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Effect of ultra-low O_2 (2%) tension on human *in-vitro* embryo development

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Abstract: The study was aimed to investigate the effect of culturing human *in-vitro* embryos in ultra-low O_2 (2%) tension. A total of 2298 oocytes from 152 patients between June 2017 and December 2017 treated with conventional *in vitro* fertilization (IVF) were harvested in this study. Oocytes were randomly assigned to the low (5%) or ultra-low (2%) O_2 tension groups on the retrieval day. We observed that the day 3 good quality embryos (43.32 versus 42.01%; *p*=.635) and available embryos (82.02 versus 83.47%; *p*=.490) rates were similar between 2% and 5% condition. No differences were observed in the D5 blastulation rate (62.79 versus 61.85%; *p*=.735) and the proportion of good quality blastocysts on Day 5 (44.51 versus 45.61%; *p*=.700), nor in the total blastulation rate (71.26 versus 70.29%; *p*=.710) between 2% and 5% condition. In the first transfer, the blastocysts had similar clinical pregnancy (68.12 versus 71.08%; *p*= .692) and ongoing pregnancy (59.42 versus 62.65%; *p*=.684) rates from 2% and 5% condition. The employ of ultra-low O_2 tension did not benefit for human *in-vitro* embryo development.

Keywords: Ultra-low O_2 culture, blastulation, clinical pregnancy, ongoing pregnancy.

INTRODUCTION

Human embryo culture was traditionally carried on under atmospheric O_2 tension of about 20%. However, it was demonstrated that the O_2 tension in the uterus and fallopian tubes generally fluctuated between 2-8% based on experimental animal models. Subsequent studies showed that low O_2 tension culture improved embryonic quality and clinical outcomes than atmospheric O_2 tension (Waldenstrom *et al*., 2009; Kovacic *et al*., 2010). Nevertheless, several systematic reviews concluded that the methodological quality of the included trials was relatively low and it seemed too early to confirm that low O_2 tension had a promising effect on IVF outcomes (Gomes Sobrinho DB *et al*., 2011; Nastri *et al*., 2016). There was much debate regarding the optimal oxygen tension in clinical embryo culture.

In recent years, the ultra-low O_2 tension (2%) for human embryo culture became a research hotpot. Past experiences with ultra-low O_2 (2%) after day 3 of development suggested that the optimal O_2tension in embryo culture might depend on the stage of development (Morin *et al*., 2017). And some investigators put forward the hypothesis which assumed that sequential O_2 exposure (5% from days 1 to 3, then 2% from days 3 to 5) would improve blastocyst yield and quality compared to continuous exposure to 5% oxygen among human preimplantation embryos (Kaser *et al*., 2017). However, a comparative study showed that human embryos culture in 3.5% O_2 tension from day 0 to 5 or 6 had higher normal fertilization and cleavage rates (Fawzy *et al*., 2017). It also showed 3.5% O_2 tension significantly decreased the blastocyst formation rates and clinical outcomes parameters than in 5% O_2 tension (Fawzy *et al*., 2017). In a recent study from 2019, the study showed that a reduction in O_2 tension from 5 to 2% after Day 3 did not improve embryo development, quality and utilization rate (De Munck N. *et al*., 2019). So it was difficult for the embryologists to determine which O_2 tension was good for the different stage of embryonic development according to the above studies.

These findings did not provide enough evidence to change current in vitro protocols, the culture of human oocytes and embryos for optimal tension of O_2 was a question that still required more data. With this in mind, we aimed to investigate the effect of culturing human *in-vitro* embryos in ultra-low oxygen (2%) tension.

MATERIALS AND METHODS

Patients and setting

This study was carried out in the Assisted Reproduction Center, Northwest Women's and Children's Hospital, Xi' an, China, from June 2017 to December 2017.

