human reproduction

ORIGINAL ARTICLE *Embryology*

The influence of oxygen concentration during embryo culture on obstetric and neonatal outcomes: a secondary analysis of a randomized controlled trial

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STUDY QUESTION: Does oxygen concentration during 3-day embryo culture affect obstetric and neonatal outcomes?

SUMMARY ANSWER: Oxygen concentration during 3-day embryo culture does not seem to affect the obstetric and neonatal outcomes measured.

WHAT IS KNOWN ALREADY: Atmospheric oxygen appears to be harmful during extended embryo culture. Embryo culture conditions might therefore be a potential risk factor for subsequent fetal development and the health of future children. No data are available concerning the obstetrics and neonatal outcomes after Day 3 transfer of embryos cultured under reduced and atmospheric oxygen tensions.

STUDY DESIGN, SIZE, DURATION: A secondary analysis of a previous randomized controlled trial assessing clinical pregnancy outcomes was carried out. This analysis included 1125 consecutive oocyte donation cycles utilizing ICSI or IVF and Day 3 embryo transfers between November 2009 and April 2012. The whole cohort of donated oocytes from patients who agreed to participate in the study were randomly allocated (1:1 ratio) to a reduced O_2 tension group (6% O_2) or an air-exposed group (20% O_2) based on a computer-generated randomization list. Fresh and vitrified oocytes were used for oocyte donation. Only those pregnancies with a live birth at or beyond 24 weeks of gestation were included.

PARTICIPANTS/MATERIALS, SETTING, METHODS: Day 3 embryos were cultured in an atmosphere of 5.5% CO_2, 6% O_2, 88.5% N_2 versus a dual gas system in air.

MAIN RESULTS AND THE ROLE OF CHANCE: From the eligible 1125 cycles, 564 were allocated to the 6% O_2 group and 561 cycles to the 20% O_2 group. However, 50 and 62 cycles did not reach embryo transfer in the 6% and 20% O_2 groups, respectively. No differences were found between 6% O_2 and atmospheric O_2 tension in the number of livebirths per embryo transfer (mean ± SD, 0.5 ± 0.7 versus 0.5 ± 0.7), pregnancy complications or neonatal outcomes. Both groups (6% and atmospheric O_2) had similar single and twin delivery rates (40.8% versus 38.1% and 10.7% versus 12.3%, respectively). Preterm delivery rates and very preterm delivery rates (10.80% versus 13.24% and 1.25% versus 2.94%, respectively), birthweight (3229 ± 561 g versus 3154 ± 731 g), low birthweight (2.92% versus 2.45%), birth height (50.18 ± 2.41 cm versus 49.7 ± 3.59 cm), head circumference (34.16 ± 1.87 cm versus 33.09 ± 1.85 cm) and 1 min Apgar scores (8.96 ± 0.87 versus 8.89 ± 0.96) were also similar between 6% and atmospheric O_2 groups, respectively.

LIMITATIONS, REASONS FOR CAUTION: The number of liveborns finally analyzed is still small and not all obstetric and neonatal variables could be evaluated. Furthermore, a small proportion of the obstetric and neonatal data was obtained through a questionnaire

filled out by the patients themselves. One reason for the lack of effect of oxygen concentration on pregnancy outcome could be the absence of trophectoderm cells at cleavage stage, which may make Day 3 embryos less susceptible to hypoxic conditions.

WIDER IMPLICATIONS OF THE FINDINGS: Nowadays many IVF laboratories use a more physiological oxygen concentration for embryo culture. However, the benefits of using low oxygen concentration on both laboratory and clinical outcomes during embryo culture are still under debate. Furthermore, long-term studies investigating the effect of using atmospheric O_2 are also needed. Gathering these type of clinical data is indeed, quite relevant from the safety perspective. The present data show that, at least in egg donation cycles undergoing Day 3 embryo transfers, culturing embryos under atmospheric oxygen concentration seems not to affect perinatal outcomes.

Introduction

The use of low oxygen concentration during embryo culture has been studied extensively owing to its implications for embryo development. Human embryos under *in vivo* conditions are exposed to low oxygen concentration that varies according to not only the section of the reproductive tract but also many other factors, such as uterine contractility, hormones, the autonomic system, cardiac pulsatility and myometrial and smooth muscle integrity (Yedwab *et al.*, 1976; Gosden and Byatt-Smith, 1986; Fischer *et al.*, 1992; Van Blerkom, 1998; Ng *et al.*, 2018). Culturing embryos under atmospheric tension is considered deleterious for embryo viability. Catt and Henman (2000) suggested that, at least in theory, three methods could ultimately help to minimize oxidative damage: decreasing the oxygen tension in the gas phase during *in vitro* culture; changing the formulation media to include antioxidant components; reducing the incubation time of male and female gametes during insemination to avoid oxidative damage caused by sperm cell metabolism. The most recent meta-analysis revealed that although a small improvement (5%) in live birth/ongoing pregnancy and clinical pregnancy rates is observed when low oxygen concentration is used during culture, the evidence was of low quality as embryologists were not blinded and some studies contained important confounding factors (e.g. different culture conditions and equipment favoring low O_2) (Nastri *et al.*, 2016).

