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ORIGINAL ARTICLE *Embryology*

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

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STUDY QUESTION: Is a reduction in the oxygen tension from 5 to 2% during extended culture from Day 3 onwards beneficial for human blastocyst development *in vitro*?

SUMMARY ANSWER: A reduction in oxygen concentration from 5 to 2% O_2 after Day 3 did not improve embryo development, quality and utilization rate.

WHAT IS KNOWN ALREADY: The human embryo leaves the fallopian tube to reach the uterine cavity around Day 3–4 post-ovulation. As the oxygen concentration ranges from 5 to 7% in the fallopian tube and decreases to 2% in the uterus, reducing the oxygen tension during extended culture from Day 3 onwards seems more physiological. We aim to mimic the *in-vivo* environment during *in-vitro* embryo culture. Therefore, we compared the effect of extended culture performed at 5% (control arm) or 2% oxygen (O_2; study arm) tension on blastocyst formation and quality.

STUDY DESIGN, SIZE, DURATION: Between December 2016 and September 2017, in two prospective studies, sibling embryos were randomized on Day 3 to either 5% O_2 (control) or 2% O_2 (study) for extended culture. In the control arms of both studies 1 and 2, the dishes with blastocyst medium were pre-equilibrated overnight in 5% O_2, 6% CO_2 and 89% N_2 at 37°C. In the 2% study groups, the overnight pre-equilibration of blastocyst media was performed in either 2% O_2 (study 1, 99 cycles) or 5% O_2 (study 2, 126 cycles). The latter provides a gradual transition from 5 to 2% O_2 environment for the study arm.

PARTICIPANTS/MATERIALS, SETTINGS, METHODS: Embryo culture until Day 3 was always performed in 5% O_2; if at least four embryos of moderate to excellent quality were obtained on Day 3, the sibling embryos were randomized to either 5% O_2 or 2% O_2 for extended culture. The endpoints were embryo development and quality on Day 5/6 and the utilization rate (embryos transferred and cryo-preserved). Statistical analysis was performed using the chi-square test, a *P*-value of <0.05 was considered significantly different.

MAIN RESULTS AND THE ROLE OF CHANCE: In study 1, 811 embryos were randomized on Day 3: 405 to the 2% O_2 and 406 to the 5% O_2 condition. No differences were observed in the blastulation rate (68.6 versus 71.9%; $P = 0.319$) and the proportion of good quality blastocysts on Day 5 (55.8 versus 55.2%; $P = 0.888$), nor in the utilization rate (53.1 versus 53.2%; $P = 1.000$). In study 2, 1144 embryos were randomized: 572 in each arm. Similarly, no significant difference was demonstrated in terms of the blastulation rate (63.6 versus 64.7%; $P = 0.758$), the proportion of good quality blastocysts (46.9 versus 48.8%; $P = 0.554$) or the utilization rate (49.8 versus 48.1%; $P = 0.953$).

LIMITATIONS, REASON FOR CAUTION: This study evaluated embryo development only until Day 5/6. The effect of oxidative stress on the developing embryo may only become evident at later stages (i.e. during implantation) and should therefore be studied in an RCT. The question also remains as to whether the switch to ultra-low oxygen tension from Day 4 onwards, when the embryo arrives in the uterus *in vivo*, would be preferential.

WIDER IMPLICATIONS OF THE FINDINGS: Based on the present study results, there is no benefit in lowering the oxygen tension from 5 to 2% from Day 3 onwards during extended human embryo culture.

STUDY FUNDING/COMPETING INTEREST(S): No funding was received for this study and the authors have no conflicts of interest to declare.

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Key words: oxygen tension / blastocyst culture / blastocyst formation / ultra-low oxygen / human

Introduction

During *in-vitro* culture of human embryos, we aim to mimic their *in-vivo* environment by a careful regulation of the temperature, oxygen (O_2), and the composition of the culture media. In the early years of IVF, embryo culture was performed under atmospheric oxygen concentrations (20%) and it was demonstrated to be more than effective by the birth of millions of children.

