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A controlled randomized trial evaluating the effect of lowered incubator oxygen tension on live births in a predominantly blastocyst transfer program†

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BACKGROUND: The potentially damaging effect of free O_2 radicals to cultured embryos may be reduced by adding scavengers to the culture media or by reducing the incubator O_2 levels. However, lowering the O_2 in the culture environment can be expensive, troublesome and may not be justifiable. The objective of this study was to evaluate the effect of lowered incubator O_2 tension on live birth rates in a predominately Day 5 embryo transfer program. METHODS: Two hundred and thirty first-cycle women undergoing routine IVF or ICSI with ejaculated sperm were randomized in a prospective clinical trial and stratified for patient age and physician. Embryos of patients were randomly assigned for culture in either a 21% O_2 (atmospheric) or 5% O_2 (reduced) environment. Clinical endpoints monitored were rates of implantation, clinical pregnancy, live birth and blastocyst cryopreservation. RESULTS: Embryos cultured in a 5% O_2 environment consistently resulted in higher rates of live birth implantation (106/247, 42.9% versus 82/267, 30.7%; difference of 12.2% with 95% confidence interval (CI) of 3.9–20.3, $P = 0.005$) and live births (66/115, 57.4% versus 49/115, 42.6%; difference of 14.8% with 95% CI of 1.9–27.0, $P = 0.043$) when compared with rates among women whose embryos were cultured in an atmospheric O_2 environment. CONCLUSIONS: The overall increase in live births demonstrated by this study indicates that the effort and expense to culture embryos in a low-O_2 environment is justified.
The study was registered at clinicaltrials.gov. NCT00708487.

Keywords: IVF; human; oxygen; blastocyst; live birth

Introduction

The embryos of various mammalian species, including the human, are not exposed to O_2 concentrations in excess of 8% *in vivo* (Yedwab *et al*., 1976; Bayatt-Smith *et al*., 1991; Fisher and Bavister, 1993). Not unexpectedly, numerous studies in animals have consistently demonstrated that embryo culture in low-O_2 concentrations markedly improves *in vitro* embryo development and subsequent pregnancy outcomes when compared with similar culture in atmospheric O_2 concentrations (Harlow and Quinn, 1979; Batt *et al*., 1991; Yuan *et al*., 2003; Karja *et al*., 2004; Leoni *et al*., 2007). However, when culturing human embryos in lower O_2 concentrations, the expected improvement in measured laboratory parameters (Dumoulin *et al*., 1995, 1999) or clinical outcomes (Kea *et al*., 2007) were not observed. One would expect to see beneficial effects on embryo development and improved clinical outcomes when culturing embryos in a lowered O_2 tension, regardless of the species.

This discrepancy between animal and human data may be partially explained by species differences in embryo physiology, characteristics of specific laboratory animal strains or a variety of culture conditions (Bavister, 2004). Furthermore, only rare opportunities exist to collect relevant human data within a prospective, randomized experimental design with an adequate number of patients. Common confounding factors observed in past human studies include patient demographics (Kea *et al*., 2007), physician effect, incubator effect and observational time period (Catt and Henman, 2000). Published investigations related to this subject often do not include meaningful clinical endpoints such as live births, but rather are limited to less important parameters such as implantations (Noda *et al*., 1994; Kea *et al*., 2007). Animal data suggest that embryo morphology or even initial implantations, after culturing in different atmospheric conditions, are not accurate

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predictors of ultimate clinical outcomes. True embryo competency should be confirmed by a live birth (Karagenc *et al.*, 2004; Waldenström *et al.*, 2008).

The human studies which found no benefit in early clinical outcomes with a lowered O_2 tension in the incubators were all associated with embryo transfers on Day 2 or on Day 3 (Dumoulin *et al.*, 1999). This is in contrast to animal studies in which blastocyst transfer was the routine (Umaoka *et al.*, 1991; Karagenc *et al.*, 2004). One could argue, therefore, that the human embryo may not be as sensitive to O_2 toxicity during the oviductal stages (Houghton *et al.*, 1996; Bavister, 2004). Since it is suggested that it is the inner cell mass (ICM) of the blastocyst that is preferentially damaged when cultured in supraphysiological O_2 concentrations (Van Soom *et al.*, 2002; Bavister, 2004), early Day 2 or Day 3 transfer may preclude O_2 damage of human IVF embryos. This argument might be used to justify the culture of embryos in a reduced O_2 environment only after Day 3 of culture. However, animal studies indicate that after *in vitro* maturation at different O_2 concentrations, mouse embryo and fetal development are negatively affected when oocytes are matured in 21% O_2 (Banwell *et al.*, 2007). In addition, the developmental potential of blastocysts can be severely impaired following fertilization in 21% O_2 (Leoni *et al.*, 2007) or after a single hour of atmospheric O_2 exposure at the 1-cell stage (Pabon *et al.*, 1989). It appears that the effects of O_2 damage may already be present in the very early animal embryo, but only be manifested at the blastocyst stage or later in the form of compromised clinical outcomes.

