Original Article

Limited Effects of Ultra-low Oxygen Concentration during Extended Embryo Culture on *In vitro* Fertilisation Outcomes in Indian Women: A Retrospective Cross-sectional Study

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Abstract

Background: Amongst various other factors, oxygen (O2) concentration in embryo culture plays an important role in determining pregnancy outcomes in women undergoing in vitro fertilisation. Some studies have reported that lowering O2 levels in embryo culture provides better results. **Aims:** To explore the effects of low- and ultra-low- O2 concentrations (5% and 2%, respectively) in extended embryo culture on various outcome parameters of pregnancy. **Settings and Design:** This was a retrospective cross-sectional study. **Materials and Methods:** In this study 382 participants had their embryos cultured in varying O2 concentrations (5% or 2%), followed by either a fresh embryo transfer (ET) or frozen embryo transfer (FET). Outcomes such as pregnancy rate, implantation rate, abortion rate, twinning rate, and live birth rate were compared between the groups. **Statistical Analysis Used:** Chi square test was applied to compare the primary and secondary outcomes between different groups. **Results:** No significant differences were observed in pregnancy rate and implantation rate between 5% and 2% O2 groups, irrespective of their mode of ET. The abortion rate was significantly higher in 5% O2 group than in 2% group during FET (24.71% vs. 11.49%, $P = 0.02$). While the proportion of good-quality embryos was higher in 5% O2 group, these did not translate to better pregnancy outcomes. Additionally, embryos cultured in 2% O2 concentration had a significantly better implantation rate when they were transferred fresh rather than frozen (71.34% vs. 61.46%, $P = 0.04$). There were no other differences observed. **Conclusion:** Only marginal benefits were observed in switching human embryos to ultra-low O2 concentration after the initial days of culture.

Keywords: *embryo culture, fresh embryo transfer, frozen embryo transfer, in vitro fertilisation, oxygen*

Introduction

In vitro fertilisation (IVF) is one of the most effective forms of assisted reproductive technology and a widely used technique for the treatment of infertility.[1] Conventionally, IVF includes hyperstimulation of ovaries, retrieval of mature eggs, fertilisation of retrieved eggs with male spermatozoa, embryo culture and implantation of a fresh embryo into the uterus.[2] Recently, the practice of freezing all embryos ('freeze all') has become more prevalent to minimise the severity of ovarian hyperstimulation syndrome at the time of pregnancy.[3,4] Several clinical studies have compared the reproductive outcomes between fresh embryo transfer (ET) and frozen embryo transfer (FET) and reported more live births and pregnancy rate in females after FET.[5-10] However, there are also reports that suggest FET to be associated

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with adverse outcomes like high birth weight and hypertensive disorders of pregnancy.[2]

Apart from the method employed for ET, the success of IVF largely depends upon embryonic development. In IVF laboratories, embryonic development and implantation are promoted by simulating the *in vitro* environment with the *in vivo* conditions with respect to pH, temperature, composition of culture medium and the composition of gases inside the incubator.[11] Oxygen (O_2) concentration is reported to play a vital role in facilitating the embryonic development.[12,13] In the past decades, standard 20% atmospheric O_2 concentration was extensively used in embryo culture.[13,14] Recent studies have revealed *in vivo* O_2 concentration in fallopian tube and uterus to be 5% and 2%, respectively.[15,16] Consequently, 60% of the embryos now are cultured in incubators having 5% O_2, as it is physiologically relevant.[17] There is also ample evidence in the field that higher O_2 concentrations can affect protein profiles, membrane potential in mitochondria, embryo metabolism, DNA methylation and gene expression.[18-21]

The effectiveness of 5% O_2 over 20% O_2 is clinically established by various studies in terms of better pregnancy rates and live births.[15,22,23] Even at ultra-low O_2 (2%), clinical studies have reported higher blastocyst numbers and quality.[12,24] The latest European Society of Human Reproduction and Embryology guidelines also recommend low O_2 tension for embryo culture.[25]

Objective

The present study aims to explore the effects of fresh ET and FET under low- and ultra-low O_2 concentrations (5% and 2%) which simulate the *in vivo* levels on various parameters of pregnancy.

