Human Reproduction Open, pp. 1–11, 2020
doi:10.1093/hropen/hoz036

human reproduction open

ORIGINAL ARTICLE

Reduced oxygen concentration during human IVF culture improves embryo utilization and cumulative pregnancy rates per cycle

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Submitted on March 9, 2019; resubmitted on September 25, 2019; editorial decision on October 16, 2019

STUDY QUESTION: Do different oxygen levels during human IVF embryo culture affect embryo utilization, cumulative IVF success rates per cycle and neonatal birthweight?

SUMMARY ANSWER: After 2 days of culture, a lower oxygen level (5%) leads to more good-quality embryos and more embryos that can be cryopreserved, and thereby to a higher cumulative live birth rate per cycle when compared to embryo culture in 20% oxygen, while birthweights are similar.

WHAT IS KNOWN ALREADY: Several studies have compared IVF outcome parameters after embryo culture in a more physiological level of 5% oxygen and the atmospheric level of 20%. Although there is consensus that embryo development improves in 5% oxygen, effects on pregnancy and live birth rates are mainly seen in blastocyst, but not cleavage-stage transfers. A major drawback of these studies is that only fresh embryo transfers were included, not taking additional frozen-thawed transfers from these cycles into account. This might have underestimated the effects of oxygen level, especially in cleavage-stage embryo transfers. Furthermore, little is known about the effect of oxygen level during culture on birthweight.

STUDY DESIGN, SIZE, DURATION: This is a cohort study in 871 consecutive patients who had an IVF cycle between January 2012 and December 2013, and 5–7 years follow-up to allow transfer of frozen-thawed embryos. Based on daily availability of positions in the incubators, all oocytes and embryos of one cycle were allocated to one of the three incubators with traditional ambient oxygen levels (6% CO_2 and 20% O_2 in air), or to a fourth incubator that was adjusted to have low oxygen levels of 5% (6% CO_2, 5% O_2 and 89% N_2). Embryos were cultured under 5 or 20% oxygen until Day 2 or 3, when embryos were transferred or cryopreserved, respectively. Clinical and other laboratory procedures were similar in both groups.

PARTICIPANTS/MATERIALS, SETTING, METHODS: To compare embryo characteristics and (cumulative) pregnancy outcomes between the two oxygen groups, for each patient only the first cycle in the study period was included in the analysis, resulting in 195 cycles in the 5% group (1627 oocytes) and 676 in the 20% oxygen group (5448 oocytes). Embryo characteristics were analysed per cycle and per embryo and were corrected for maternal age, cycle rank order, fertilization method (IVF or ICSI) and cause of subfertility. Perinatal data from the resulting singletons (n = 124 after fresh and 45 after frozen-thawed embryo transfer) were collected from delivery reports from the hospitals or midwife practices.

MAIN RESULTS AND THE ROLE OF CHANCE: In the 5% oxygen group, there were significantly more embryos of good quality (45.8 versus 30.9% in the 20% group, adjusted odds ratio (OR) [95% CI] = 1.9 [1.6–2.4]). This did not result in higher live birth rates per cycle, but after fresh transfers more good-quality spare embryos could be cryopreserved (46.1 versus 29.7%, adjusted OR [95% CI] = 2.0 [1.7–2.5]).

After a follow-up period of 5–7 years, in which 82.4% of the cryopreserved embryos from the 5% oxygen group and 85.4% from the 20% oxygen group were thawed, the percentage of patients with at least one live birth resulting from the study cycle was significantly higher in the low oxygen group (adjusted OR [95% CI] = 1.5 [1.01–2.2]). In 124 live born singletons from fresh embryo transfers and in 45 from transfers of cryopreserved embryos, birthweight was similar in both oxygen groups after correction for confounding factors.

LIMITATIONS, REASONS FOR CAUTION: This is a retrospective study, and treatment allocation was not randomised. The study was not powered for a predefined birthweight difference. With the number of live births in our study, small differences in birthweight might not have been detected. The selection of embryos to be cryopreserved was based on embryo morphology criteria that might be different in other clinics.

WIDER IMPLICATIONS OF THE FINDINGS: Improved embryo utilization by more cryopreservation leading to higher cumulative live birth rates per cycle favours the use of 5% instead of 20% oxygen during human IVF embryo culture. This study also demonstrates that for comparison of different IVF treatment regimens, the cumulative outcome, including transfers of fresh and frozen-thawed embryos, is to be preferred instead of analysis of fresh embryo transfers only.

STUDY FUNDING/COMPETING INTEREST(S): No external funding was received for this study. None of the authors has a conflict of interest to declare.

TRIAL REGISTRATION NUMBER: NA

Key words: fertilization in vitro / embryo culture techniques / oxygen / pregnancy rate / embryo utilization / birthweight

WHAT DOES THIS MEAN FOR PATIENTS?

During an IVF treatment, embryos are kept for a few days at the laboratory in conditions that support embryonic growth. This is called embryo culture. When culturing embryos, one can use either atmospheric air that contains ~20% oxygen or use a gas mixture that contains 5% oxygen. Five percent oxygen resembles the oxygen level in the human body. It is still unclear whether 5% oxygen improves the chance of a live born child. Previous studies on this topic have looked only at transfers of fresh embryos, while in most IVF treatments good-quality embryos remain that can be frozen for later use. Transferring these frozen embryos gives an extra chance for a live birth. In this study, we investigated whether different oxygen levels during human IVF embryo culture makes a difference to the chance of a live born child after transferring fresh and frozen embryos.

In this study, embryos from IVF patients were cultured for several days under either 5 or 20% oxygen. One or two 'fresh' embryos were transferred into the uterus, and remaining good-quality embryos were frozen for later use.