There are increasing concerns about the possible long-term consequences of any type of change we may introduce during ART, such as changes in oxygen concentration during embryo culture, as it may impact the health of the next generation. Thus, embryo culture conditions might be a potential risk factor for subsequent fetal development and the health of future children (Fleming *et al.*, 2018). Since birthweight, among other factors, has historically been used as a marker of neonatal health, we wanted to investigate if atmospheric oxygen conditions could have affected obstetric and neonatal outcomes of children conceived after fresh and cryopreserved Day 3 transfer of embryos cultured under either atmospheric (20%) or low (6%) oxygen concentration, by performing a secondary analysis of our previously published randomized controlled trial (RCT) (NCT 01532193).

Materials and methods

Participants and study design

The present study was approved by the Institutional Review Board at the Instituto Valenciano de Infertilidad (IVI, Valencia, Spain). This secondary analysis of a previous RCT was carried out on 1125 consecutive oocyte donation cycles undergoing ICSI or IVF and Day 3 embryo transfers between November 2009 and April 2012, to compare the effect of 5.5% CO_2, 6% O_2 and 88.5% N_2 (5.5/6/88.5) versus 5.5% CO_2 and 20% O_2 in air. From the initial 1125 cycles eligible for the study, 564 cycles were randomly allocated to the 6% O_2 group and 561 cycles were allocated to the 20% O_2 group. However, in the 6% O_2 group, 50 cycles did not reach embryo transfer (35 due to fertilization failure/bad oocyte quality, 13 due to endometrial bleeding and two for other reasons), and in the 20% O_2 group, 62 cycles did not reach embryo transfer (37 due to fertilization failure/bad oocyte quality, 13 due to endometrial bleeding and 12 for other reasons).

Within the 4-year follow-up period, the maximum number of frozen embryo transfers (FETs) was four in the low O_2 group and five in the atmospheric O_2 tension group. The mean numbers of embryos transferred in the low O_2 and atmospheric O_2 groups were 2.46 ± 1.21 and 2.28 ± 1.15 (mean $\pm$ SD), respectively ($P = 0.101$), while the mean number of frozen embryos transferred was $2.14 + 2.29$ and $2.01 + 2.20$ (mean $\pm$ SD), respectively ($P = 0.267$).

Finally, a total of 1013 cycles with fresh embryo transfers were included in the study (514 in the 6% O_2 group and 499 in the 20% O_2 group). In each allocation group, those women who did not achieve pregnancy or had an early miscarriage became pregnant following cryotransfer (FET) with the surplus embryos (734 FETs) (Fig. 1).

The inclusion criteria for oocyte recipients were women with good physical and mental health, aged between 25 and 45 years; first or second oocyte recipient treatment; Day 3 embryo transfers; BMI between 18 and 30 kg/m^2. The exclusion criteria were uterine pathology or hydrosalpinx, recurrent miscarriage and chronic illness. Furthermore, the proportion of smokers did not differ between groups (3.2% in the 6% O_2 group versus 1.9% in the 20% O_2 group).

The whole cohort of donated oocytes from patients that agreed to participate in the study were randomly allocated (1:1 ratio) to a reduced O_2 tension group (6% O_2) or an air-exposed group (20% O_2)
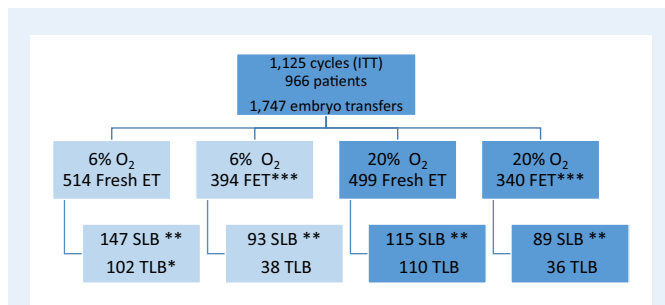
Figure 1. Flowchart showing the origins of all live births studied. *TLBs correspond to the number of babies born from twin pregnancies. **SLBs correspond to the number of babies born from single pregnancies. ***Number of total fresh embryo transfers performed (including all attempts per group). ITT, intention to treat; ET, embryo transfer; FET, frozen embryo transfer; SLB, single liveborn; TLB, twin liveborn.

based on a computer-generated randomization list. Both fresh and vitrified oocytes were used for oocyte donation.