Methods

Study design

This was a retrospective study conducted at Sunflower Women's Hospital, Ahmedabad, India from January 2021 to January 2022. The study was approved by our institute's ethical committee (ECR/1435/Inst/GJ/2020) with waiver of patient consent. The study was conducted in accordance with the Declaration of Helsinki (2013) and Ethical Guidelines for Biomedical Research on Human Subjects issued by the Indian Council of Medical Research (2017). Participants received either a fresh embryo transfer or a FET during their IVF cycle. In both these groups, participants had their embryos cultured either under 5% O_2 concentration or 2% O_2 concentration. As this was a retrospective exploratory study, we did not calculate sample sizes formally but only considered similar time period for enrolment in both the groups.

Study population

Eligible participants were adult women in the age range of 22 years to 53 years, with a history of infertility for at least 1 year and who received ovum from donors. All participants have provided written informed consent for the procedures. Intracytoplasmic sperm injection was performed for all the cycles, and surgical sperm retrieval was included in the protocol. Patients undergoing pre-implantation genetic testing were excluded from the study. Embryos (day 3 to day 5) were cultured in 5% O_2 for participants enrolled from January 2021 to May 2021 (fresh ET and FET) and in 2% O_2 for those enrolled from June 2021 to January 2022 (fresh ET and FET).

Embryo culture and transfer protocol

The embryos were cultured in MINC incubator (Cook, Australia) under standard conditions (temperature 37°C, high humidity and Tri-gas mixture of 5% CO_2, 5% O_2 and balance N_2). Using single-step media (SAGE, Origio, Denmark), group culture was performed with 3–5 embryos per droplet. As shown in Figure 1, after successful fertilisation, embryos were graded as good/average/poor quality on day 3 based on the number and symmetry of blastomeres, percentage of fragmentation, vacuolisation, granulation and multinucleation.[26] All the embryos were cultured in 5% O_2 till day 3. Subsequently, the embryos were evaluated, regrouped based on quality and moved for extended culture (day 5/day 6). After appropriate overnight equilibration, embryos were cultured with either 5% O_2 or 2% O_2. On day 5, blastocysts were graded using Gardner and Schoolcraft grading system,[27] based primarily on their morphology. It takes into account the blastocoele expansion, the appearance of inner cell mass (ICM) or compaction and the number and appearance of trophectoderm (TE). In the extended embryo culture (day 5/day 6), blastocysts were scored as excellent to good (Grade 1), moderate (Grade 2) and poor (Grade 3).[28]

In our study, fully hatched or expanded blastocysts or fully compacted embryos with visible ICM and TE were selected for transfer in the subjects on day 5. Additional blastocysts reaching at least full blastocyst Stage 3 (BL3) and with visible ICM and TE (Type A or B) were cryopreserved on day 5 or 6. The embryos were vitrified using Kitazato Vitrification Kit (Kitazato, Japan) and Cryotop device (one or two blastocysts per Cryotop). For FET, the embryos were thawed in the morning and ET was performed by late noon.

Recipient endometrium was prepared by using hormonal supplementation to synchronise the donor and recipient cycles in cases of fresh ET. In FET cases, a gonadotropin-releasing hormone agonist (Inj. Lupride depot) was used to cause pituitary downregulation and ovarian function suppression. After this, oestrogen