Patients with >12 oocytes of IVF cycles were selected for this study. All females were not beyond 35 years old to eliminate possible age-related cycle characteristics and all the cycles were first attempt down-regulated ovarian stimulation cycle. The patients with severe endometriosis and polycystic ovary syndrome were not included in this study. Cycles with oocyte donation transfer, rescue ICSI or half rescue-ICSI were not included in this study.

Only single blastocyst transfer was included in this study. For not less two good quality embryos obtained on day 3, the cleavage-stage embryo transfer was performed. So

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these patients were not included. And no blastocyst (expansion stage ≥ 4) obtained on day 5, the patients were also excluded.

26 patients were excluded due to rescue ICSI (n=9), <2 D3 good quaity embryos collected (n=8), and no D5 blastocysts (expansion stage ≥4) obtained (n=9). Ultimately, 152 patients were eligible for the trial.

After the oocyte retrieval, the oocyte cumulus complex (OCC) was numbered and divided into 2 groups (odd-number and even-number groups) for each patient. Then, each of the odd- and even-numbered oocyte groups was randomly assigned to culture condition with either 2% or 5% O_2 until day 5 or 6. In this study, one incubator with 2% O_2 and three incubators with 5% O_2 were included.

Ovarian stimulation protocol

All the studied patients used the standard long protocols with GnRH agonist (GnRH-a, Decapeptyl, Germany) and recombinant FSH (GONAL-f, Merck, Serono, Italy; Puregon, Organon, the Netherlands) for controlled ovarian hyperstimulation (COH). A total of 10,000 units of human chorionic gonadotrophin (hCG) was administered when >3 follicles were >18 mm. Oocyte retrieval was performed 36h later by transvaginal ultrasonography-guided aspiration (Li *et al*., 2016).

Fertilization and embryo culture

The OCCs were cultured in the media (IVF; Vitrolife, Sweden) after retrieval. Fertilization was performed 39 to 40 hours (39-42 hours for ICSI) after HCG administration while incubated in fertilization media (IVF; Vitrolife). The zygotes were shifted to the cleavage media (G-1; Vitrolife) 5 hours after the fertilization of IVF. The embryos to culture blastocyst on day 3 were transferred to the blastocyst media (G-2; Vitrolife) until day 6. All the medium were covered with paraffin oil in a humidified atmosphere at 37°C for prior 24 h.

The same brand of incubators (Model c200; Labotect, Germany) was used for both 2% and 5% O_2 tension to culture oocytes and embryos. The 5% O_2 tension was to culture in 6% CO_2, 5% O_2 and 89% N_2. The 2% O_2 culture condition had also 6% CO_2 in air. All other conditions were equal except O_2 tension between 2 systems.

In this study, the embryo of fresh transfer was from day 5 and the embryo of frozen embryo was from day 5 or 6. Only the data of first transfer was counted and all the first transfer was single blastocyst transfer.

Embryo morphology assessment

The cleavage-stage embryo scoring system used a combination of blastomere number, blastomere size, and degree of fragmentation. The details were presented in the previous articles from our work team (Shi *et al*., 2013). The scoring system for blastocyst was a combination of the stage of development (Early, Blastocyst, Full blastocyst, Expanded, Hatching/Hatched) and of the grade of the inner cell mass (ICM; good, fair, poor) and of the trophectoderm (TE; good, fair, poor) (Gardner *et al*., 2000).

ET and pregnancy confirmation

The details of ET protocol was performed accoding to our pevious studies (Li *et al*., 2019). Clinical pregnancy was defined as an intrauterine gestational sac and fetal heart on transvaginal ultrasound 4 weeks after ET. Ongoing pregnancy was defined as fetal cardiac activity at 12 weeks.

Ethical approval

The procedures followed in this study were in accordance with the ethical standards of Northwest Women' s and Children' s Hospital ethics committee.