However, the physiological oxygen concentration of the mammalian oviduct ranges from 5 to 8.7% O_2 (Fischer and Bavister, 1993). Already in the early 70s, Steptoe *et al.* (1971) described the successful use of 5% O_2 leading to improved human embryonic development to the blastocyst stage. Other mammalian studies have also shown a beneficial effect of reduced oxygen (5%) on embryo development (Umaoka *et al.*, 1992; Olson and Seidel, 2000; Yuan *et al.*, 2003; Lequarre *et al.*, 2003; Wale *et al.*, 2010). While only small improvements in human preimplantation development were described when reducing the oxygen tension from 20 to 5% O_2 (Gomes Sobrinho *et al.*, 2011; Bontekoe *et al.*, 2012; Nastri *et al.*, 2016), live birth rates seemed increased (Bontekoe *et al.*, 2012).

During *in-vivo* preimplantation development, the human embryo reaches the uterine cavity around the morula stage, Day 3.5. The human intrauterine oxygen tension has been measured to be around 2% O_2 (Yedwab *et al.*, 1976; Ottosen *et al.*, 2006). This decreasing oxygen gradient coincides with the metabolic shift from oxidative phosphorylation, during the pre-compaction stage in the oviduct, to glycolysis, which is initiated post-compaction in the uterus (Leese, 1995; Thompson *et al.*, 1996). The use of an oxygen gradient from 7 to 2% O_2, mimicking the physiological oxygen tension in the female reproductive tract, has been shown to improve the development of larger embryos, like bovine embryos (Thompson *et al.*, 2000). Not unimportantly, these shifts in oxygen from 7 to 2% may have a significant influence on the expression of genes involved in embryonic metabolism, both for mouse and bovine embryos (Kind *et al.*, 2005; Harvey *et al.*, 2007).

The effect of this more physiological, ultra-low oxygen during prolonged *in-vitro* culture was recently reviewed by Morin (2017a). A reduction in oxygen tension from atmospheric to more physiologic levels is beneficial for the mammalian blastocyst, in terms of a higher cell number and decreased apoptosis, less DNA fragmentation and less oxidative stress. During this highly metabolically active period of growth of the embryo and differentiation into trophectoderm (TE) and inner cell mass (ICM), this decrease in oxygen may protect against oxidative stress (Guerin *et al.*, 2001).

At the start of this study, only a few reports were available on extended culture at 2% O_2, one study on frozen–thawed research embryos (Yang *et al.*, 2016), one on fresh embryos (Morin *et al.*, 2017b) and one on frozen or abnormally fertilized oocytes (Kaser *et al.*, 2018). While the first study did not observe any difference in blastulation or utilization rate, the latter two studies showed a higher blastulation rate after culture in 2% O_2, though this was not accompanied by a higher utilization rate. Before introducing a 'new technique or procedure' in the IVF lab (Harper *et al.*, 2012), we decided to perform a study randomizing sibling Day 3 embryos to ultra-low (2%) or low oxygen (5%) in a clinical setting. Therefore, the current study aimed to analyse blastocyst development after exposure of Day 3 embryos to 2 or 5% oxygen in each of two sibling studies: the first with direct, and the second with gradual, exposure to 2% O_2.

Material and Methods

For these studies, approval was obtained by the Local Ethical Committee of the Universitair Ziekenhuis Brussel (B.U.N. 143201630022 and B.U.N. 143201733715, respectively). All patients signed the informed consent forms for assisted reproductive treatment including participation in studies aiming to improve the quality of processes in the IVF laboratory.

Study protocol

The inclusion criteria were: patients scheduled for extended embryo culture; ICSI or IVF as insemination method; ejaculated sperm (fresh or frozen, autologous or heterologous); all ages; fresh embryo transfer (ET) on Day 5 or a freeze all strategy on Day 5; and all stimulation protocols. Exclusion criteria were: surgically obtained sperm; cycles with PGT or IVM cycles; or cycles with combined IVF and ICSI as the insemination method. All embryos were cultured in G185 incubators (K systems) in which the individual chambers were set at a constant temperature of 37.0 ± 0.2°C.