Data source and outcome measures

In the low O_2 group, the loss to follow-up was 5.9% whereas it was 11.1% in the high-tension group. For frozen cycles, the loss to follow-up was 4.9% in the low O_2 group, whereas in the high O_2 tension group, this rate was 2.7%.

For assessment of pregnancy follow-up, we only included in our study those pregnancies with a livebirth at or beyond 24 weeks of gestation. Information on the past medical and obstetric history of the women participating in this study, as well as data on the IVF cycle, was obtained from computerized clinical charts at IVIRMA Valencia, Spain. Data on pregnancy follow-up and deliveries were collected mostly from medical records or reports sent by the doctors caring for the pregnant women and, in 15% of the cases, through a survey sent to the patients.

A total of 730 babies were included in the study. There were 444 singleton liveborns (60.8% of the total). A total of 380 (52.1%) resulted from embryos that had grown under low oxygen tension (6% O_2) and 350 (47.9%) from embryos that had grown under atmospheric air conditions (20% O_2). There were 286 twin liveborns (32.8% of the total), 140 (48.9%) under low oxygen conditions and 146 (51.0%) under atmospheric oxygen concentration (Fig. 1).

The outcome measures were rate of positive β-hCG, clinical pregnancy rate, miscarriage rate, ongoing pregnancy rate, obstetric complications and neonatal outcome. The positive β-hCG/embryo transfer rate was defined as a serum β-hCG level of at least 50 mIU/ml 12 days after embryo transfer. Clinical pregnancy was defined by the presence of a gestational sac and fetal heartbeat confirmed by transvaginal ultrasound examination at 6–8 weeks of gestation. The miscarriage rate was defined as the early miscarriage rate (i.e. pregnancy losses before 12 weeks of gestation). Ongoing pregnancy was defined as a viable intrauterine pregnancy after 12 weeks of gestation. The number of livebirths/embryo transfer was defined as the number of live newborns per embryo transfer.

The obstetric and neonatal outcome measures were pregnancy complications (diabetes, hemorrhage, pregnancy-induced hypertension and premature rupture of membranes), weeks at delivery, type of delivery, sex, birthweight (and other related measures), height at birth, head circumference (cm), Apgar scores, congenital malformations and puerperal problems including hemorrhagic events and infections.

Information on pregnancy outcome was obtained in all cases but data on problems occurring during pregnancy could be retrieved in only 306 patients, 136 from the low O_2 group and 170 from the normoxic condition group.

We defined small for gestational age (GA), very small for GA, large for GA and very large for GA as a birthweight <10th, <3rd, >90th and >95th percentile, respectively. Low birthweight and very low birthweight were defined as <2500 g and <1500 g, respectively. Large birthweight was defined as >4500 g. Birthweight Z-scores were also calculated. In the case of twin pregnancies, the inter-twin birthweight disparity was calculated in grams. Preterm births and very preterm births were defined as those occurring <37 and <32 weeks of gestation, respectively.

Stimulation protocol for donors

The controlled ovarian stimulation protocol for donors consisted of administering a daily dose of a GnRH-agonist (decapeptyl, 0.1 mg; Ipsen Pharma, Barcelona, Spain) in the luteal phase after menses. Controlled ovarian stimulation was started with 225 IU/day of recombinant FSH (Gonal F, Merck Serono; Barcelona, Spain or Puregon, Shering Plough; Madrid, Spain) or hMG (Menopur, Ferring Pharmaceutical; Madrid, Spain). The daily dose was adjusted to ovarian response. Stimulation was continued until the mean diameter of leading follicles was >18 mm. Recombinant hCG (Ovitrelle, Merck Serono) was administered and oocyte retrieval was carried out 36 h later. Anonymous donors were matched to oocyte recipients according to phenotype and blood groups. As the use of vitrified oocytes yields similar clinical outcomes compared to fresh oocytes (Cobo et al., 2010), both were used for oocyte donation purposes.

Endometrial preparation and oocyte and embryo handling

The protocol for endometrial preparation differed according to the presence or absence of ovarian function, as previously described (de los Santos et al., 2013).

IVF procedures

Four hours after oocyte retrieval, or 2 h after oocyte warming, oocytes were injected as previously described (Rubio et al., 1997). ICSI was performed at 400× magnification with a IX7 Olympus microscope. Based on the allocation group, both oocytes and embryos were culture equilibrated under oil in a 50 μl drop of Sydney IVF cleavage media (COOK) (Limerick, Ireland), which had been previously equilibrated overnight in the respective gas mixtures and then incubated in Sanyo MCI 5M incubators (Etten-Leur, the Netherlands) set at either 6% O_2 or 20% O_2 under a humidified environment.