The damaging effects that accrue in animal embryos cultured at atmospheric O_2 concentrations have been attributed to the generation of reactive oxygen species that can cause organelle and membrane damage (Fujitani *et al.*, 1997; Kwon *et al.*, 1999), direct and indirect DNA damage (Iwata *et al.*, 2000) and altered gene expression (Harvey *et al.*, 2004). These negative effects may potentially be reduced by adding free radical scavengers (Umaoka *et al.*, 1992; Orsi and Leese, 2001) and antioxidants (Umaoka *et al.*, 1991) to the culture media or by reducing the incubator O_2 tension (Dumoulin *et al.*, 1999). In the absence of proven clinical benefits of lowered incubator O_2 tension in the human, some have chosen to add antioxidants to the embryo culture media (Umaoka *et al.*, 1991, 1992; Catt and Henman, 2000). If the damaging effects of supraphysiological O_2 concentrations on human embryos were reliably and innocuously subverted by the addition of antioxidants, it may be a less expensive and less labor-intensive alternative. Intuitively, it seems more rational to prevent the formation of damaging agents in and around growing embryos in the first place by culturing embryos in reduced O_2 concentrations, rather than to attempt neutralization of these agents after their formation (Catt and Henman, 2000). Relying only on free radical scavengers and antioxidants to protect embryos in a supraphysiological O_2 culture environment also assumes that these protective substances can reach their target molecules inside the embryonic cells before damage ensues. Furthermore, there are mechanisms by which O_2 may cause damage to embryos such as altered gene expression that may not be prevented by the simple addition of antioxidants to the culture media (Harvey *et al.*, 2004).

Because the culture of human embryos in a reduced O_2 environment appears to be the most physiological alternative and the overwhelming evidence that supports such a thesis in many other mammalian species, a study was designed to test the effect of a lowered O_2 embryo culture environment in a private, predominantly Day 5 transfer, human IVF program. The study design attempted to minimize common confounding factors and to measure live births as the ultimate clinical outcome.

Materials and Methods

Study design

Only first-cycle patients undergoing routine IVF or ICSI with ejaculated sperm were recruited for this study over a 1-year period following local Institutional Review Board approval. Thus, the number of cycles was equal to the number of patients enrolled. A total of 230 patients were randomly assigned to one of two treatment groups and prospectively stratified for physician, patient age ($\leq$34, 35–37, 38–40, $>$40 years) and source of oocytes (homologous, donor) to exclude any possible effect of physician or patient age on the results (Tables I and II, Fig. 1). Gametes and embryos were cultured at 37.1°C either in an atmosphere of 5% CO_2 in air (21% O_2) (high-O_2 treatment group, $n = 115$) or in 5% CO_2, 5% O_2, 90% N_2 (low-O_2 treatment group, $n = 115$). Patients were randomly assigned to a treatment group within physician and patient age group (donor oocytes when applicable) 2 days before the oocyte retrieval so that the complete treatment cycle could be performed in the pre-assigned atmospheric conditions to include setting up of all retrieval and culture dishes. Exclusion criteria were as follows: one or more previous IVF cycles without a live birth, the need to use non-ejaculated sperm (epididymal or testicular), no oocytes retrieved or no fertilization, PGD or cryobanking of all embryos for any reason.