After transfer of fresh embryos only, no difference in live birth rate was seen between culture under 5 or 20% oxygen. However, in the 5% oxygen group more good-quality embryos were obtained that were suitable for freezing. When the transfers of these frozen embryos were taken into account as well, more women achieved a live birth when the embryos were cultured under 5% oxygen as compared to 20% oxygen.

Our results support the use of 5% oxygen during culture above 20%.

Introduction

During IVF treatment, the oxygen concentration in the incubator is an important determinant of the *in vitro* environment that an embryo encounters. The preimplantation embryo consumes oxygen at a relatively constant level from the one-cell through the morula stage, while at the blastocyst stage the consumption is considerably increased (Houghton *et al.*, 1996). Oxygen plays a role in energy production, but in too high concentrations it may have a toxic effect by the formation of reactive oxygen species (Catt and Henman, 2000). While *in vivo* oxygen levels range from 1.5 to 8.7% in the fallopian tubes and uterus of several species (Fischer and Bavister, 1993), in most laboratories *in vitro* culture of human embryos was traditionally performed under atmospheric oxygen concentrations (~20%). The technologies available in the early days of IVF, and most likely also for financial reasons, led to the widespread implementation of incubators that used atmospheric air (i.e. 20% oxygen) (Wale and Gardner, 2016). Around the turn of the last century, more advanced incubators capable of creating an environment of 5% oxygen, which better resembles the natural environment for oocytes and embryos, were developed. Currently, in around 60% of the IVF cycles performed worldwide, the embryos are cultured under 5% oxygen (Christianson *et al.*, 2014).

Over the years, many groups investigated the effect of the relatively high atmospheric oxygen concentration on the intrinsic properties of embryos in several species including human and found that high oxygen levels affect gene expression (Rinaudo *et al.*, 2006; Mantikou *et al.*, 2016), DNA methylation (Li *et al.*, 2016), mitochondrial membrane potential (Ma *et al.*, 2017), protein profiles (Katz-Jaffe *et al.*, 2005) and embryo metabolism (Wale and Gardner, 2012; Wale and Gardner, 2013) (see Wale and Gardner, 2016, for a review on this topic). It is therefore not surprising that a high oxygen level also alters embryo development in humans (Dumoulin *et al.*, 1999; Kovacic *et al.*, 2010; Kirkegaard *et al.*, 2013; Peng *et al.*, 2016), in mouse (Quinn and Harlow, 1978; Karagenc *et al.*, 2004; Wale and Gardner, 2010) and in sheep and cattle (Thompson *et al.*, 1990).

The effect of oxygen level during embryo culture on pregnancy and live birth rates is less straightforward. Although most studies found an improvement in embryo quality when culturing embryos under 5% oxygen, pregnancy or live birth rates were not always better (Dumoulin *et al.*, 1999; Bahceci *et al.*, 2005; Kea *et al.*, 2007; Meintjes *et al.*, 2009; Waldenstrom *et al.*, 2009; Kovacic *et al.*, 2010; de los Santos *et al.*,

2013; Peng *et al.*, 2015). This discrepancy is partly explained by lack of power due to small sample sizes in some studies. When data are pooled in meta-analyses, the overall effect favours culture under 5% oxygen (Gomes Sobrinho *et al.*, 2011; Bontekoe *et al.*, 2012; Nastri *et al.*, 2016). Another explanation for the inconsistent results among the studies could be due to the differences in duration of exposure, until cleavage or blastocyst stage. When data in the meta-analyses were stratified for duration of culture, there was no effect of oxygen on pregnancy or live birth rates after 2–3 days of culture. After culture to the blastocyst stage, however, culture under 5% oxygen resulted in improved ongoing pregnancy and live birth rates.

The discrepancy in the oxygen effect on pregnancy outcomes according to embryonic stage suggests that a longer exposure might be needed to observe an effect. However, as the embryo is more vulnerable to oxygen in the pre-compaction stage as compared to the post-compaction stage (Wale and Gardner, 2010; Kirkegaard *et al.*, 2013), the duration might not be the problem. It is also possible that the morphology-based embryo scoring systems can better distinguish between a good- and lower-quality embryo at cleavage than at blastocyst stage, especially regarding cell number.

In an IVF programme with embryo culture either under high or low oxygen, the embryos that can cope best (i.e. the high-quality embryos) will be transferred, resulting in only small or no detectable effects of oxygen on pregnancy rates in fresh embryo transfer cycles. Better outcome parameters are embryo utilization rate and cumulative pregnancy rate after transfers of fresh and frozen-thawed embryos, since these reflect whether low oxygen moves overall embryo development to a more favourable side. The shift towards single fresh embryo transfer has made embryo cryopreservation and subsequent frozen-thawed embryo transfer of greater importance; therefore, now the additional pregnancies from these transfers can no longer be neglected and should be incorporated in the calculation of IVF success rates (Maheshwari *et al.*, 2015). In most published studies, only fresh embryo transfers were taken into account, which might underestimate the effect of oxygen.

Another drawback of the published studies is that the data analyses have not always been carried out in a consistent and proper way. In studies in which patients were allocated to one of the two oxygen groups, embryo outcomes such as fertilization or cleavage have been analysed either as mean numbers or as mean proportions per cycle (Dumoulin *et al.*, 1999; Waldenstrom *et al.*, 2009), while in other papers the oocyte or embryo is the unit of analysis (Kea *et al.*, 2007; de los Santos *et al.*, 2013). This impedes a proper comparison. Furthermore, in a per embryo analysis it should be taken into account that oocytes and embryos from one patient do not reflect independent observations. This has not been done in any of the studies. Kirkegaard *et al*. showed that up to one-third of the variation in timing of embryo development is explained by patient characteristics (Kirkegaard *et al.*, 2016). Failure to correct for this interdependency may lead to overestimation of potential effects.