STATISTICAL ANALYSIS

The statistical analysis was performed using SPSS software (version 17.0, SPSS Inc., Chicago, IL, USA). Continuous variables were presented as mean ± standard deviation (SD) and subjected to paired-samples t test, and the categorical data were analyzed using $\chi 2$-tests analysis or Fisher exact probability test. After statistical consulting, the minimum sample size was 398. p value <0.05 was considered statistically significant.

RESULTS

Table 1 showed basic characters of the patients such as female age, basal serum FSH, AMH, Gn administration, Gn does, infertility duration and the number of retrieved oocytes (table 1).

Table 1: Characteristics of the patients.

Characteristics	Values
Female age (yr)	30.13 ± 3.46
Basal serum FSH (IU/L)	6.48 ± 1.57
AMH (ng/mL)	4.81 ± 2.69
Gn administration (d)	10.45 ± 1.91
Gn does (IU)	28.12 ± 10.25
Infertility duration (y)	3.47 ± 2.19
Oocytes retrieved (n)	15.13±3.51

Table 2 showed the zygotes characteristics and embryo development between 2% and 5% O_2 condition. Neither normal fertilization (2pn) rate nor abnormal fertilization (0pn, 1pn, γ3pn) rate showed statistical differences between two groups. The day 3 good quality embryos (43.32 versus 42.01%; p=0.635) and available embryos (82.02 versus 83.47%; p=0.490) rates were similar

Table 2: The zygotes characteristics and embryo development between 2% O_2 and 5% O_2 condition

	2% O_2	5% O_2	*p*
No. of oocytes allocated	1138	1160	/
5 hours postinsemination			
MII matured oocytes, n (%)	1020 (89.63%)	1074 (92.58%)	0.015
Second polar body, n (%)	737 (64.76%)	763 (65.78%)	0.630
Nonhomogeneous cytoplasm, n (%)	53 (4.66%)	62 (5.34%)	0.503
Large vacuoles, n (%)	96 (8.44%)	92 (7.93%)	0.704
Abnormal zona pellucid, n (%)	12 (1.05%)	17 (1.47%)	0.456
Fertilization check			
1pn, n (%)	42 (3.69%)	47 (4.05%)	0.667
3pn (including pn≧3), n (%)	102 (8.96%)	109 (9.40%)	0.773
2pn (normal fertilization with 2 pronuclei), n (%)	734 (64.50%)	738 (63.62%)	0.664
Cleavage rate (% per 2pn)	718 (97.82%)	720 (97.56%)	0.863
Available embryos on day 3, n (% per 2pn)	602 (82.02%)	616 (83.47%)	0.490
Good quality embryos on day 3, n (% per 2pn)	318 (43.32%)	310 (42.01%)	0.635
No. of embryos cultured for blastocyst on day 3	602	616	/
D5 blastulation rate, n (%)	378 (62.79%)	381 (61.85%)	0.735
D5 good quality blastocyst rate, n (%)	268 (44.51%)	281 (45.61%)	0.700
Total balstulation rate, n (%)	429 (71.26%)	433 (70.29%)	0.710

Table 3: Summary of results of the blastocyst for d5 blastocyst and clinically used blastocyst (transferred and frozen)

	2% O_2		5% O_2		*P*	
	D5	Transferred and Frozen	D5	Transferred and Frozen	D5	Transferred and Frozen
ICM						
Good	1	1	2	2	0.102[a]	0.598
Fair	251	264	226	230		
Poor	106	133	98	122		
Total	358	398	326	354		
TE						
Good	2	2	2	2	0.457	0.376
Fair	134	136	106	118		
Poor	222	260	208	234		
Total	358	398	326	354		

[a]Mann-Whitney U test. Abbreviations: ICM, inner cell mass; TE, trophectoderm.