Embryo culture until Day 3 was performed under low oxygen (5% O_2) for all embryos. On Day 3 of development, embryos were evaluated and an embryo quality score (EQ1-4) was given (see below). Once four embryos of EQ1-3 were obtained, the cycle was included in the study. The embryos were allocated to the study group (2%) or control group (5%) based on a computer-generated randomization list. The first half of the embryos with EQ1-3 was assigned to the arm indicated by the randomization list; the second half were assigned to the other arm. In cases where an odd number of embryos were available, the extra embryo was allocated to the first arm. The primary outcome measures were embryo development on Day 5 and utilization rate (number of embryos transferred or cryopreserved on Day 5/6 per number of embryos undergoing extended culture from Day 3 to Day 5). The secondary outcome measures were implantation rate and clinical pregnancy rate.

Design of study 1: direct exposure

Between December 2016 and February 2017, 881 embryos from 99 patients were selected for inclusion. Embryo culture till Day 3 was performed under low oxygen (5% O_2) in cleavage medium. On Day 2 of development, dishes with blastocyst medium were prepared and equilibrated overnight in 2% O_2 (for the study arm) or in 5% O_2 (for the control arm). On Day 3, the embryos were transferred into the respective dishes.

Design of study 2: gradual exposure

Between February 2017 and September 2017, 1144 embryos from 126 patients were selected for inclusion. As in study 1, embryo culture till Day 3 was performed under low oxygen (5% O_2) in cleavage medium. On Day 2 of development, dishes with blastocyst medium were prepared and equilibrated overnight in 5% O_2 for both the study and the control arm. On Day 3 of development, embryos were transferred to the blastocyst medium. Control embryos were further cultured in an incubator with 5% O_2, while study embryos were placed in the incubator with 2% O_2, allowing a more gradual exposure from 5% O_2 to 2% O_2. The pO_2 was 70 mmHg after overnight equilibration at 5% oxygen. After changing the culture dishes to a 2% O_2 atmosphere, the pO_2 decreased at a rate of 0.33 mmHg /minute and stabilized at 50 mmHg after one hour.

Preparation of the dishes and embryo culture

All culture dishes (IVF round dish, Ø 60 mm, 35 Falcon) were prepared at room temperature the day before use. The dishes were prepared as follows: 12 individual droplets (25 µl) were placed in a circular and four drops were placed in the centre with an automated dispenser (multipetteplus, Eppendorf, VWR), after which they were covered with 7 ml paraffin oil (Ovoil, Vitrolife, Sweden). After preparation, culture dishes were equilibrated overnight in 5% O_2, 6% CO_2 and 89% N_2 at 37°C or in 2% O_2, 6% CO_2 and 92% N_2. (The latter was only for study 1). Embryo culture was performed in cleavage medium (83040010 A, Origio, Denmark) from Day 0 post injection till Day 3 for ICSI embryos. For IVF embryos, fertilization was performed in fertilization medium (83020060 A, Origio, Denmark) from Day 0 till Day 1; cumulus cells were removed in the morning of Day 1 after which the zygotes were placed in cleavage medium. On Day 3, embryos were transferred to blastocyst medium (83060010 A, Origio, Denmark) till Day 5 or 6. Embryos were always cultured individually.

Oocyte retrieval and insemination

After oocyte retrieval, cumulus–oocyte complexes (COC) were incubated in fertilization medium in G185 incubators until insemination for IVF or denudation for ICSI. Denudation and ICSI were performed as previously described (De Vos *et al.*, 2008). IVF was performed by the addition of 10 000 progressive motile sperm cells per 25 µl droplet with fertilization medium containing one or two COC.

Fertilization and embryo quality

Evaluation of all embryos from one patient was always performed in a sequential order, in which the maximum time between the first and last embryo was restricted to 10 min. Fertilization was assessed the day after insemination by the presence of two pronuclei under an inverted microscope at ×200 magnification. Evaluation of Day 3 embryos was based on the number and symmetry of the blastomeres, percentage of fragmentation, vacuolization, granulation and multinucleation. Based on all these parameters, an embryo quality (EQ) score was assigned to all normally fertilized embryos based on a predefined algorithm, which divides them into four categories: excellent, good, moderate or poor (De Munck *et al.*, 2015). On Day 5, blastocysts (BL) were scored according to the grading system developed by Gardner and Schoolcraft (1999), based on: (i) the expansion stage, (ii) estimation of the number of cells joining the compaction or blastulation; and (iii) the appearance of the TE and ICM. The following blastocyst quality scores were given to the Day 5 or 6 embryos: excellent, good, moderate or poor (De Munck *et al.*, 2015). On Day 5, fully compacted embryos, early blastocysts or fully expanded or hatching blastocysts with a visible TE and ICM were considered adequate for transfer. Supernumerary blastocysts were cryopreserved on Day 5 or 6 if they reached at least the full blastocyst stage (BL3) with an ICM and TE type A or B. Early blastocysts stage 1 and 2 were further cultured until Day 6 and cryopreserved if they developed to a full blastocyst with an ICM and TE type A or B. Specifically for this study design, the embryo utilization rate was calculated as the blastocysts that were transferred or cryopreserved per number of embryos undergoing extended culture to Day 5. The expansion of the TE was semi-quantitatively defined as the number of blastocysts (at least Bl3) with a type A TE.