In contrast to the numerous reports on the effects of different oxygen levels on embryo and pregnancy outcomes, the effects of oxygen levels on human offspring health are hardly investigated (Nastri *et al.*, 2016), even though the effect of the preimplantation environment on offspring health is an emerging topic. In several studies, both in animals and in humans, certain IVF culture media or the protein source added to these media led to altered birthweights (Zandstra *et al.*, 2015). This weight difference was found to be maintained at least until the age of 9 years (Zandstra *et al.*, 2018). Birthweight is negatively related to the risk for adult diseases such as cardiovascular disease and diabetes (Barker, 2004). Whether this relation also accounts for IVF is not known yet, but the lower birthweight found in IVF children should raise awareness that the environment during very early life might have long-lasting effects.

Regarding the scarcity of data on oxygen concentration effects on cumulative pregnancy outcomes per started IVF cycle, the primary aim of our study was to investigate whether 5% oxygen as compared to 20% oxygen concentration leads to higher cumulative pregnancy rates per IVF cycle, if the data from additional transfers of cryopreserved embryos are included. In the 5- to 7-year follow-up period in this study, around 85% of the cryopreserved embryos were thawed and could be included in the analyses. The data are analysed on a per cycle and per embryo level separately, correcting for the interdependency between embryos from the same patient. Next to this, our secondary aim was to assess whether different oxygen concentrations during human embryo culture affect birthweight.

Materials and Methods

Study Design

Data for this retrospective cohort study were collected from patients who underwent an IVF cycle between January 2012 and December 2013 at the University Medical Centre Groningen (UMCG) (Fig. 1). Frozen embryo transfers from these cycles were included until December 2018, i.e. 7 years since the first and 5 years since the last oocyte retrieval in this study. Patients either had their full IVF cycle in the UMCG or were (part of) the clinical phase in the affiliated satellite clinic (Wilhelmina Hospital Assen (WZA)) or one of the transport clinics (Medical Center Leeuwarden (MCL) or Scheper Hospital Emmen (SZE)) and the laboratory phase at the UMCG. In the satellite clinic, only the ovarian stimulation is regulated and monitored, while all other procedures including oocyte retrieval, all laboratory procedures and embryo transfer are performed at the UMCG. In the transport clinics, all procedures up to and including the oocyte retrieval are performed, after which the oocytes within the follicular fluid are transported to the IVF laboratory at the UMCG, where all laboratory procedures, including embryo culture, and embryo transfer are performed. All clinical protocols were similar in all four hospitals. All laboratory procedures were always performed at the UMCG.

The study took place during a transition period in the UMCG, in which a change from culture with 20% oxygen to 5% oxygen was introduced in a stepwise manner. Three out of the four incubators were kept at the traditional ambient oxygen levels (Forma Scientific 3121 [Forma Scientific Inc., Marietta, OH, USA), 6% CO_2 and 20% O_2 in air], and one incubator was adjusted to have low oxygen levels of 5% [Sanyo MCO-18M (Sanyo, Osaka, Japan), 6% CO_2, 5% O_2 and 89% N_2]. Cycles were arbitrarily allocated to one of the four incubators, based on daily available positions, and all oocytes and embryos from that cycle were fertilized and cultured under 5 or 20% oxygen, respectively. Both type of incubators had eight workspaces and were used at equal intensity. All clinical and laboratory procedures were similar in both groups. Only cycles with at least one inseminated or injected oocyte