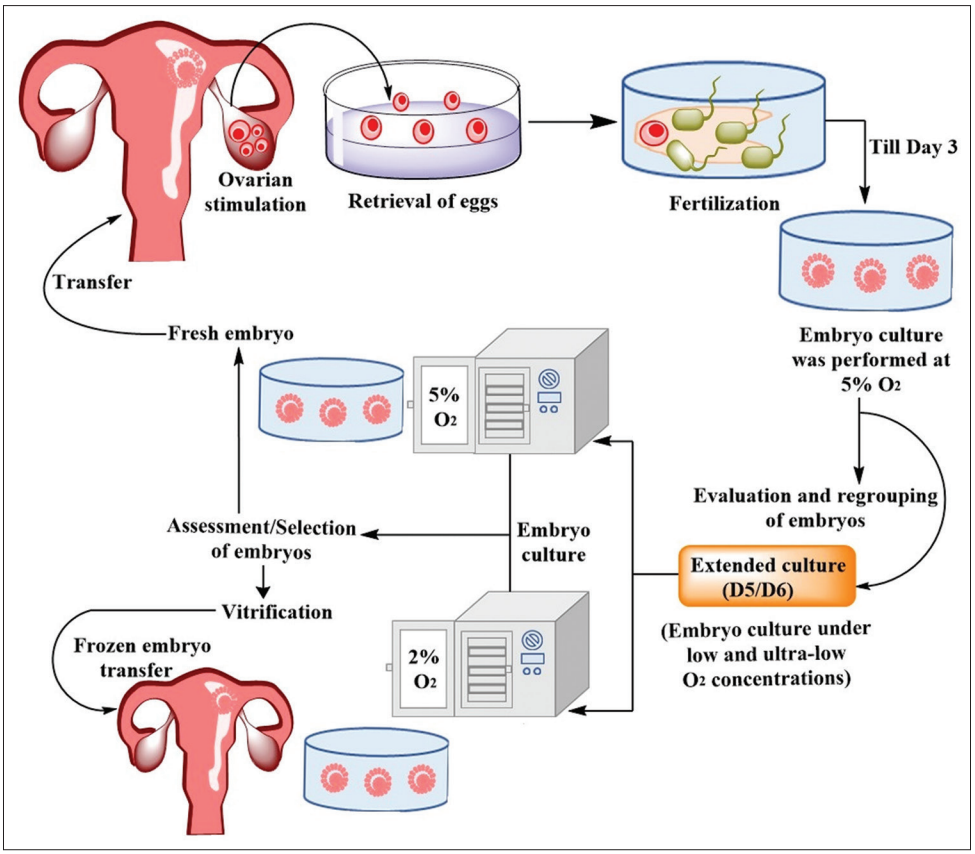

Figure 1: Schematic representation of fresh embryo transfer and frozen embryo transfer under 5% and 2% O_2 concentrations

was given from day 2/3 of period (one tablet thrice a day, up to 6 tablets in a day). To monitor endometrial thickness, transvaginal ultrasounds were routinely done. Progesterone was initiated and ET procedure was booked when the endometrial thickness was found to be 9 mm.

Study outcomes

The primary outcomes of the study were the differences in pregnancy and implantation rates between embryos cultured in 5% O_2 or 2% O_2 concentrations, with fresh as well as FETs. A positive pregnancy was defined as a positive beta-human chorionic gonadotropin (β-hCG) blood test 14 days after fresh/frozen ET. Implantation rate was defined as the percentage of gestational sacs at ultrasonographic visualisation out of the total embryos that were transferred.

The secondary outcomes determined were abortion rate, twinning rate and live birth rate. Abortion rate in a group was the percentage of participants who had an abortion out of the total number of participants with a positive pregnancy test. Twinning rate is the presence of multiple gestational sacs (>2) in a pregnant woman and was calculated as the percentage of participants with multiple sacs out of all the pregnant participants. Live births included the number of participants in whom their pregnancy continued until successful birth of neonate (s).

Statistical analyses

Statistical analyses were performed using GraphPad Prism version 8.0 for Windows (GraphPad Software, Boston, Massachusetts USA). Chi-square test was applied to compare the primary and secondary outcomes at two different levels (5% vs. 2% O_2 concentration for fresh and frozen ET and fresh vs. frozen ET under 5% and 2% O_2 concentration). P <0.05 was considered statistically significant.

To alleviate bias at the level of patient selection, we included all participants who underwent ovum donation. Additionally, since the missing data were random, we performed a complete-case analysis and included only those participants who had their complete outcome data available.

Results

Participant distribution and characteristics

From January 2021 to January 2022, 382 women fulfilled the inclusion criteria and were enrolled in the study. Of these, 382 women fulfilled the inclusion criteria and were enrolled in the study. A total of 65 participants underwent fresh ET and 111 subjects underwent FET with embryos cultured in 5% O_2 concentration, while 95 participants received fresh ET and 111 participants

received FET with embryos cultured in 2% O_2 concentration [Figure 2].

The baseline characteristics of the participants in all the groups are presented in Table 1. The ages of participants ranged from 22 to 53 years. The number of participants over the age of 40 years was similar for groups with embryos cultured in 5% O_2 or 2% O_2 in both fresh ET and FET. Other physical parameters such as weight, height and body mass index (BMI) were comparable for all women across all groups. The causes of infertility varied within the group as well as between the groups, where almost half of the women had low levels of anti-Müllerian hormone. High BMI was also identified as a cause for infertility affecting both men and women. In majority of the men, the cause of infertility was not identified.