Pregnancy outcomes

Pregnancy rates after fresh and frozen ET were calculated. Pregnancy was defined as a positive βhCG blood test 14 days after transfer. Clinical pregnancy was defined as the presence of at least one gestational sac at ultrasonographic visualization; multiple gestational sacs were counted as one clinical pregnancy. Clinical pregnancy with foetal heart beat was defined as the presence of at least one viable foetus 5–7 weeks after ET. Since the primary outcome parameter was the EQ on Day 5/6, mixed ET, i.e. transfer of embryos derived from both groups, were allowed. This provides inconclusive data in case of a single implantation after a multiple ET. Because of the sibling-oocyte study design, pregnancy data are only descriptive.

Sample size calculations and statistical analysis

Group sample sizes of at least 403 Day 3 embryos undergoing extended culture to Day 5 in group 1 (the treatment group) and at least 403 Day 3 embryos undergoing extended culture to Day 5 in group 2 (the control group) achieve 80% power to detect a difference between the group proportions of 0.1. The proportion in group 1 is assumed to be 0.5 under the null hypothesis and 0.6 under the alternative hypothesis. The proportion in group 2 is 0.6. The test statistic used is the two-sided Fisher's Exact test. The significance level of the test was targeted at 0.05.

Results

Study 1: direct exposure to 2% O_2 or standard 5% O_2

A total of 99 cycles were included: 8 IVF cycles and 91 ICSI cycles. Table I shows the fertilization and embryo development up to Day 3

Table I Study 1 with direct exposure to 2% O_2 or standard 5% O_2: fertilization and embryo quality on Day 3. Data are presented separately for IVF and ICSI cycles.

	ICSI	IVF
No. of cycles	91	8
No. of cumulus oocyte complexes	1231	122
No. of mature oocytes (%)	999 (81.2)	
Fertilization		
No. of fertilized oocytes (%)	809 (81.0)	66 (54.1)
Day 3 embryos undergoing extended culture to Day 5		
No. of embryos	752	59
EQ1 (%)	459 (61.0)	33 (55.9)
EQ2 (%)	211 (28.1)	18 (30.5)
EQ3 (%)	80 (10.6)	8 (13.6)
EQ4 (%)	2 (0.3)	0

EQ, embryo quality.

Table II Study 1 with direct exposure to 2% O_2 or standard 5% O_2: blastocyst development.

	2%	5%	*P* value
No. of Day 3 embryos	405	406	
EQ1 (%)	257 (63.5)	235 (57.9)	
EQ2 (%)	100 (24.7)	129 (31.8)	
EQ3 (%)	47 (11.6)	41 (10.1)	
EQ4 (%)	1 (0.2)	1 (0.2)	
Day 5 Evaluation			
Blastulation rate	278/405 (68.6)	292/406 (71.9)	0.319
EQ1+2 (%)	88 + 138/405 (55.8)	63 + 161/406 (55.2)	0.888
EQ3+4 (%)	63 + 116/405 (44.2)	75 + 107/406 (44.8)	
Utilization rate			
No. of embryos transferred	34	35	
No. of embryos cryopreserved on Day 5 + 6	123 + 58	111 + 70	
Utilization rate	215/405 (53.1)	216/406 (53.2)	1.000
Utilization rate based on Day 3 EQ			
EQ1 (%)	164/257 (63.8)	155/235 (66.0)	0.637
EQ2 (%)	43/100 (43.0)	56/129 (43.4)	1.000
EQ3 (%)	8/47 (17.0)	5/41 (12.2)	0.563
EQ4 (%)	0/1 (0.0)	0/1 (0.0)	1.000
Trophectoderm expansion			
At least Bl3 with TE type A	93	65	
Per No. embryos undergoing extended culture (%)	93/405 (23.0)	65/406 (16.0)	0.013
Per No. of blastulating embryos (%)	93/278 (33.5)	65/292 (22.3)	0.004

EQ, embryo quality; TE, trophectoderm; Type A according to Gardner and Schoolcraft (1999).