Statistical analysis

The sample size estimation for the initial published study (de los Santos *et al.*, 2013) was based on an expected 10% increase in the clinical pregnancy rate per cycle following culture with a reduced O_2 concentration. Analysis based on a two-sided significance level with α of 0.05 and a β error of 0.2% showed that at least 380 cycles were required per arm.

From the total number of births achieved in our first publication, rates of neonatal and obstetric outcomes and the corresponding risk ratios (RRs) with 95% CI were calculated. A Poisson regression was performed to calculate the RRs.

Data are presented as mean $\pm$ SD or n (%). The differences in outcomes between groups were tested by the Student's *t*-test for continuous variables and Fisher's exact test for binomial variables. A *P* value of <0.05 was considered statistically significant. R statistical software package version 3.5.3 was used for statistical analysis.

Results

IVF outcomes

A total of 524 pregnant women participated in this study (Supplementary Table SI). Small but significant differences were found in their BMIs. No differences were found regarding the cause of infertility between women in the reduced and atmospheric oxygen groups. The IVF outcomes after fresh embryo transfer were similar in both study groups. Long-term follow-up of the initial pregnancies showed no significant impact of culture conditions on the mean LBs per transfer and the LB rate per intention to treat (ITT) cycle (Table I).

In FET cycles (Supplementary Table SII), no statistically significant differences were found after transfer of cryopreserved embryos cultured under either oxygen concentration. Only the number of embryos transferred appeared to be marginally significant ($P = 0.05$). Similar results were found when taking fresh embryo transfers and FETs into consideration. Embryos cultured under low oxygen did not yield higher pregnancy, ongoing pregnancy and delivery rates per ITT compared to embryos grown under atmospheric oxygen tension (Table II).

In the low O_2 group, the loss to follow-up was 5.9%; loss was 11.1% in the high-tension group. For frozen cycles, the loss to follow-up in the low O_2 group was 4.9%; in the high O_2 tension group, this rate was 2.7%.

Obstetric outcomes

In this study, we analyzed twin and singleton pregnancies together because we had the same proportion of twin pregnancies in each group (10.5% in the 6% oxygen group versus 12.3% in the 20% oxygen group; P = NS). In the fresh embryo transfers and FET groups with singleton deliveries, no differences were found in the cesarean section rate (70.33% and 68.57%, respectively, in the 20% oxygen group versus 58.47% and 55.13%, respectively, in the 6% oxygen group, P = NS). However, the proportion of cesarean sections was higher in twin deliveries with both fresh embryo transfers and FET (Supplementary Table SII, SIII, SIV, SV and SIV).

The incidence of diabetes in pregnancy (11.8% versus 11.8%), first, second and third trimester bleeding (39.7% versus 38.8%), premature rupture of membranes (9.6% versus 7.1%), pregnancy-induced hypertension (12.5% versus 15.2%) and puerperal problems (8.1% versus 10%) was similar in the 20% and 6% oxygen groups, respectively (P = NS).

Table I IVF outcomes following fresh embryo transfers.

	6% O_2	20% O_2	RR [95% CI]	*P*
Number of cycles (ITT)	564	561		
Number of fresh ETs	514	499		
Positive β-hCG/fresh ET	318 (61.9%)	303 (60.7%)	1.02 [0.92, 1.12]	0.71
Clinical pregnancy/fresh ET	276 (53.7%)	263 (52.7%)	1.02 [0.91, 1.14]	0.75
Ongoing pregnancy/fresh ET	233 (45.3%)	227 (45.5%)	1.00 [0.87, 1.14]	1
Miscarriage/fresh ET	83 (16.2%)	73 (14.6%)	1.10 [0.83, 1.47]	0.50
Clinical miscarriage/fresh ET	41 (8.0%)	34 (6.8%)	1.17 [0.76, 1.81]	0.48
Multifetal pregnancy/fresh ET	84 (16.34%)	105 (21.04%)	0.77 [0.59, 1.00]	0.06
Reduction of yolk sac/fresh ET	15 (3.2%)	14 (3%)	1.04 [0.51, 2.14]	0.91
Number of embryos transferred/fresh ET	1.9 ± 0.3	1.9 ± 0.3		0.45
LB mean/fresh ET	0.5 ± 0.7	0.5 ± 0.7		0.88
Perinatal mortality/fresh ET	0 (0%)	1 (0.2%)		0.99
Total deliveries/fresh ET	198 (40.8%)	170 (38.1%)	1.07 [0.91, 1.26]	0.40
Twin deliveries/fresh ET	51 (10.5%)	55 (12.3%)	0.85 [0.59, 1.22]	0.38
Total deliveries/ITT	198 (37.0%)	170 (33.5%)	1.11 [0.94, 1.30]	0.24
Liveborns/fresh ET	249 (0.44)	279 (0.40)		0.88

ET, embryo transfer; ITT, intention to treat; LB: live birth; RR, risk ratio.
Data are expressed as n (%) or mean ± SD. The RR was calculated with the 20% oxygen group as the reference group. The *P* values were calculated with Fisher's exact test.
Deliveries per ITT were calculated considering the cases of loss to follow-up.