Effect of low and ultra-low oxygen conditions on pregnancy rate, implantation rate, abortion rate and multiple sacs with fresh embryo transfer versus frozen embryo transfer

In participants who received fresh ET or FET, no significant difference was observed in pregnancy rate or implantation rate whether embryos were cultured in 5% O_2 or 2% O_2 concentrations. However, at 2% O_2 concentration, the implantation rate was significantly higher in participants who received fresh ET as compared to FET (71.34 vs. 61.46; $P = 0.04$) [Figure 3].

In participants who underwent fresh ET, we observed a higher proportion of multiple sacs in pregnancies where embryos were cultured in 2% O_2 as compared to 5% O_2 concentration (50.6% vs. 33.3%; $P = 0.047$).

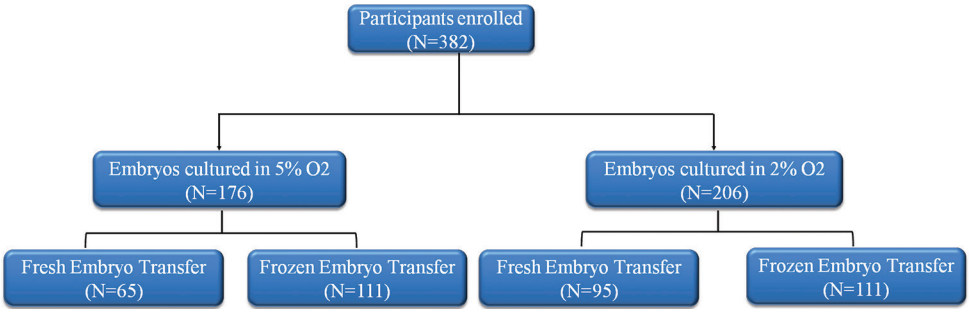

Figure 2: Participant disposition

Table 1: Baseline characteristics of the participants undergoing embryo transfer

	Fresh embryo transfer		Frozen embryo transfer	
	5% O_2	2% O_2	5% O_2	2% O_2
Number of participants	65	95	111	111
Age (years), median (minimum–maximum)	38 (25–53)	35 (22–49)	37 (26–51)	36 (24–49)
Age ≥40 (years), frequency	27	28	34	33
Weight (kg), median (minimum–maximum)	60 (41–154)	62 (32–161)	61 (35–103)	58 (29.6–155)
Height (cm), median (minimum–maximum)	155 (140–175)	155 (69–175)	155 (143–175)	155 (39.7–180)
BMI (kg/m^2), median (minimum–maximum)	25 (18–38)	26 (14–39)	25 (17–46)	24 (12–35.8)
Duration of infertility (months), median (minimum–maximum)	11 (1–28)	10 (0.8–35)	9.5 (1–36)	12 (1–34)
Causes of infertility (female)*, frequency (%)				
Low AMH	37 (56.9)	52 (53.7)	46 (41.4)	49 (44.1)
High BMI	11 (16.9)	10 (10.5)	22 (19.8)	7 (6.3)
PCOD	5 (7.7)	15 (15.8)	4 (3.6)	18 (16.2)
Unexplained	20 (30.8)	23 (24.2)	49 (44.1)	38 (34.2)
Others	-	6 (6.3)	2 (1.8)	4 (3.6)
Causes of infertility (male)*, frequency (%)				
Asthenozoospermia	3 (4.6)	7 (7.3)	4 (3.6)	1 (0.9)
Oligoasthenozoospermia	3 (4.6)	9 (9.4)	1 (0.9)	-
High BMI	4 (6.1)	10 (10.5)	5 (4.5)	10 (9)
Unexplained	54 (83)	58 (61)	79 (71.1)	87 (78.3)
Others	2 (3.1)	13 (13.7)	21 (18.9)	12 (13.5)

*Many participants had more than one cause of infertility. BMI=Body mass index, AMH=Anti-Müllerian hormone, PCOD=Polycystic ovarian disease

In participants with FET, we observed significantly higher abortion rates in those where embryos were cultured in 5% O_2 than in 2% O_2 (24.71 vs. 11.49; $P = 0.02$) [Table 2].

