Board of Chung Shan Medical University IRB.

Controlled ovarian stimulation protocols. Both gonadotropin-releasing hormone (GnRH) agonist (Lupron; Takeda Chemical Industries, Tokyo, Japan) and antagonist (cetorelix acetate; Merck Serono, Darmstadt, Germany) protocols were used for controlled ovarian stimulation (COS). For ovarian stimulation, all patients received a recombinant follicle-stimulating hormone (FSH, Gonal-F; Merck Serono) from cycle day 3 until the diameter of the dominant follicle exceeded 18 mm, followed by a dual trigger, including 250 µg of human chorionic gonadotropin (hCG) (Ovidrel; EMD Serono, Rockland, MA, USA), and 0.2 mg of triptorelin (Decapeptyl; Ferring, Schleswig-Holstein, Germany) at 36 h before oocyte retrieval. Finally, COS procedures involving GnRH agonist and antagonist protocols, oocyte collection, and denudation were performed as previously described⁵⁶.

Embryo culture. Before fertilization, the retrieved oocytes were cultured in Quinn's Advantage Fertilization Medium (Sage BioPharma, Trumbull, CT, USA) supplemented with 15% serum protein substitute (SPS; Sage BioPharma) in a triple gas phase under 5% CO₂, 5% O₂, and 90% N₂. The insemination methods in this study were including ICSI (110 cycles) and half-ICSI (70 cycles, oocytes from the same patient are divided into half of the oocytes for conventional insemination (IVF) and the other half for ICSI). Conventional insemination (IVF) or intracytoplasmic sperm injection (ICSI) was then performed 38–41 h after the dual trigger. Following conventional insemination or ICSI, all embryos were further cultured in microdrops of cleavage medium (Quinn's Advantage Cleavage Medium; Sage BioPharma) supplemented with 15% SPS. Embryo culture was then performed until day 3 (at 70–72 h after insemination or ICSI) in cleavage medium under 5% O₂. On day 3, all cleaved embryos were group-cultured in microdrops of blastocyst medium (Quinn's Advantage Blastocyst Medium; Sage BioPharma) supplemented with 15% SPS. The culture systems were divided into monophasic O₂ (5%) and biphasic O₂ (5–2%) tension groups. In the monophasic O₂ (5%) tension group, the embryos were cultured in a 5% O₂ incubator until day 5/6. In the biphasic O₂ (5–2%) tension group, the embryos were transferred into 2% O₂ incubators on day 3 for culturing until day 5/6. All embryos were individually cultured before day 3 and then group-cultured thereafter. Laser-assisted hatching was performed on day 4. Once the embryos reached the blastocyst stage, trophectoderm (TE) biopsy was performed as described by Chen et al.⁵⁶. The quality of the blastocysts was then immediately assessed before TE biopsy according to the criteria of Gardner and Schoolcraft⁵⁷. Only high-quality expanded blastocysts (grade 4, 5, 6, AB, BA, or BB) with a blastocyst diameter ≥ 150 µm were defined as QBs suitable for TE biopsy. On day 5, fully expanded or hatching blastocysts with a qualified TE and inner cell mass (ICM) were considered adequate for TE biopsy. Blastocysts without expansion (< 150 µm) on day 5 were further cultured until day 6, and TE biopsy was then performed on the expanded blastocysts with ICM and TE grade A or B. The biopsied TE cells were then immediately placed in an RNase/DNase-free polymerase chain reaction tube and amplified using a SurePlex DNA Amplification System (Illumina, San Diego, CA, USA). The extracted cells were then added to 2 µL of buffer and shipped frozen to the genetics laboratory (Genesis Genetics) for PGT-A on a high-resolution next-generation sequencing platform. After TE biopsy, the blastocysts were cryopreserved through vitrification conducted by Cryotech⁵⁶. According to the next-generation sequencing results, blastocysts without aneuploidy were defined as "transferable blastocysts", and cycles with least one transferable blastocyst were defined as "cycles with transferable blastocysts".

Statistical analysis. Wilcoxon's signed-rank test was used to perform pair comparisons of nonparametric and count data. The chi-squared test was then used to compare the percentage results of the biphasic O₂ (5–2%) and monophasic O₂ (5%) groups. On the basis of Spearman rank correlation coefficients, the variables were analyzed through a generalized estimating equation (GEE) and univariable regression was conducted. The outcome variables included the probability of cycles with transferable blastocysts (yes/no) and predictors such as the age of the women (per year), the total FSH dosage (per 1,000 IU), the number of retrieved oocytes (per number), the number of MII oocytes (per number), the number of 2PN (per number), the blastocyst formation rate (per 10%), the insemination method (ICSI/half ICSI), and the O₂ tension type (biphasic/monophasic O₂ tension). Logistic and Poisson's regression analyses were then performed to evaluate the effect of confounding factors on binary data representing the probability of cycles with transferable blastocysts and the count data of QBs, respectively. The total number of 2PN per cycle served as the offset for the Poisson regression model, and a difference with $p < 0.05$ was regarded as statistically significant. All calculations were performed using SPSS version 23.0 (IBM SPSS, Armonk, NY).

Data availability

The data underlying this article will be shared on reasonable request to the corresponding author.

Received: 23 December 2022; Accepted: 13 March 2023

Published online: 15 March 2023

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Acknowledgements

We would like to thank the Lee Women's Hospital and embryologists for their careful work on reporting the data.

Author contributions

M.-S.L., H.-H.C., C.-I.L. and C.-H.C. contribute to the conception and design of the work. H.-H.C., and C.-H.C. contribute to the collection, analysis, or interpretation of data. H.-H.C., C.-H.C. and M.-S.L. contribute to the draft preparation of the manuscript. All authors contribute to the critical revision of the manuscript for important intellectual content and approved the final version. All authors reviewed the results and approved the final version of the manuscript.

Competing interests

The authors declare no competing interests.

Additional information

Supplementary Information The online version contains supplementary material available at <https://doi.org/10.1038/s41598-023-31472-4>.

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