Culture conditions

All media, oil and prepared micro-droplet culture dishes were equilibrated overnight in their respective atmospheric conditions. Oocytes were retrieved and washed in P-1 medium (Irvine Scientific, Santa Ana, CA, USA) supplemented with 3 g/l of human serum albumin (HSA, 0.3% weight/volume) using an isolette (37.1°C, 5% CO_2 in air). The washed oocytes were then placed in 750 μl of HSA-supplemented P-1 medium under oil and incubated at 37.1°C in their respective gas phase for 6 h before insemination or ICSI. *In vitro* insemination took place in 200 μl droplets of equilibrated, HSA-supplemented P-1 medium under oil. Oocytes that were sperm-injected were cultured overnight in 40 μl microdroplets of equilibrated HSA-supplemented G-1 medium (Vitrolife, Götheburg, Sweden) under oil. After 15–19 h of insemination or ICSI, all normally [2 pronuclei (PN)] fertilized oocytes were washed and transferred to fresh 40 μl microdroplets of equilibrated, HSA-supplemented G-1 medium under oil and returned to their designated incubator. On Day 3, embryos were evaluated, washed and transferred to 40 μl microdroplets of equilibrated, HSA-supplemented G-2 medium (Vitrolife) for transfer to the patient or for further culture to the blastocyst stage. All embryos were again transferred to fresh 40 μl microdroplets of equilibrated, HSA-supplemented G-2 medium on Day 5.

Embryo transfer and cryopreservation

Embryos were transferred to patients on Day 2–3 or on Day 5, depending on the number and quality of embryos available for transfer. Should either the low-O_2 or high-O_2 treatment have significantly disadvantaged the embryos early on, one would expect more Day 2–3

Table I. Implantation outcomes by patient age and donor status for the atmospheric-O_2 (21%) and low-O_2 (5%) treatment groups.

Age group (years) (patients per treatment group)	Implantion[a]			Live birth implantation[b]		
	21% Oxygen	5% Oxygen	*P*-value[c]	21% Oxygen	5% Oxygen	*P*-value[c]
≤34 (60)	62/138 (44.9%)	72/125 (57.6%)	0.039	58/138 (42.0%)	61/125 (48.8%)	0.174
35–37 (23)	14/53 (26.4%)	18/54 (33.3%)	0.56	11/53 (20.8%)	16/54 (29.6%)	0.373
38–40 (18)	7/47 (14.9%)	14/37 (37.8%)	0.073	3/47 (6.4%)	13/37 (35.1%)	0.011
>40 (2)	0/4 (0%)	1/5 (20.0%)	0.166	0/4 (0%)	1/5 20.0 (%)	0.166
Donor (12)	12/25 (48.0%)	17/26 (65.4%)	0.205	10/25 (40.0%)	15/26 (57.7%)	0.204
Total (115)	95/267 (35.6%)	122/247 (49.4%)	0.003	82/267 (30.7%)	106/247 (42.9%)	0.005

[a]Number of gestational sacs identified by ultrasound by 8 weeks post-embryo transfer divided by the number of embryos transferred.
[b]Number of babies born alive divided by the number of embryos transferred.
[c]General linear models for proportions, controlling for day of transfer, patient age and the number of embryos per transfer.

Table II. Clinical pregnancy and live birth outcomes by patient age and donor status for the atmospheric-O_2 and low-O_2 treatment groups.

Age group (years) (patients per treatment group)	Clinical pregnancy[a]			Live birth[b]		
	21% Oxygen	5% Oxygen	*P*-value[c]	21% Oxygen	5% Oxygen	*P*-value[c]
≤34 (60)	36/60 (60.0%)	45/60 (75.0%)	0.099	34/60 (56.7%)	38/60 (63.3%)	0.525
35–37 (23)	9/23 (39.1%)	11/23 (47.8%)	0.639	7/23 (30.4%)	11/23 (47.8%)	0.289
38–40 (18)	4/18 (22.2%)	8/18 (44.4%)	0.291	2/18 (11.1%)	8/18 (44.4%)	0.060
>40 (2)	0/2 (0%)	1/2 (50.0%)	1.000	0/2 (0%)	1/2 (50.0%)	1.000
Donor (12)	7/12 (58.3%)	9/12 (75.0%)	0.382	6/12 (50.0%)	8/12 (66.7%)	0.406
Total (115)	56/115 (48.7%)	74/115 (64.3%)	0.027	49/115 (42.6%)	66/115 (57.4%)	0.043

[a]Number of patients with the presence of a gestational sac in the uterus identified by ultrasound by 8 weeks post embryo transfer divided by the number of patients with an oocyte retrieval.
[b]Number of patients with a live-born infant divided by the number of patients with an oocyte retrieval.
[c]General linear models for proportions, controlling for day of transfer, patient age and the number of embryos per transfer.