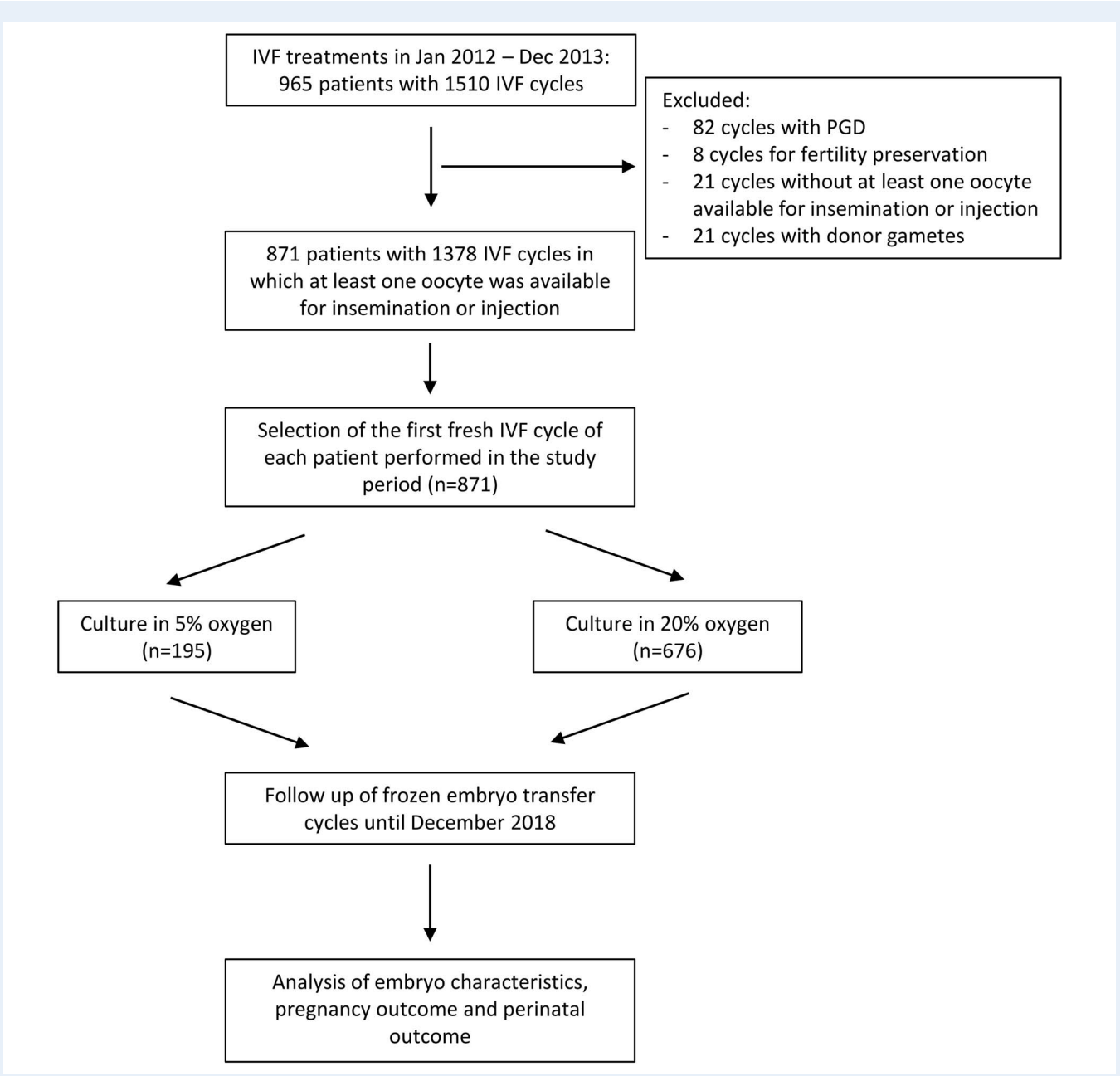

Figure 1 Flowchart depicting the inclusion of 965 patients and the selection to arrive at 871 cycles for the analyses. Only the patient's first cycle performed after the start of the study was included. This was not necessarily the first cycle for the patient. Allocation to culture in 5% or 20% oxygen was based on daily available positions in the incubators.

were included, and cycles in which donor gametes were used or PGD or fertility preservation was performed were excluded.

To compare embryo and (cumulative) pregnancy outcomes between the two oxygen groups, only one cycle per patient was included in the analysis. This cycle was the patient's first cycle performed after the start of the study in January 2012, but the cycle could have a higher rank order (Fig. 1).

At the start of their IVF treatment, all patients at the UMCG and affiliated clinics sign a universal consent form in which they allow the use of their data obtained during routine medical care from medical records for research purposes, under the condition that the patients' identity remains untraceable and the data remain confidential. The Institutional Review Board of the UMCG approved the study (file no M19.225065).

IVF procedures

At the UMCG as well as in the affiliated clinics, controlled ovarian stimulation (COS) was performed according to the same standard long GnRH agonist protocol. For ovarian stimulation, recombinant FSH or urinary FSH was used. Follicular growth was monitored by ultrasound and as soon as at least three of the dominant follicles were >18 mm diameter, 5000 IU of hCG was given. Oocyte retrieval was performed

36 h after hCG administration. The luteal phase was supported by intravaginal progesterone capsules starting from the evening of the day of oocyte retrieval until 14 days after embryo transfer.

Retrieved oocytes were collected in a G-IVF-PLUS medium (Vitrolife, Göteborg, Sweden) that was pre-equilibrated in the respective gas mixture and allocated to one of the four incubators (with either 5 or 20% oxygen) as described under study design, which was used throughout the whole laboratory phase. Approximately 4 h after follicular aspiration, oocytes were either inseminated with 5000–10 000 motile spermatozoa in G-IVF-PLUS medium (conventional IVF) or injected with one spermatozoon and cultured in pre-equilibrated G1-PLUS version5 medium (Vitrolife) in case of ICSI. The next morning, around 17 h post-injection or 19 h post-insemination, the oocytes were checked for the presence of two pronuclei (2PN), and the IVF oocytes were transferred to a pre-equilibrated G1 PLUS version5 culture medium. Embryonic development (number of blastomeres, fragmentation, size and shape of the blastomeres and presence of multinucleated blastomeres) was recorded daily, around 8 a.m. Embryo transfer was performed on Day 2 after oocyte retrieval. In 2012, a single embryo was transferred when female age was less than 38 years and at least one embryo of good morphology was available (2PN on Day 1 and four cells on Day 2, with less than 20% fragmentation and no multinucleated blastomeres). Due to a change in refunding policy by the national government, from 1 January 2013, one embryo was transferred irrespective of embryo quality, except for women aged 38 years or older, or women in their third cycle. In these cases, the transfer of two embryos, if available, is allowed.

At Day 3, supplementary good-quality embryos (2PN at day 1, $\geq$6 blastomeres, $\leq$20% fragmentation and no multinucleated blastomeres on Day 3, and growth progression between Days 2 and 3) were cryopreserved using slow freezing with dimethylsulfoxide. If no pregnancy occurred in the fresh cycle, these embryos could be thawed and transferred in an artificial cycle created with orally administered estradiol and vaginal progesterone. Only embryos with at least 50% of the cells surviving directly after thawing were transferred, and if the thawed embryo did not meet this criterion, additional (if any) embryos were thawed until at least one surviving embryo was obtained. After September 2015, thawed embryos were cultured overnight at 5% oxygen before transfer ($n = 59$ out of 719 transfers of cryopreserved embryos). The policy for the number of frozen embryos to be transferred was similar to that for the transfer of fresh embryos.

Pregnancy and perinatal data collection

A biochemical pregnancy was determined by a urine hCG test that was performed 14–16 days after the embryo transfer. An ongoing pregnancy was defined as the presence of foetal heartbeat on ultrasound at 12 weeks' gestation. Live birth was defined by the birth of at least one living child. A delivery report of each patient, made by a midwife or obstetrician, was requested shortly after delivery from the General Practitioner of the patient to obtain pregnancy data from the mother as well as perinatal data from the child.