When participants were stratified according to their ages (>35 years and < 35 years), no difference was observed in any of the pregnancy parameters in the > 35-year age group with respect to the embryo culture conditions or mode of ET (data not shown). However, in < 35-year category, we observed that participants whose embryos were cultured in 5% O_2 concentrations showed a significantly higher pregnancy rate when these embryos were transferred fresh as compared to frozen (94.12% vs. 69.44%; $P = 0.045$).

Effect of low and ultra-low oxygen conditions on embryo quality with fresh embryo transfer versus frozen embryo transfer

The percentage of good-quality embryo (GQE) was found significantly higher in participants whose embryos were cultured in 5% O_2 than those in 2% O_2 for both groups [Table 3]. There was no significant difference in the quality of embryos that were used for fresh ET or FET.

Effect of low and ultra-low oxygen conditions with Grade 1 embryo transfer on pregnancy parameters

No significant difference was observed in pregnancy rate and abortion rate between participants who received Grade 1 embryos cultured in 5% or 2% O_2 concentrations [Table 4].

Effect of low and ultra-low oxygen conditions on live birth with fresh embryo transfer versus frozen embryo transfer

In both fresh ET and FET, we observed no significant difference between the number of live births in participants with embryo culture done under 5% or 2% O_2 concentrations [Table 5].

Discussion

The present study aimed to evaluate IVF outcomes in subjects with fresh ET and FET under low and ultra-low O_2 concentrations (5% and 2%, respectively). Owing to the widespread success of IVF in terms of live births, modifications in its techniques have been taken under investigation in order to further improve perinatal outcomes. The levels of O_2 used during the culture of embryo and the mode of ET have been explored

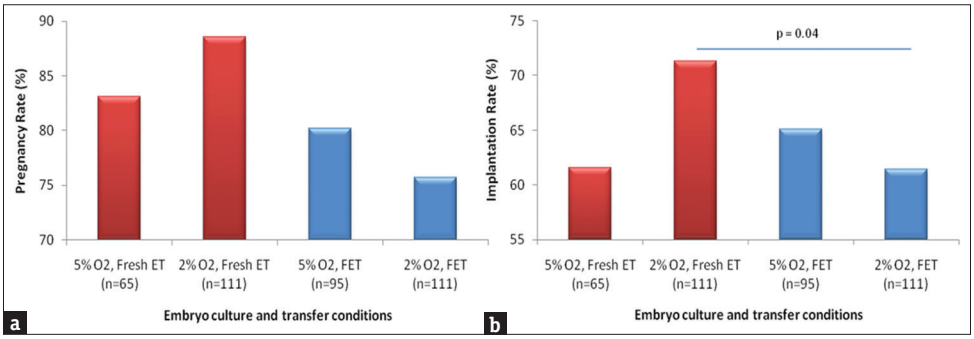

Figure 3: Comparison of (a) Pregnancy rate, (b) Implantation rate, in participants receiving fresh embryo transfer or frozen embryo transfer where embryos were cultured in 5% O_2 or 2%

Table 2: Comparison of pregnancy parameters in participants receiving fresh or frozen embryos with varying O_2 in embryo culture

	Fresh embryo transfer			Frozen embryo transfer			P-value fresh ET versus FET	
	5% O_2	2% O_2	p	5% O_2	2% O_2	p	5% O_2	2% O_2
Number of participants	65	95		111	111			
Number of participants with positive pregnancy	54	81	0.71	89	87	0.64	0.72	0.2
Pregnancy rate (%)	83.08	85.26		80.18	78.37			
Number of embryo transfers	112	171		192	205			
Number of sacs observed	69	122	0.09	125	126	0.45	0.54	0.04
Implantation rate (%)	61.60	71.34		65.10	61.46			
Number of abortions	6	7	0.38	22	10	0.02	0.14	0.54
Abortion rate (%)	11.11	8.64		24.71	11.49			
Number of multiple sacs	18	41	0.047	36	43	0.23	0.39	0.87
Multiple sacs (%)	33.33	50.6		40.45	49.4			

Statistical test – Chi-square test, $P<0.05$ significance. ET=Embryo transfer, FET=Frozen embryo transfer