No differences were found between the study groups in the GA at delivery, preterm birth and very preterm birth rates in singleton and twin pregnancies (Tables III and IV).

Neonatal outcomes

Regarding the neonatal endpoints of this study, no statistical differences were observed between the study groups. The prevalence of congenital malformations was similar between O_2 groups. Apart from those registered at birth (Tables III and IV), there were three terminations of pregnancy because of fetal abnormalities: two in the low oxygen group (a case of acrania and a case of chromosome 2 microdeletion—Feingold syndrome) and one in the high oxygen group (a case of acrania). Similar sex ratios, birthweight (analyzed in different ways), head circumference and Apgar scores were also found in singletons and twins in the two O_2 groups (Tables III and IV). Likewise, no differences in sex ratio, birthweight, head circumference or Apgar scores were observed when analyzing the fresh embryo transfer and FET groups separately (Supplementary Tables SIII, SIV, SV and SVI).

Discussion

The concentrations of oxygen *in vivo* (in follicular fluids, fallopian tubes and uterine cavities) (Yedwab *et al.*, 1976; Fischer *et al.*, 1992;

Table II Cumulative IVF outcomes of all ETs.

	6% O_2	20% O_2	RR [95% CI]	P
Number of cycles	564	561		
Positive β-hCG/ITT	411 (72.87%)	390 (69.52%)	1.048 [0.97, 1.13]	0.24
Clinical pregnancy/ITT	381 (67.55%)	357 (63.64%)	1.062 [0.96, 1.16]	0.17
Ongoing pregnancy/ITT	328 (58.16%)	310 (55.26%)	1.052 [0.95, 1.166]	0.34
Total deliveries/ITT	277 (49.11%)	247 (44.03%)	1.115 [0.984, 1.265]	0.094

Poisson regression was used to calculate the proportions, RRs and P values. Data are expressed as n (%).

Table III Pregnancy outcomes of liveborn singletons from fresh ET and frozen embryo transfers.

	6% O_2	20% O_2	RR [95% CI]	P
Number of SLBs	240	204		
Females	105 (43.75%)	93 (45.59%)	0.95 [0.77, 1.18]	0.69
Preterm births (<37 w)	26 (10.8%)	27 (13.24%)	0.81 [0.49, 1.35]	0.43
Very preterm births (<32 w)	3 (1.25%)	6 (2.94%)	0.43 [0.11, 1.67]	0.22
Weeks at delivery	39.04 ± 2.03	38.67 ± 3.0	0.36 [−0.11, 0.85]	0.13
Birthweight (g)	3229 ± 561	3154 ± 731	75.85 [−0.60, 1.05]	0.26
Low birthweight (<2500 g)	17 (7.08%)	22 (10.78%)	0.65 [0.35, 1.20]	0.17
Low birthweight at term	7 (2.92%)	5 (2.45%)	1.19 [0.38, 3.69]	0.76
Very low birthweight (<1500 g)	2 (0.83%)	7 (3.43%)	0.24 [0.05, 1.15]	0.07
Large birthweight (>4500 g)	2 (0.83%)	3 (1.47%)	0.57 [0.09, 3.35]	0.53
Small for gestational age (birthweight <p10)	19 (9.36%)	17 (10.69%)	0.87 [0.47, 1.62]	0.67
Very small for gestational age (birthweight <p3)	9 (4.43%)	5 (3.14%)	1.41 [0.48, 4.21]	0.52
Large for gestational age (birthweight >p90)	33 (16.26%)	28 (17.61%)	0.75 [0.45, 1.26]	0.28
Very large for gestational age (birthweight >p95)	17 (8.37%)	11 (6.92%)	1.21 [0.58, 2.51]	0.60
Birthweight Z-score	−0.03 ± 1.15	0.08 ± 1.47	0.04 [−0.21, 0.31]	0.72
Height (cm)	50.18 ± 2.41	49.67 ± 3.59	0.49 [−0.21, 1.20]	0.17
Head circumference (cm)	34.16 ± 1.87	33.90 ± 1.85	0.26 [−0.30, 0.83]	0.36
1 min Apgar score	8.96 ± 0.87	8.89 ± 0.96	0.07 [−0.17, 0.31]	0.57
5 min Apgar score	9.71 ± 0.61	9.65 ± 0.63	0.06 [−0.12, 0.24]	0.50
10 min Apgar score	9.98 ± 0.43	9.75 ± 0.43	0.08 [−0.08, 0.26]	0.33
Cesarean sections	96 (40.00%)	98 (48.04%)	0.92 [0.66, 1.02]	0.08
Congenital malformations (major and minor)	8 (3.35%)	6 (2.96%)	1.13 [0.39, 3.21]	0.81

Data are expressed as n (%) or mean ± SD. The RRs were calculated with the 20% oxygen group as the reference group. The P values were calculated with Fisher's exact test or the Student's t-test, where appropriate.
p3, third percentile; p10, 10th percentile; p90, 90th percentile; p95, 95th percentile; SLB, singleton liveborn; w, weeks.