Statistical analysis

Statistical analyses were performed using SPSS Statistics 23 (IBM, Chicago, IL, USA). In general, differences in continuous variables were analysed with a Student *t* test and a linear regression analysis to correct for potential confounders. Differences in categorical variables were analysed with a χ^2 test and a logistic regression analysis to correct for potential confounders. A *P* value of <0.05 was considered to indicate a significant difference.

For the comparison of the oocyte and embryo characteristics (fertilization, cleavage, development into a good-quality embryo), and embryo utilization (i.e. the embryo was either transferred or cryopreserved), two analyses were conducted: an analysis per cycle and an analysis at oocyte/embryo level. In the analysis per cycle, fertilization, cleavage, good-quality embryo and utilization rate were calculated per cycle and then averaged by oxygen group, as was done for the number of oocytes retrieved and injected or inseminated, transferred and cryopreserved. A linear regression analysis was conducted to correct for potential confounding factors (maternal age, cycle rank order, fertilization method (IVF or ICSI) and cause of subfertility).

For the per oocyte/embryo analysis, general estimating equation (GEE) binary logistic models with an exchangeable correlation matrix were used to analyse the effect of oxygen on oocyte and embryo parameters while correcting for the correlation between oocytes or embryos within one patient. Subsequently, maternal age, cycle rank order, fertilization method (IVF or ICSI) and cause of subfertility were added to this model as potential confounders.

To compare birthweights of singletons of different genders, of different birth rank order and with different gestational ages at birth, a *Z* score was assigned to each child using the data from the Dutch Perinatal Registry (Visser *et al.*, 2009). The *Z* score reflects the number of SDs the birthweight of an individual child deviates from the mean of a reference population with the same gender, birth rank order and gestational age at birth. The association between oxygen level during culture and birthweight was further analysed by multiple linear regression analysis. Gestational age at birth, gender, fertilization method (IVF or ICSI), number of transferred embryos, primiparity (yes or no), cause of subfertility and duration of subfertility were included as potential confounders.

Results

Incubator comparison

The aim of the study was to compare the effects of two different oxygen levels in the four available incubators. However, during the study period, the four incubators were of two different brands, Forma Scientific 3121 and Sanyo MCO-18M. To verify that the type of incubator did not affect the outcome parameters, data from the year preceding this study (i.e. 2011) when all incubators had 20% oxygen were compared. The fertilization, cleavage, good-quality embryo and embryo utilization rate were all comparable between the two types of incubators (Supplementary Table SI).

Study population

During the study period from January 2012 to December 2013, 871 out of the 965 consecutive patients could be included and underwent 1378 IVF or IVF/ICSI treatment cycles in which at least one oocyte was available for injection or insemination (Fig. 1). Only one cycle per patient was included in the analysis and this cycle was the patient's first cycle performed after the start of the study in January 2012. One

Table I Patient characteristics at the time of inclusion in 871 women who participated in the study and whose embryos were cultured under 5% or 20% oxygen.

		5% O_2 (*n* = 195)	20% O_2 (*n* = 676)	*P* value
Maternal age (years)		33.9 ± 4.0	34.0 ± 4.5	NS
Duration of subfertility (years)		3.8 ± 2.6	3.9 ± 2.6	NS
COS cycle order	1	114 (58.5%)	378 (55.9%)	NS
	2	55 (28.2%)	182 (26.9%)	
	3	19 (9.7%)	83 (12.3%)	
	≥4	7 (3.6%)	33 (4.9%)	
Fertilization method	IVF	52 (26.7%)	198 (29.3%)	NS
	ICSI	143 (73.3%)	478 (70.7%)	
Subfertility	Primary subfertility*	123 (63.1%)	380 (56.9%)	NS
	Secondary subfertility	72 (36.9%)	288 (43.1%)	
Cause of subfertility	Female factor	49 (25.1%)	172 (25.4%)	NS
	Male factor	109 (55.9%)	362 (53.6%)	
	Unexplained	37 (19.0%)	142 (21.0%)	
Clinic	UMCG	118 (60.5%)	352 (52.1%)	NS
	WZA (Satellite)	8 (4.1%)	42 (6.2%)	
	MCL, SZE (Transport)	69 (35.4%)	282 (41.7%)	

Data are presented as mean ± SD or as number (%).
*For eight cases in the 20% group, primary or secondary subfertility was not known.
COS = controlled ovarian stimulation, UMCG = University Medical Centre Groningen, WZA = Wilhelmina Hospital Assen, MCL = Medical Centre Leeuwarden, SZE = Scheper Hospital Emmen.
NS=Not significant, $P > 0.05$.

hundred ninety-five cycles from 195 patients were arbitrarily allocated based on daily available positions to the only incubator with 5% oxygen, and 676 cycles from 676 patients to one of the three incubators with 20% oxygen. The characteristics of the groups are shown in Table I and were comparable.

IVF outcomes

In Table II, the embryo characteristics and outcomes in both oxygen groups are shown. These characteristics are expressed and analysed per cycle and per embryo. In both analyses, there were significantly more embryos with 2PN that cleaved, and more embryos of good quality (2PN on Day 1 and four cells on Day 2, with less than 20% fragmentation and no multinucleated blastomeres) in the lower oxygen group. Therefore, more embryos could be cryopreserved, and a significantly higher embryo utilization rate was achieved in the lower compared to the higher oxygen group. These differences remained significant after correction for maternal age, cycle rank order, fertilization method (IVF or ICSI) and cause of subfertility. The mean number of embryos transferred per cycle was significantly lower in the 5% oxygen group (see Table II, analysis per cycle), while in the analysis per oocyte/embryo, the percentage of embryos that was transferred from the total number of embryos was similar in both groups (see Table II).