(in 5% O_2). On Day 3 of development, 752 ICSI embryos and 59 IVF embryos were randomly split between further culture at 2% O_2 or further culture at 5% O_2 (Table II). On Day 5, no significant difference was obtained in the blastulation rate (68.6 versus 71.9%; $P = 0.319$), nor in the high EQ scores (55.8 versus 55.2%; $P = 0.888$) and the utilization rate (53.1 versus 53.2%; $P = 1.000$). When analysing the utilization rate on Day 5/6 stratified according to the EQ on Day 3, no differences were found between the two arms. Interestingly, when analysing the expansion of the TE, defined as a TE type A from at least a full blastocyst on Day 5, a significant difference was found in favour of the 2% O_2 (23.0 versus 16.0%; $P = 0.013$). Of the 99 cycles, 58 patients received a fresh ET, one patient had insufficient EQ for transfer and for 40 patients a 'freeze all' strategy was applied with at least one frozen embryo. Supplementary Table SI presents the pregnancy outcomes after the fresh and subsequent frozen ET cycles. After a single ET, pregnancy rates of 59.2 and 54.5% were obtained in the 2 and 5% O_2 groups.

Study 2: gradual exposure to 2% O_2 or standard 5% O_2

A total of 126 ICSI cycles were included, allowing 1144 Day 3 embryos to be randomized between 2% O_2 and 5% O_2 (Table III). Table IV shows the blastocyst development of the randomized embryos. No significant difference was observed between the blastulation rate (63.6 versus 64.7%; $P = 0.758$), the high blastocyst quality scores (46.9 versus 48.8%; $P = 0.554$) or the utilization rate (49.8 versus 48.1%; $P = 0.953$). There was also no difference when the utilized embryos were stratified according to their EQ on Day 3. As compared to the direct exposure to 2% O_2, gradual exposure to 2% O_2 did not increase the expansion of the TE (15.2 versus 15.0%; $P = 1.000$). The pregnancy outcomes after fresh and subsequent frozen ETs are presented

Table III Study 2 with gradual exposure to 2% O_2 or standard 5% O_2: fertilization and embryo quality on Day 3.

No. of cycles	126
No. of cumulus oocyte complexes	1798
No. of mature oocytes (%)	1494 (83.1)
Fertilization	
No. of fertilized oocytes (%)	1230 (82.3)
Day 3 embryos undergoing extended culture to Day 5	
No. of embryos	1144
EQ1 (%)	693 (60.6)
EQ2 (%)	284 (24.8)
EQ3 (%)	163 (15.2)
EQ4 (%)	4 (0.3)

EQ, embryo quality.

Table IV Study 2 with gradual exposure to 2% O_2 or standard 5% O_2: blastocyst development.

	2%	5%	*P* value
No. of Day 3 embryos	572	572	
EQ1 (%)	348 (60.8)	345 (60.3)	
EQ2 (%)	142 (24.8)	142 (24.8)	
EQ3 (%)	81 (14.2)	82 (14.3)	
EQ4 (%)	1 (0.002)	3 (0.01)	
Day 5 evaluation			
Blastulation rate	364/572 (63.6)	370/572 (64.7)	0.758
EQ1+2 (%)	83 + 185/572 (46.9)	83 + 196/572 (48.8)	0.554
EQ3+4 (%)	142 + 162/572 (53.1)	121 + 172/572 (51.2)	
Utilization rate			
No. of embryos transferred	52	54	
No. of embryos cryopreserved on Day 5 + 6	121 + 112	123 + 98	
Utilization rate	285/572 (49.8)	275/572 (48.1)	0.953
Utilization rate based on Day 3 EQ			
EQ1 (%)	217/348 (62.4)	212/345 (61.4)	0.815
EQ2 (%)	59/142 (41.5)	53/142 (37.3)	0.544
EQ3 (%)	9/81 (10.0)	9/82 (11.0)	1.000
EQ4 (%)	0/1 (0.0)	1/4 (25.0)	1.000
Trophectoderm expansion			
At least Bl3 with TE type A	87	86	
Per No. embryos undergoing extended culture (%)	87/572 (15.2)	86/572 (15.0)	1.000
Per No. of blastulating embryos (%)	87/364 (23.9)	86/370 (23.2)	0.862