Table 3: Comparison of good-quality embryo in participants receiving fresh or frozen embryos with varying O_2 in embryo culture

	Fresh embryo transfer			Frozen embryo transfer			*P*-value fresh ET versus FET	
	5% O_2	2% O_2	*P*	5% O_2	2% O_2	*P*	5% O_2	2% O_2
Number of participants	65	95		111	111			
Number of embryos classified as Grade 1, 1 and 1, 1 and 2*	60	76	0.03	101	85	0.003	0.76	0.55
Number of embryos classified as Grade 2, 2 and 2, 2 and 3*	5	19		10	26			
GQE (%)	92.3	79.16		90.99	76.57			

*Embryo grading according to Gardner classification – excellent to good (Grade 1), moderate (Grade 2) and poor (Grade 3) statistical test – Chi-square test, $P<0.05$ significance. ET=Embryo transfer, FET=Frozen embryo transfer, GQE=Good-quality embryo

Table 4: Comparison of pregnancy parameters in participants with at least one of the embryos in Grade 1 with varying O_2 in embryo culture

	Fresh embryo transfer			Frozen embryo transfer			*P*-value fresh ET versus FET	
	5% O_2	2% O_2	*P*	5% O_2	2% O_2	*P*	5% O_2	2% O_2
Number of participants	65	95		111	111			
Number of embryos classified as Grade 1, 1 and 1, 1 and 2*	60	76		101	85			
Number of participants with positive pregnancy	51	67	0.59	80	68	0.89	0.36	0.16
Pregnancy rate (%)	85.00	88.16		79.21	80.00			
Number of miscarriages	6	3	0.14	7	9	0.38	0.57	0.3
Miscarriage rate (%)	11.76	4.48		8.75	13.23			

*Embryo grading according to Gardner classification – excellent to good (Grade 1) and moderate (Grade 2) statistical test – Chi-square test, $P<0.05$ significance. ET=Embryo transfer, FET=Frozen embryo transfer

Table 5: Comparison of live births in participants receiving fresh or frozen embryos with varying O_2 in embryo culture

	Fresh embryo transfer			Frozen embryo transfer			*P*-value fresh ET versus FET	
	5% O_2	2% O_2	*P*	5% O_2	2% O_2	*P*	5% O_2	2% O_2
Number of participants	65	95		111	111			
Number of embryo transfers	112	171		192	205			
Number of live births	53	93	0.24	90	94	0.83	0.94	0.1
Live births (%)	47.32	54.38		46.87	45.85			

Statistical test – Chi-square test, $P<0.05$ significance. ET=Embryo transfer, FET=Frozen embryo transfer

across various studies.[24,29,30] Recent studies have established that embryo culture under low O_2 levels results in improved number and quality of blastocyst than the embryo cultured under atmospheric O_2 concentration.[31-33] These findings opened new avenues for the researchers to evaluate different approaches like using low or ultra-low O_2 concentrations in culture or monophasic or biphasic O_2 availability for better IVF outcomes.

Once the embryos are cultured, the choice between fresh ET and FET becomes a subject of great deliberation and research. As compared to fresh ET, the use of FET has increased dramatically due to its reported advantages in terms of maternal and neonatal outcomes.[34] Several studies associate FET with a decreased risk of low birth weight, babies born small for their gestational age, preterm birth, placental abnormalities and perinatal mortality.[34-36] Even in terms of outcomes such as implantation rates, pregnancy rates and live births, studies have reported FET to be superior.[37,38] However, few studies and a recent systematic review suggest that both fresh ET and FET are quite similar in terms of pregnancy rates as well as number of live births.[2,10,39]

In order to explore differences in both the approaches, we designed our study to understand the effects of O_2 levels as well as mode of ET on subsequent pregnancy-related outcomes. It has been reported that the use of biphasic O_2 concentration (5% from day 0 to 3 and 2% from day 3 to 5/6) improved the embryo quality as well as cumulative live birth rate.[12,24] However, when the same strategy was used by us, we found the percentage of GQEs to be higher in the group where 5% O_2 was used