The improved average embryo quality did not affect the ongoing pregnancy and live birth rates in the fresh embryo transfer cycles (Table III). However, after including the frozen-thawed embryo transfer cycles, performed in a follow-up period of 5–7 years in which 82.4% of the cryopreserved embryos from the 5% oxygen group and 85.4% from the 20% oxygen group were thawed (see Supplementary Table SII), the percentage of patients with at least one ongoing pregnancy (26.2 versus 19.4% in the 5 and 20% groups, respectively) or one live birth (25.1 versus 18.8%) was significantly higher in the low oxygen group (Table III, adjusted odds ratio = 1.5, both significant). Seven patients had two live births from one started fresh cycle (i.e. six times one live birth after transfer of a fresh embryo and one after transfer of frozen-thawed embryos and one time two live births after two consecutive frozen embryo transfers). At the end of the follow-up period, 75 patients (23 (18.1%) in the 5% group and 52 (16.3%) in the 20% group) still had embryos cryopreserved (Supplementary Table SII, range 1–11 embryos), of which 57 patients already had at least one live birth from the transfer of fresh or frozen-thawed embryos from this cycle.

Perinatal outcomes

Data on the perinatal outcome parameters (gender, gestational age at birth and birthweight) was obtained from 100, 100 and 89%, respectively, of the 124 live born singletons after fresh embryo transfers. There was no difference in perinatal outcome between the 5 and 20% oxygen group regarding birthweight, birthweight corrected for gestational age at birth, gender and parity (*Z* score), gestational age at birth and gender (Table IV).

There were 47 live births after the transfer of frozen-thawed embryos. One of these was a twin and 46 were singletons. From 38 (82.6%) live born singletons (11 in the 5% and 27 in the 20% oxygen group), birthweight data were obtained. Gender and gestational age were known for all live born singletons. The perinatal outcome

Table II Embryo characteristics and outcomes after culture under either 5 or 20% oxygen, analysed by comparing averages per cycle by using linear regression analysis and by comparing the outcomes per oocyte/embryo by using GEE binary logistic models.

	5% O_2	20% O_2	*P* value	Adjusted *P* value[6]
Analysis per cycle	***n* = 195**	***n* = 676**		
Number of oocytes	8.3 ± 5.1	8.1 ± 5.0	NS	NS
Number of patients with >10 oocytes	64 (32.8%)	229 (33.9%)	NS	-
Number of patients with >20 oocytes	7 (3.6%)	25 (3.7%)	NS	-
Number of injected or inseminated oocytes	7.4 ± 4.5	7.1 ± 4.5	NS	NS
Fertilization rate[1]	62.4 ± 26.5	62.7 ± 26.7	NS	NS
Cleavage rate[2]	97.0 ± 10.4	94.2 ± 16.2	$P < 0.05$	$P < 0.05$
Good-quality embryo rate[2,4]	48.0 ± 33.8	30.4 ± 32.5	$P < 0.001$	$P < 0.001$
Number of embryos transferred[3]	1.2 ± 0.4	1.3 ± 0.5	$P < 0.05$	$P < 0.05$
Number of embryos cryopreserved[3]	2.1 ± 2.3	1.4 ± 2.0	$P < 0.001$	$P < 0.001$
Embryo utilization rate[2,5]	76.1 ± 25.7	66.2 ± 31.6	$P < 0.001$	$P < 0.001$
Number of thaw cycles with cryopreserved embryos	1.10 ± 1.4	0.8 ± 1.2	$P = 0.002$	-
Analysis per oocyte/embryo	***n* = 1627**	***n* = 5448**	**Crude GEE-OR [95% CI]**	**Adjusted GEE-OR [95% CI][6]**
Number of injected or inseminated oocytes	1448/1627 (89.0%)	4779/5448 (87.7%)	1.1 [0.88–1.48]	1.2 [0.90–1.49]
Normal fertilization rate (2PN)	877/1448 (60.6%)	3031/4779 (63.4%)	0.9 [0.78–1.09]	0.9 [0.79–1.10]
Cleavage rate	849/877 (96.8%)	2865/3031 (94.5%)	1.8 [1.18–2.86]	1.8 [1.17–2.85]
Good-quality embryo rate	402/877 (45.8%)	936/3031 (30.9%)	2.0 [1.59–2.39]	1.9 [1.59–2.39]
Embryos transferred	226/877 (25.8%)	842/3031 (27.8%)	0.9 [0.76–1.05]	0.9 [0.76–1.06]
Embryos cryopreserved	404/877 (46.1%)	900/3031 (29.7%)	2.0 [1.66–2.47]	2.0 [1.67–2.49]
Embryo utilization rate	630/877 (71.8%)	1742/3031 (57.5%)	1.8 [1.50–2.20]	1.8 [1.50–2.20]

Data are presented as mean ± SD or as number (%).
[1]Expressed as the proportion of the number of injected or inseminated oocytes.
[2]Expressed as the proportion of the number of two pronuclei (2PN) zygotes per cycle.
[3]Expressed as mean per cycle with zygotes.
[4]Defined as having 2PN on Day 1 and four blastomeres on Day 2 with less than 20% fragmentation and no multinucleated blastomeres.
[5]Defined as the proportion of zygotes per cycle that is transferred or cryopreserved as an embryo.
[6]The difference between the oxygen groups was corrected for maternal age, cycle rank order, cause of subfertility and fertilization method, except at number of oocytes and number of injected or inseminated oocytes where fertilization method was left out of the analysis.
NS = not significant, $P > 0.05$.
GEE-OR = odds ratio derived from the general estimating equations (GEE) binary logistic models.

parameters did not differ between the singletons in the 5 and 20% oxygen group (Table IV).