Waldenström *et al.*, 2009) are much lower than those currently used in many IVF laboratories, which are based on atmospheric oxygen concentration and may provoke a certain degree of toxicity to the embryos (Catt and Henman, 2000). Although improvements have been incorporated into *in vitro* embryo culture and, as a result, clinical outcomes have significantly improved over the years (Swain, 2010, 2012; Nastri *et al.*, 2016), oxygen concentration is considered a critical parameter in *in vitro* culture systems. In fact, some studies in mice revealed that embryos generated under 5% oxygen more closely resemble *in vivo*-developed embryos (Garrisi *et al.*, 1993; Rinaudo *et al.*, 2006). In rhesus monkeys, the O$_2$ partial pressure is lower in the uterus ($\sim$18 mmHg) than in the fallopian tubes ($\sim$58 mmHg), but data comparing these compartments in humans are limited. However, what is reported so far is that the uterine measurements of oxygen partial pressure (ppO$_2$) vary from 6.6 to 32 mmHg and increase by the time of ovulation and in the luteal phase. In any case, these values are far from the atmospheric ppO$_2$, which is 750 mmHg (Ottosen *et al.*, 2006).

The underlying mechanism by which the use of atmospheric oxygen may be harmful for human embryos was inferred from animal studies. These studies revealed that low oxygen concentration during embryo culture could be beneficial to gene expression patterns, the proteome, energy metabolism or the methylome (Katz-Jaffe *et al.*, 2005; Rinaudo *et al.*, 2006; Wu and Zhang, 2010; de los Santos *et al.*, 2015; Kaser *et al.*, 2018). In the early stages, 20% oxygen was associated with an overall increase in amino acid turnover and pyruvate uptake by mouse

embryos. In contrast, after compaction, 20% oxygen was related to a decrease in amino acid turnover and glucose uptake (Kaser *et al.*, 2018). Some studies show clear evidence suggesting that global loss of DNA methylation of the paternal genome is attributable to Tet-mediated oxidation. Tet proteins contribute to dynamic changes in DNA methylation, and gene expression studies will enhance our understanding of epigenetic regulation (Wu and Zhang, 2010; de los Santos *et al.*, 2015; Kaser *et al.*, 2018). There are few studies comparing reduced and atmospheric oxygen culture conditions in humans. Studies looking at the metabolic fingerprint of good quality Day 3 human embryos showed that, at least in terms of net depletion or release of some relevant analytes such as glucose or fatty acids (Katz-Jaffe *et al.*, 2005; de los Santos *et al.*, 2015), no differences were found between low O$_2$ and normoxic embryo culture conditions. More recent data in human blastocysts have demonstrated that, at the blastocyst stage, even small variations of oxygen during culture (from 5% to 2%) impact the relative abundances of anabolic amino acids and metabolites involved in redox homeostasis by upregulating mucin 1, showing that oxygen influences embryo metabolism and may be important, at least at this more differentiated stage of development (Kaser *et al.*, 2018).

Therefore, the question remains of whether the plasticity or adaptability that embryos are able to demonstrate *in vitro* may also account for their oxygen resilience, at least at preimplantation stages, and their ability to implant after embryo transfer. In addition, the use of ultralow oxygen concentrations, which is an even more physiological approach for embryo culture, yields inconclusive results (Kaser *et al.*, 2016,

Table IV Pregnancy outcomes of liveborn twins from fresh ET and frozen embryo transfers.