EQ, embryo quality; TE, trophectoderm; Type A according to Gardner and Schoolcraft (1999).

in Supplementary Table SII. Taking into account all single ETs, a pregnancy rate of 60.5% and 48.7% was obtained after culture under ultra-low or low oxygen respectively.

Discussion

This study evaluated the effect of direct or gradual exposure of cleavage stage sibling embryos to 2 or 5% O_2 on blastocyst development. Whether the exposure from 5 to 2% O_2 was direct or gradual, the 2% O_2 condition did not affect the blastulation rate, blastocyst quality on Day 5 or the embryo utilization rate. When direct exposure from 5 to 2% O_2 was applied, a significantly higher TE expansion was observed with 2% O_2. This difference was not seen after gradual exposure.

Only limited data is available on the effect of ultra-low oxygen on blastocyst development. The study of Yang (2016) compared the exposure to ultra-low (2%), low (5%) or atmospheric (20%) oxygen on frozen–thawed Day 3 embryos. Their results showed no significant differences in the blastocyst formation rate nor in the blastocyst cell numbers between the three groups. However, the apoptosis rate was significantly lower with 5% O_2. Although their first cleavages were performed under atmospheric oxygen conditions, a blastulation rate of 58.7, 63.6 and 66.7% was obtained after blastocyst culture in increasing oxygen concentrations and 23.9, 22.7 and 20.0% good quality blastocysts, respectively. This non-significant difference in blastulation rate between 2 and 5% O_2 was also obtained in our study, after direct exposure (68.6 and 71.9%) and after gradual exposure (63.6 and 64.7%). Also, the percentages of good quality blastocysts were not different between our two groups, neither after direct exposure (55.8 versus 55.2%), nor after gradual exposure (46.9 versus 48.8%). While the blastulation rate in our study was similar to the blastulation rate in the study by Yang (2016), the percentage of good quality blastocysts is twice as high in our study. This may be attributed to multiple differences between the two studies: (i) Yang (2016) included partially survived frozen–thawed embryos which may affect blastocyst development; (ii) different culture media were used (G series by Yang) which may have a different composition of antioxidants and free radical scavengers and thus a different response to stress; and (iii) the culture until Day 3 was performed under 20% oxygen and not under 5% oxygen as in our study. Exposure to 20% oxygen during the early cleavage stages has been shown to impair blastocyst development in mouse (Wale and Gardner, 2010).

Kaser *et al.* (2018) analysed the blastulation potential of normally and abnormally (>2PN) fertilized embryos after culture in 2 or 5% O_2 from Day 3 onwards. Culture under 2% oxygen led to a higher blastulation rate (40.2 versus 22.5%) and a higher utilization rate (36.8 versus 21.3%). Interestingly, all blastocysts derived from 3PN zygotes had a significantly lower cell count after culture at 2% O_2. Morin *et al.* (2017b) presented the effect of sequential oxygen changes as an oral presentation at ASRM; up to Day 3, the embryos were cultured in 5% O_2 and randomized to 2% or 5% O_2 from Day 3 onwards. Although

the blastulation rate was significantly higher in 2% O_2 (74 versus 66%; $P < 0.05$), the blastocyst utilization rates were not different (59 and 54%). These results are comparable to the utilization rates obtained in our study: 53.1 versus 53.2% and 49.8 versus 48.1%. Importantly, the aneuploidy rate was equal in the two oxygen tension arms (Morin *et al.*, 2017b).