A multivariate regression analysis on birthweight and oxygen level while correcting for several potential confounding factors, such as gestational age at birth, gender, fertilization method (IVF or ICSI), number of transferred embryos, primiparity (yes or no), cause of subfertility and duration of subfertility, showed that birthweight was not related to oxygen level during embryo culture, neither after fresh nor frozen-thawed embryo transfer.

Discussion

The results from this study show that embryo culture under 5% oxygen level as compared to 20% for as little as 2 days already leads to a higher number of good-quality embryos, and thus more embryos suitable for cryopreservation. Consequently, the cumulative ongoing pregnancy and live birth rate after transfer of fresh and frozen embryos from one cycle was significantly higher in the 5% oxygen group as compared to the 20% group. The oxygen level did not affect birthweight of the resulting singletons.

The improved embryo quality in the 5% oxygen group was no surprise as a better embryo development in low oxygen has been shown before. Kirkegaard and colleagues compared human embryo development by using time-lapse monitoring and found that timing of the third cleavage division was delayed in culture under 20% oxygen as compared to 5% The timing of blastocyst stages was similar, however, suggesting stage-specific oxygen effects (Kirkegaard *et al.*, 2013). These data confirm earlier time-lapse data in mice where continuous

Table III Pregnancy outcomes in 195 cycles with embryo culture under 5% oxygen and 676 cycles with culture under 20% oxygen.

	5% O_2 (n = 195)	20% O_2 (n = 676)	P value	Adjusted OR[2] [95% CI]
After fresh embryo transfer				
Biochemical pregnancy	50/195 (25.6%)	142/676 (21.0%)	NS	1.3 [0.90–1.90]
Implantation[1]	45/226 (19.9%)	135/842 (16.0%)	NS	1.3 [0.88–1.88]
Ongoing pregnancy	40/195 (20.5%)	104/676 (15.4%)	NS	1.4 [0.95–2.16]
Live birth	37/195 (19.0%)	100/676 (14.8%)	NS	1.4 [0.90–2.07]
Multiple pregnancy	2/37 (5.4%)	11/100 (11.0%)	NS	0.4 [0.08–2.05]
Cumulative after fresh and frozen-thawed embryo transfer				
At least one ongoing pregnancy	51/195 (26.2%)	131/676 (19.4%)	0.04	1.5 [1.04–2.19]
At least one live birth	49/195 (25.1%)	127/676 (18.8%)	0.052	1.5 [1.01–2.17]

Data are presented as number (%) or as mean ± SD. NS = not significant, $P > 0.05$.
[1] Defined as the total number of foetal sacs divided by the total number of embryos transferred.
[2] The difference between the oxygen groups was corrected for maternal age, cycle rank order, cause of subfertility and fertilization method.

Table IV Perinatal outcome in 124 and 46 singleton live births after fresh and frozen-thawed embryo transfers respectively, with embryo culture under either 5 or 20% oxygen.

After fresh embryo transfer		5% O_2 (n = 35)	20% O_2 (n = 89)	P value
Birthweight (grams)		3464 ± 616	3421 ± 538	NS
Z score[1]		0.29 ± 1.0	0.19 ± 1.0	NS
Gestational age at birth (weeks)		39.3 ± 2.2	39.1 ± 1.6	NS
Gender	Male	20 (57.1%)	51 (57.3%)	NS
	Female	15 (42.9%)	38 (42.7%)	
After frozen-thawed embryo transfer		5% O_2 (n = 14)	20% O_2 (n = 32)	
Birthweight (grams)		3513 ± 778	3527 ± 454	NS
Z score[1]		0.64 ± 0.9	0.23 ± 1.1	NS
Gestational age at birth (weeks)		37.5 ± 5.9	39.7 ± 1.3	NS
Gender	Male	7 (50.0%)	15 (46.9%)	NS
	Female	7 (50.0%)	17 (53.1%)	

Data are presented as mean ± SD or as number (%).
[1] Z score indicates birthweight corrected for gender, parity and gestational age at birth.
NS = not significant, $P > 0.05$.

culture under 20% oxygen for 118 h delayed timing of the first, second and third cleavage divisions as well as blastocyst development (Wale and Gardner, 2010). A switch at 48 h from 20 to 5% oxygen level did not alter the outcomes, again suggesting a specific oxygen effect at pre-compaction stage that is not reversible.

The improved embryo quality in the 5% oxygen group indeed resulted in more suitable embryos for cryopreservation and better cumulative ongoing pregnancy and live birth rates. Reporting the cumulative pregnancy rates per cycle after transfer of fresh and frozen embryos, as assessed in our study, is to be preferred when comparing IVF treatments. So far, only a few studies had cumulative pregnancy rates as an endpoint, but have reported contradictory results. Kovacic *et al.* (2010) reported a significantly improved cumulative pregnancy rate after culturing under low oxygen, while De los Santos *et al.* (2013) did not find a difference in cumulative pregnancy rates between the 5 and 20% oxygen groups in ovum donation cycles. In both studies, it is unclear, however, how many embryos were still cryopreserved at the end of the study period and whether transfer of these embryos could have led to a divergence in cumulative live birth rate between the two oxygen groups.

Our study had a long follow-up time of 5–7 years in which 84.5% of all frozen embryos were thawed. At the end of the follow-up period, in the 5% group 23 patients still had 71 embryos cryopreserved and in the 20% group were 52 patients with 131 embryos. Twelve and 15 patients in the 5 and 20% groups, respectively, had not achieved a live birth in the fresh embryo transfer cycle in which remaining embryos had been cryopreserved and, although not very likely after 5–7 years, they might still return to our clinic for an additional transfer. However, a similar rate of patients achieving a live birth in each group will not change the outcome of the study. For

example, if one-third of these patients would achieve a live birth, the four and five extra live births in the 5 and 20% groups would result in cumulative live birth rates of 27.2 and 19.5%, respectively ($P = 0.021$).