Table 4: Clinical outcomes of the blastocysts from 2% O_2 and 5% O_2 condition

	2% O_2	5% O_2	*p*
No. of transferred cycles	69	83	/
Clinical pregnancy rate, n (%)	47 (68.12%)	59 (71.08%)	0.692
Ongoing pregnancy rate, n (%)	41 (59.42%)	52 (62.65%)	0.684

between two groups. And more importantly we observed no differences in the D5 blastulation rate (62.79 versus 61.85%; *p*=.735) and the proportion of good quality blastocysts on Day 5 (44.51 versus 45.61%; *p*=.700), nor in the total blastulation rate (71.26 versus 70.29%; *p* = .710) between 2% and 5% condition (table 2).

Table 3 showed the quality of blastocyst (ICM and TE) either on day 5 or transferred and frozen was also similar between day 3 embryos generated from 2% and 5% O2 condition (table 3).

Table 4 showed the blastocysts had similar clinical pregnancy (68.12 versus 71.08%; *p*=.692) and ongoing pregnancy (59.42 versus 62.65%; *p*=.684) rates from 2% and 5% condition in the first transfer (table 4).

DISCUSSION

In the early years of IVF, human embryo culture was performed under atmospheric O_2 tension. Soon after some years, there was an abundance of data on several mammalian species, including humans, showing that

atmospheric O_2 tension made negative effects on the preimplantation-stage embryo (Wale *et al*., 2016). Although current meta-analyzes on the subject did not show important and convincing evidence, the majority of modern IVF labs had accepted 5% O_2 tension for human embryo culture.

It was no doubt that extended embryo culture and blastocyst-stage embryo transfers had been related with increased clinical pregnancy rates and reduced rates of multiple gestations. So it was significant to improve blastocyst formation rate and the proportion of good quality blastocysts by regulating O_2 tension for human *in-vitro* embryo development.

O_2 tension was in fact lower in the uterus than in the oviduct and the human intrauterine O_2 tension had been measured to be around 2% when the human embryo reached the uterine cavity on day 3.5. Recently, a new debate has emerged regarding whether a further reduction after day 3 of development represents the most physiologic system. In 2017, the hypothesis that "sequential oxygen exposure (5% from days 1 to 3, then 2% from days 3 to 5) improved blastocyst yield and quality compared to continuous exposure to 5% O_2 among human preimplantation embryos." was also proposed (Morin *et al*., 2017; Kaser *et al*., 2017). In 2018, it was confirmed that a reduction in O_2 tension to 2% was beneficial to extended culture which might mean more blastocysts available for transfer (Morin *et al*., 2018). In a recent study from 2019, the study showed that a reduction in O_2 tension from 5 to 2% after Day 3 did not improve embryo development, quality and utilization rate (De Munck N *et al*., 2019). Above all, a new debate has emerged regarding whether a further reduction after day 3 of development represents the most physiologic system.

In a second study, a reduction in O_2 tension was to 3.5% from day 0 to day 5 or 6, but they observed lower blastocyst formation rate and clinical outcomes parameters (Fawzy *et al*., 2017). Interestingly, they observed higher rates of fertilization and cleavage which suggested a reduction in O_2 tension from day 0 to day 3 might be beneficial to early embryo development (Fawzy *et al*., 2017).

In this study, a reduction in O_2 tension was to 2% from day 0 to day 5 or 6, but we observed that it made no effect on early embryo development from day 0 to day 3. And we also observed that it did not improve blastocyst development. More importantly, we made further statistics on the clinical outcomes of these blastocysts in the first transfer. For 152 patients in this study, 83 blastocysts from 5% condition and 69 blastocysts from 2% condition were selected in the first transfer. Our data showed the blastocysts had similar clinical pregnancy and ongoing pregnancy rates from 2% and 5% condition. It suggested that the clinical outcomes might not be affected once the good quality blastocysts formed whether in 5% or 2% condition. Currently, we did not own reliable data regarding subsequent neonatal outcomes following ultra-low O_2 tension culture.

CONCLUSIONS

In summary, we still suggested culturing human embryos *in vitro* in 5% O_2 tension from day 0 to day 5 or 6. The employ of ultra-low O_2 tension did not benefit for human in-vitro embryo development.

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