transfers in the affected treatment group due to more patients with poor morphology embryos on those days. Embryos were transferred on Day 2–3 only if the physician and patient were comfortable transferring all embryos that reached an acceptable developmental stage (e.g. three 6–8-cell embryos on Day 3 in a 40-year old patient). Day 2–3 transfers were typically performed when the patients were older, when a limited number of oocytes had been retrieved, there were low numbers of fertilized oocytes or when there was a poor prognosis based on the Day 2/Day 3 embryo morphology. Transferring the embryos of these patients on Day 2–3, rather than forcing 5 days of culture is the standard clinical approach in our program. This approach has been very successful in reducing the likelihood that there will be no viable embryo(s) to transfer on Day 5. In contrast, good prognosis patients were encouraged to transfer a single blastocyst to reduce the probability of twins.

Should the patient and physician not feel comfortable with the transfer of all sufficiently advanced embryos (e.g. 2 or 3 perfect 8-cell embryos in a 30-year old patient), the embryo transfer procedure was delayed until Day 5. On Day 5, one or two blastocysts were chosen for transfer and supernumerary blastocysts were cryopreserved. Blastocysts were frozen after IVF or ICSI if they met the standard criteria for cryopreservation in this program. A blastocyst had to be fully expanded (>150 μm in diameter) with an ICM grade of A, B or C (A being the best, F being the worst) and a trophoblast grade of A or B (A being the best, D being the worst). Cryopreservation took place on Day 5 and/or Day 6 and did not occur before 112 or after 140 h post-insemination.

Incubators

Two identical Forma model 3120 incubators were dedicated to this study which differed only in the preset O_2 concentrations (5 and 21%) to rule out subtle differences between different models or brands of incubators such as incubator recovery time, internal incubator temperature gradients or any other incubator-related confounding factor. The embryos of a maximum of three patients were incubated at any one time in one incubator to optimize temperature and gas-phase recovery times after opening. Incubator CO_2 and O_2 concentrations were confirmed on a daily basis throughout this study period, using a manual CO_2 and O_2 fyrite gas analyzer with a measuring range of 0–7.6% (Bacharach, New Kensington, PA, USA). In addition, the partial pressures of CO_2 (normal arterial range 32–40 mm Hg) and O_2 (normal arterial range 83–108 mm Hg) were measured using a calibrated blood-gas analysis machine. The partial pressures of CO_2 and O_2 were confirmed to be within these normal human arterial blood-gas ranges for each new batch of medium (approximately once per month) for each incubator dedicated to this study. Similarly, the pH equivalence (study normal of 7.30–7.40) for each batch of medium in each study-dedicated incubator was confirmed during the same blood-gas analysis procedure, regardless of the O_2 concentration.

Statistical analyses

Laboratory and clinical endpoints included fertilization rate, cleavage rate, number of embryos per transfer, frequency of cryopreservation, implantation rate, live-birth implantation rate, clinical pregnancy rate, live birth rate, singleton and twin birth weights. The two primary endpoints targeted in this study were live-birth implantation rate and live birth rate. All other endpoints were considered to be secondary. The anticipated sample size to detect a minimum difference of 10% in live birth rate with a power of 80% and an α-error level of 5% was determined to be ~150 patients in each treatment group (300 patients in total). The live birth rate expected for this population of patients in our program at the commencement of this study was ~45%. Therefore, the power calculation was based on a 10% increase