Besides the long 5- to 7-year follow-up, another strength of this study is that the analyses of embryo outcomes were conducted both per cycle and per oocyte/embryo and that the analyses per oocyte/embryo were corrected for the interdependency of data from embryos from the same patient. In a per cycle analysis, the number of oocytes and embryos per cycle is not taken into account and therefore the interpretation of the outcomes is different. That outcomes can differ when analysed on a per cycle or per embryo level becomes apparent with the average number of embryos transferred. Per cycle, there were on average more embryos transferred in the 20% oxygen when compared to the 5% group, while at the embryo level there was no difference between the two groups regarding the percentage of embryos selected for transfer.

As this was a retrospective study, allocation to the two oxygen groups was not randomized. Furthermore, the selection of the embryos to be cryopreserved was based on morphological criteria, which might be different from those applied in other clinics, although cell number, degree of fragmentation and absence of multinucleation are widely used. Another limitation of this study is that besides the two different oxygen levels, two different types of incubators were also used. Therefore, we analysed data from the year preceding the transition from 20 to 5% oxygen and showed that in 20% oxygen the embryo outcome parameters were similar between the two incubator types.

Recently, it was suggested to reduce the oxygen level after day 3 to 2%, as this would mimic the decreasing oxygen gradient in the female reproductive tract from the tubes towards the uterus (Morin, 2017). However, in a recently published randomized controlled trial, no better blastulation and blastocyst utilization rates were achieved when compared to continuous culture under 5% (De Munck *et al.*, 2018). Bolnick and colleagues suggested, based on culture of embryonic and trophoblastic stem cells, that 2% could be the optimal oxygen level for human embryo culture, although in order to optimize embryo culture at 2% oxygen, other elements of culture should be adjusted as well (Bolnick *et al.*, 2017).

We found no differences in birthweights, and our results are comparable to those in two other studies that reported on human birthweight after culturing under two different oxygen conditions (Meintjes *et al.*, 2009; Kasterstein *et al.*, 2013). In these studies, however, the number of included live births was small (ranging from 6 to 26 per group) and no correction for possible confounding factors was done. In studies investigating trends in birthweight over time, one study found an increase in birthweight over time, especially after the implementation of bench top incubators with 5% oxygen (Castillo *et al.*, 2019), while another study did not see an effect (Maas *et al.*, 2016). However, in these kinds of retrospective analyses in which many patient, clinic and laboratory factors are subject to change over time, it is difficult, if not impossible, to pinpoint changes in birthweight to a specific item. In animal studies, opposing results have been found. Culture of bovine embryos in 5% as compared to 20% oxygen levels resulted in a reduced weight at birth (Fischer-Brown *et al.*, 2005) and 14 days post-partum (Iwata *et al.*, 2000), which might have been due to a reduced cotyledon surface area reported in the placentas of the 5% group (Fischer-Brown *et al.*, 2005). Exposure of mouse embryos to 2% oxygen levels during the post-compaction stage only led to a reduced foetal weight at embryonic day 18 when compared to exposure to 7 or 20% oxygen and to an *in vivo* group (Feil *et al.*, 2006). This reduction might also be caused by the reduced placental surface available for exchange that was reported in the 2% group. In another study in mice comparing the effect of culture under 5 and 20% oxygen, foetal weights were similar, but the increased number of resorption sites in the 20% oxygen group might have biased the results (Karagenc *et al.*, 2004).

No difference in birthweight does not necessarily mean that health in the longer term is unaffected. It has been reported that atmospheric 20% oxygen during embryo culture affects repressive and permissive histone modifications in bovine blastocysts (Gaspar *et al.*, 2015) and gene expression in mouse blastocysts (Rinaudo *et al.*, 2006). Whether these epigenetic changes are adaptive or deregulatory, and whether these changes only alter the embryonic developmental potential or lead to long-term alterations in the offspring, is not yet known. On the other hand, in a study in mice in which IVF with two different oxygen levels and natural conception were compared, an increased frequency of stochastic epigenetic errors at imprinted genes in placental tissue was detected after IVF: there were, however, no differences in epigenetic errors between the oxygen levels (de Waal *et al.*, 2014).

As far as we know, this study is the largest study reporting on 5 versus 20% oxygen levels during human embryo culture and birthweight. Despite the retrospective design of the study, correction for major confounding factors such as gender, gestational age at birth and parity was possible. However, information on other possible confounding factors, including pregnancy complications, parental weight and height and maternal smoking during pregnancy, were missing. Furthermore, this study was not powered for a predefined birthweight difference. It should therefore be kept in mind that with the number of live births in our study, small differences in birthweight might not have been detected.

In conclusion, after culturing embryos for 2 days under either 5 or 20% oxygen, the cumulative ongoing pregnancy rates and live birth rates per cycle were higher in the 5% group owing to more good-quality embryos available for cryopreservation and subsequent transfer. There was no difference in birthweights of the live born singletons. Our results support the implementation of 5% oxygen during culture instead of 20%, even for short culture protocols. Long-term follow-up of IVF offspring is needed to ascertain the safety of embryo culture in general, regardless of culturing under 5 or 20% oxygen.

Supplementary data

Supplementary data are available at *Human Reproduction Open* online.

Acknowledgements

The authors would like to thank Karin Heerlijn for her dedicated work in managing the clinical data base and gathering the delivery reports.

Authors' roles

A.P.A.v.M., J.v.E.A. and J.A.L initiated and designed the study. A.P.A.v.M., E.G.J.M.A., J.v.E.A., L.W., A.S. and M.J.P. contributed to the data collection. A.P.A.v.M. did a quality control of the data and

analysed the data. All authors interpreted the data. A.P.A.v.M. wrote the report with input from the other authors.

Funding

No external funding was received for this study.

Conflict of interest

The authors have no conflict of interest to declare.

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