throughout in culture medium. This trend was seen in the embryos lined up for fresh transfers (92.3% vs. 79.2% GQEs; $P = 0.03$) as well as for frozen transfers (90.1% vs. 76.6% GQEs; $P = 0.003$). Similar findings were reported in a study where embryos cultured in 2% O_2 were worse in quality than those cultured in 5% O_2.[40] Another study reported no significant difference in the embryo development and quality based on the O_2 concentration in the culture.[22,29] In our study, the high number of GQEs in 5% O_2 group did not translate to better pregnancy outcomes. The embryos cultured in low- or ultra-low O_2 concentration showed no statistical difference in pregnancy rate, implantation rate or in the number of live births. The patterns were the same whether these embryos were transferred fresh or frozen. Other studies have reported similar findings where despite differences in embryo quality owing to varying O_2 in culture conditions, it had no effect on the clinical outcomes.[11,40,41] Even in participants where only GQEs were transferred, no differences were observed in the pregnancy rates.

Furthermore, we observed a higher abortion rate in embryos cultured in 5% O_2 as compared to 2% O_2 (24.7% vs. 11.5%; $P = 0.02$) in women who underwent FET. While higher abortion rates are reported in women who undergo FET as compared to fresh ET,[42,43] a study where embryos were exposed either to 5% O_2 or 2% O_2 reported no significant difference in the rate of miscarriage.[40] However, the numbers in our study in each of these groups are too small to draw any conclusion regarding the abortion rate. We also reported higher multiple sacs in pregnancies with fresh ET at 2% O_2 as compared to 5% O_2 concentration (50.6% vs. 33.3%; $P = 0.047$). To our knowledge, a comparative effect of 5% and 2% O_2 concentrations on multiple gestational sacs has not been studied. Many studies conducted on fresh ET vs. FET reported a higher proportion of multiple pregnancies in the fresh ET group compared to FET group.[44,45] A recent study contradicts these findings by reporting significantly higher multiple pregnancies and abortion rate in women with fresh ET as compared to those who undergo FET.[46] However, in our study, no differences were observed in these parameters based on the mode of ET.

In IVF, optimisation of pregnancy rate has always remained an unsolved issue, particularly in participants with poor prognosis, namely one or more failed IVF cycles and age near or above 35 years.[47] In line with previous reports, our study also evaluated women's age as a critical factor affecting pregnancy outcomes following fresh ET and FET.[31,48,49] In our study, we observed that in case of participants under the age of 35 years, embryos cultured in 5% O_2 showed higher pregnancy rates with fresh ET as compared to FET (94.1% vs. 69.4%; $P = 0.045$). Similar results were not observed in participants above 35 years of age. To our knowledge, the effect of O_2 levels in embryo culture medium has not been explored by stratifying women's age.

While the superiority of using 5% O_2 over atmospheric O_2 in embryo culture medium has been established, there is insufficient and contradictory evidence towards the use of 2% O_2 in culture medium. Although it is physiologically relevant owing to the O_2 levels reported in female reproductive tract, its use in laboratory-based practices warrants further research. There are now reports that suggest that as long as the first couple of days of embryo culture are under low O_2 concentration, subsequently limiting O_2 availability does not improve pregnancy outcomes.[41] Older studies done in rodents have even suggested that the oxidative stress-associated damage in embryo formation seems to be limited only in the initial stages of cleavage and that switching to even conventional atmospheric O_2 levels would yield similar results as maintaining a state of hypoxia.[50]

Limitations

The present study has certain limitations owing to its retrospective design and small sample size. Furthermore, the heterogeneity of the study population in terms of the causes of infertility also makes it difficult to draw inferences from the results. IVF outcomes are reported to differ according to the cause as well as duration of infertility.[51] The interpretation of our findings might thus be limited and confounded by several variables. In light of these, further research is warranted to determine the most effective strategy for optimising potential IVF outcomes.

Conclusion

In our study, we find limited differences in using biphasic O_2 in culture medium wherein we switch human embryos to ultra-low O_2 concentration after the initial days of culture. These limited effects do not translate to improved pregnancy outcomes in our study population.

Author's contribution:

All the authors were involved in data collection and interpretation of the sequencing data. They drafted the manuscript and it was critically revised by the authors. All the authors have seen the final draft and take full responsibility for the manuscript's contents.

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Nil.

Conflicts of interest

There are no conflicts of interest.

Data availability statement

The datasets generated and analysed during the current study are available from the corresponding author on reasonable request.

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