	6% O$_2$	20% O$_2$	RR [95% CI]	P
Number of TLBs	140	146		
Females	69 (49.2%)	70 (47.9%)	1.02 [0.81, 1.30]	0.82
Preterm births ($<$37 w)	86 (61.43%)	84 (57.5%)	1.06 [0.88, 1.29]	0.50
Very preterm births ($<$32 w)	9 (5.71%)	10 (6.85%)	0.83 [0.33, 2.05]	0.69
Weeks at delivery	35.88 $\pm$ 2.56	35.87 $\pm$ 2.73	0.0 [$-$0.61, 0.63]	0.97
Birthweight (g)	2312 $\pm$ 498	2318 $\pm$ 569	$-$5.4 [$-$14.2, 131.2]	0.93
Low birthweight ($<$2500 g)	69 (49.2%)	73 (50.0%)	0.98 [0.78, 1.24]	0.90
Low birthweight at term	13 (9.29%)	20 (13.7%)	0.67 [0.35, 1.31]	0.24
Very low birthweight ($<$1500 g)	8 (5.71%)	10 (6.85%)	0.83 [0.39, 2.05]	0.69
Large birthweight ($>$4500 g)	1 (0.88%)	3 (2.05%)	0.35 [0.0, 3.41]	0.37
Small for gestational age (birthweight $<$p10)	34 (30.09%)	40 (27.40%)	0.91 [0.62, 1.34]	0.65
Very small for gestational age (birthweight $<$p3)	18 (15.9%)	15 (10.27%)	1.49 [0.76, 2.90]	0.23
Large for gestational age (birthweight $>$p90)	1 (0.88%)	3 (0.22%)	0.35 [0.03, 3.41]	0.37
Very large for gestational age (birthweight $>$p95)	0 (0%)	1 (0.7%)	NA	1
Birthweight Z-score	$-$1.00 $\pm$ 1.18	$-$0.94 $\pm$ 1.00	$-$0.15 [$-$0.44, 0.11]	0.26
Height (cm)	46.96 $\pm$ 2.77	45.99 $\pm$ 4.03	0.96 [$-$0.21, 2.14]	0.11
Head circumference (cm)	32.44 $\pm$ 1.50	32.45 $\pm$ 2.23	$-$0.01 [$-$0.79, 0.77]	0.97
1 min Apgar score	8.70 $\pm$ 0.94	8.46 $\pm$ 1.34	0.28 [$-$0.19, 0.67]	0.28
5 min Apgar score	9.48 $\pm$ 0.72	9.58 $\pm$ 0.62	$-$0.10 [$-$0.35, 0.14]	0.41
10 min Apgar score	9.70 $\pm$ 0.72	9.62 $\pm$ 0.65	0.04 [$-$0.46, 0.53]	0.88
Cesarean sections	13 (38.1%)	2 (10%)	3.81 [0.91, 15.81]	0.07
Congenital malformations (major and minor)	5 (3.57%)	6 (4.11%)	0.86 [0.27, 2.78]	0.81

Data are expressed as n (%) or mean $\pm$ SD. The RRs were calculated with the 20% oxygen group as the reference group. The *P* values were calculated with Fisher's exact test or the Student's *t*-test, where appropriate.
TLB, liveborn twins.

2018; Morin 2017). Kaser et al. (2016) suggested that blastocyst yield and quality may be superior when O_2 tension is reduced from 5% to 2% on Day 3. Thus, they proposed sequential oxygen exposure (5% from Days 1 to 3, then 2% from Days 3 to 5) (Kaser et al., 2016).

Regarding clinical outcomes, the use of a low oxygen environment showed the benefits of a more physiological approach, especially for blastocyst culture (Nastri et al., 2016). However, this recent meta-analysis found low-quality evidence that low O_2 is better for live birth/ongoing pregnancy [risk ratio (RR) = 1.1, 95% CI 1.0–1.3] and clinical pregnancy (RR = 1.1, 95% CI 1.0–1.2) compared to atmospheric O_2 concentration. Some examples of study flaws include the use of different incubators (a new model for the low O_2 group and an old model for the normoxic group), the unblinding of embryologists and the lack of rigor regarding random sequence generation and group allocation (Nastri et al., 2016).

Studies on the long-term effect of using atmospheric O_2 are missing and gathering these types of data is important, especially when there are increasing concerns about the possible long-term consequences of any type of change we may introduce during in vitro human embryo culture, as has been suggested regarding the use of culture media or some of its components (Dumoulin et al., 2010; Zandstra et al., 2015; Marianowski et al., 2016; Thompson et al., 2016).

In this study, we wanted to know whether the use of low or atmospheric O_2 concentration in our previous RCT had an impact on the pregnancies achieved during the study and their neonatal outcomes.

As far as we know, no previous report has looked at the relevant obstetric and neonatal outcomes following use of two different oxygen concentrations during embryo culture.

Our singleton and twin oocyte donation pregnancies following normoxic embryo culture had similar pregnancy complications, GA at delivery, preterm birth rate, birthweight, low birthweight rate, small for GA rate and Apgar scores as those previously reported in pregnancies following vitrified oocyte donation (Cobo et al., 2014). The cesarean section rates were similarly high. Likewise, although limited data on obstetric complications were gathered, our data are comparable with previous reports by our group (Cobo et al., 2014) and others (De Munck et al., 2016) describing oocyte donation programs using fresh and/or vitrified oocytes.