The theoretical model of Byatt-Smith *et al.* (1991) on oxygen diffusion suggested that human embryos may become marginally hypoxic when cultured under (ultra-) low oxygen conditions, especially when cultured in small medium droplets. Considering that convection, driven by temperature differences, is able to homogenize the culture drops, it could prevent the development of anoxia (Baltz and Biggers, 1991). However, in our study, the embryos were cultured under very stable temperature conditions (37.0 ± 0.2°C; G185 incubators, K systems) and, more importantly, embryos were not taken out of the incubator between Days 3 and 5. This diminishes (if not excludes) the effect of convection to control for anoxia, thus indicating that the diffusion of oxygen, passing the two liquid phases medium and oil, appears to be sufficient in ultra-low oxygen exposure in 25 µl culture medium.

We chose to perform a 'sequential' oxygen tension strategy in which culture prior to Day 3 is performed under low oxygen (5%) and culture after Day 3 is under ultra-low oxygen (2%). The sequential strategy, though different from atmospheric oxygen to 5% O_2, has been shown to be detrimental for mouse embryo development compared to single culture at 5% O_2 (Wale and Gardner, 2010). This detrimental effect was not seen in our study; however, it is unknown how the embryo development would be affected by a continuous culture at 2% O_2. Fawzy *et al.* (2017) recently analysed the impact of continuous culture at a lower oxygen level (3.5% O_2) versus culture at 5% O_2 on the clinical pregnancy rate. Not only was the embryo utilization rate lower after culture in 3.5% O_2 (921/2549, 36% versus 1005/1823, 55%) also the clinical pregnancy rate after transfer on Day 5 was significantly lower in 3.5% O_2 culture (40 versus 52%; $P = 0.003$). Despite some flaws in the design (no RCT), this study indicates that a sequential low oxygen strategy is probably more favourable than continuous culture below 5% O_2 and that the early embryo development favours at least 5% O_2.

Hypoxic conditions have been described to stimulate the proliferation of the TE cells (Smith *et al.*, 2012). This proliferation or induced expansion was also observed in the present study after direct exposure to 2% O_2 but not after gradual exposure. On the contrary, Kaser *et al.* (2018) found a significantly lower mean cell count for the blastocysts (at least full blastocyst) after culture at 2% O_2 (62.0 versus 83.4; $P = 0.04$). However, their embryos were derived from 3PN zygotes, which might not be a fully reliable model to test whether hypoxic conditions are able to induce a beneficial proliferation of the TE or not. As TE proliferation has been linked with implantation potential (Hill *et al.*, 2013), it was speculated that 2% O_2 would increase the implantation potential in our study. Although the numbers are very low and only descriptive, the implantation potential of a single fresh blastocyst tended to be lower after direct exposure to 2% O_2. However, considering all subsequent frozen ET cycles, this trend disappeared. Besides stimulation of proliferation, low oxygen levels (5% versus atmospheric) also play an important role in reducing ROS, that may further influence gene expression and reduce mosaicism (Kind *et al.*, 2005). It would be interesting to study whether a reduction to 2% O_2 is translated into a lower rate of mosaicism and a subsequent lower miscarriage rate.

This study is a sibling-embryo study, excluding patient and cycle variation, and an important step to determine any influence of ultra-low oxygen on preimplantation development in a clinical setting. However, only a patient-by-patient RCT can evaluate whether or not ultra-low oxygen is in favour of higher implantation and live birth rates. Also unresolved is the question on how different culture media, be it sequential or single step, are influenced by a lower oxygen tension. Besides this, estrogen and progesterone also appear to affect the uterine pO_2 (Ng *et al.*, 2018). Therefore, it remains to be determined to what extent different stimulation protocols affect the implantation potential when using different oxygen concentrations.

To conclude, this study evaluated the effect of exposure of cleavage stage sibling embryos (Day 3) to ultra-low (2% O_2) or low (5% O_2) oxygen on blastocyst development. Except for a higher TE expansion rate after direct exposure to 2% O_2, no differences were observed between all of the analysed groups. Further studies (RCTs) are definitely needed to test whether this more physiological culture conditions have a clinical benefit.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

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Authors' roles

N.D.M.: conception and design of the study, construction of the database, analysis and interpretation of the data and writing of the article; R.J.: conception and design of the study, and critical review of the article; I.S., H.T., H.V.V. and G.V.: study design and critical review of the article.

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Conflict of interest

The authors have no conflicts of interest to declare.

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