Placenta-mediated pregnancy complications, including pre-eclampsia, birth of a small for GA child, placental abruption or late pregnancy loss, are common (Maheshwari et al., 2013; Bidarimath and Tayade, 2017; Kingdom et al. 2018; Sultana et al., 2018). Oxygen also seems to be very important in placental development and function (Pinborg et al., 2013). Trophoblast culture under different oxygen concentrations has an impact on the invasive phenotype in vitro (Udayashankar et al., 2011). However, based on our results, culturing cleavage-stage human embryos at atmospheric O_2 concentration did not influence these placenta-derived complications. One reason for the lack of effect of oxygen concentration on pregnancy outcome in this study could be the absence of trophectoderm cells (known precursors of the placenta), which may make Day 3 embryos less susceptible to hypoxic conditions. Future inquiries will be necessary to address the impact of low and high oxygen concentration at the blastocyst stage, when the initial steps of trophoblast differentiation have already started, although embryonic genome activation and metabolism changes start at cleavage stages (Devreker and Englert, 2000; Stokes et al., 2005; Ménézo et al., 2010). Another possibility may be that placenta-associated

pregnancy complications are not affected by the culture conditions and are more influenced by maternal related factors.

Additionally, no differences were observed in live birth rates or neonatal outcomes between the two study groups. Neonatal outcomes were analyzed according to the multiplicity of the pregnancies. In the present study, we did not find any significant statistical dissimilarities between the 6% and 20% O_2 groups, either among singleton or twin pregnancies. Transfer of more than one embryo may decrease the birthweight of singletons (Kliegman et al., 1990; Grady et al., 2012; Pinborg et al., 2013). In our case, a similar number of embryos was transferred in both groups.

We do not have an exact explanation for the high proportion of cesarean deliveries for twin pregnancies in both oxygen groups, as deliveries took place in different hospitals. However, many of these pregnancies were considered high-risk owing to other factors (advanced maternal age, oocyte donation, multiple pregnancy, cryopreservation, etc.) and this might have also influenced the mode of delivery. Additionally, a previously published series of oocyte donation pregnancies also showed a high prevalence of cesarean deliveries (Cobo et al., 2014).

Birthweight has historically been used as a marker of neonatal health (Kliegman et al., 1990). Neonatal outcomes of singletons after assisted reproduction, such as congenital malformations, perinatal mortality and birthweight among others, are poorer than those of children conceived spontaneously (Kliegman et al., 1990; Grady et al., 2012; Henningsen and Pinborg, 2014; De Munck et al., 2016; Qin et al., 2016). Birthweight seems to be lower after ART than with spontaneous conception, but there are differences between ART techniques. For instance, thawed embryo transfers seem to result in higher birthweight (Qin et al., 2016) and better perinatal outcomes than fresh embryo transfers (Maas et al., 2016). Apart from the use of cryopreservation, other variables such as the culture medium, the stage of the embryo at transfer, or the number of embryos being transferred should also be considered.

Our data show that, in singletons, not only does the oxygen concentration during culture have no effect on birthweight, but the cryopreservation procedure has no consequence either. These data do not support previous published data comparing fresh and cryopreserved cycles (Pinborg et al., 2013; Maas et al., 2016) but agree on the fact that birthweight does not seem to be affected by culture conditions when fresh transfers are performed (Maas et al., 2016), despite differences in pregnancy rates.

We need to acknowledge some limitations of our study. The number of pregnancies analyzed was small and data on pregnancy problems could not be retrieved for all patients. Furthermore, a small proportion of the obstetric and neonatal data was obtained through a questionnaire filled out by the patients themselves. Despite these limitations, we believe this is the largest series published so far on this issue.

In conclusion, our limited data show promising results suggesting no apparent effect on live birth and neonatal outcomes when a high oxygen concentration is used at early stages (Day 3) of embryo development, at least in the oocyte donation cycle. Data from blastocyst transfers under normoxic and hypoxic conditions need to be reported to better understand the long-term consequences of the atmospheric O_2 environment during culture.

Given the above, our results are interesting because presently, despite the recommendations of single blastocyst transfer, many patients come to the ART clinics to achieve pregnancy through oocyte

donation and Day 3 embryo transfers. Thus, it is important to analyze the potential impact, if any, of embryo culture conditions on the long-term health of the children conceived through these *in vitro* techniques.

Supplementary data

Supplementary data are available at *Human Reproduction* online.

Authors' roles

M.R.A. exploited the data base, participated in the elaboration of tables and figures and data analyses. V.S. supervised the clinical data, wrote the manuscript and gave the final approval of the manuscript. P.G. cultured embryos, participated in the laboratory and clinical data entry. J.M.D.L.S. participated in the quality control of the laboratory equipment and in the clinical data entry. J.R. gave the final approval of the manuscript. A.T.N. made the statistical analyses. M.J.D.L.S. conceived, supervised the project, wrote the manuscript.

Conflict of interest

The authors declare that they have no conflict of interest with regard to the content of this manuscript.

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