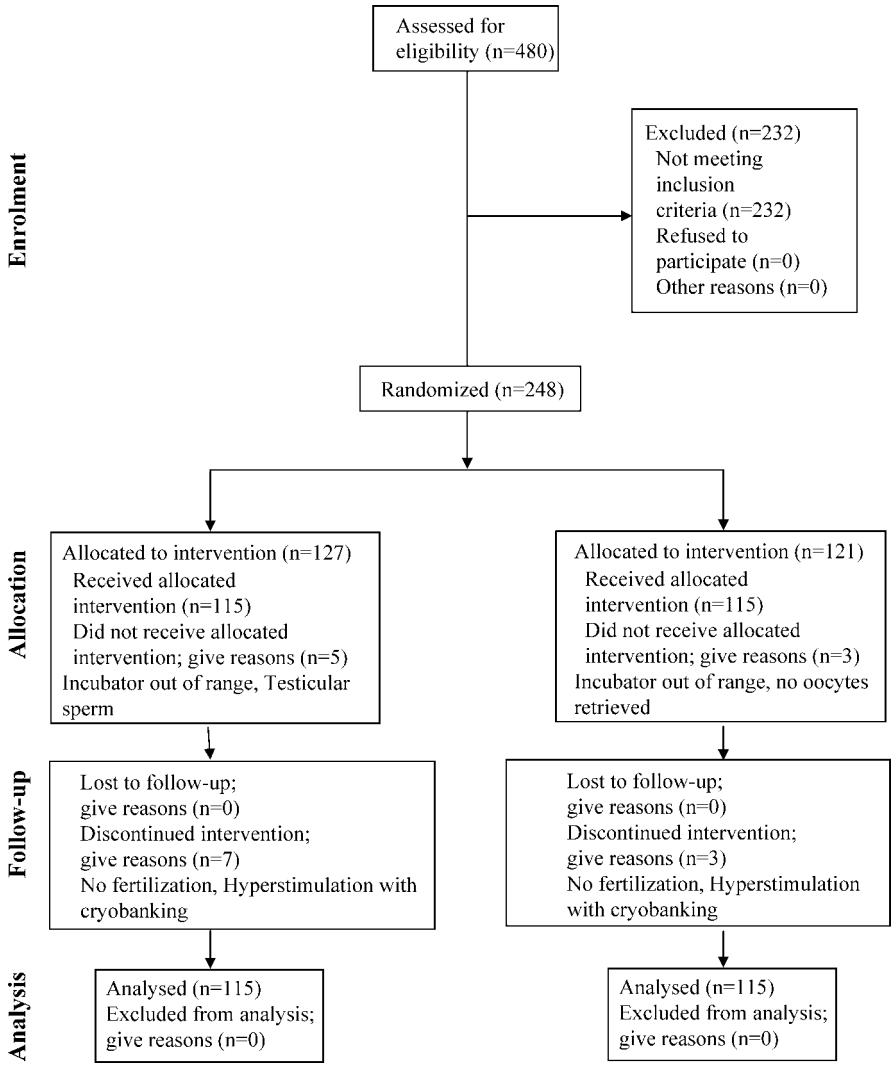

Figure 1: CONSORT statement flow diagram.

in live birth rate from 45 to 55%. Differences in implantation rates could be detected earlier (more statistical power with more embryos transferred than patients enrolled). For this reason, analyses of implantations were performed continually throughout this study to detect a possible treatment effect earlier and to ensure ongoing feasibility. Normal fertilization rate was calculated by the number of 2 PN embryos obtained by IVF and ICSI divided by the number of mature oocytes retrieved. Cleavage rate was the percentage of 2 PN embryos that divided into two or more cells on Day 2 of embryo culture. The implantation rate is defined as the number of intrauterine sacs observed by ultrasound within 8 weeks of embryo transfer divided by the number of embryos transferred. The live-birth implantation rate is the number of babies born alive divided by the number of embryos transferred. The clinical pregnancy rate was calculated as the number of patients with at least one intrauterine sac observed by ultrasound within 8 weeks of embryo transfer divided by the number of patients with an oocyte retrieval. The live birth rate is the number of patients with one or more live-born infants divided by the number of patients with an oocyte retrieval. Multi-variate analyses were performed to evaluate the overall treatment effect on clinical success rates, while controlling for the effects of covariates. Specifically, a logistical regression (LR) analysis was used to test for the effect of O_2 concentration on live births, while controlling for covariates. Specific covariates considered included the age group of the patient, day of transfer and the number of embryos per transfer. Furthermore, the primary and secondary endpoints were analyzed with general linear models for proportions, controlling for the same covariates. Differences in frequencies of fertilization, cleavage, and cryopreservation were evaluated with Pearson's χ^2 test with Yates' continuity correction as independent variables ($P < 0.05$). A two-tailed *t*-test was used to test for differences in singleton and twin birth weights.

Results

The results of the LR showed that the model was highly significant (LR < 0.001) while rejecting the null hypothesis of no effect. The O_2 treatment ($P = 0.032$), age group ($P = 0.042$) and day of transfer ($P = 0.004$) were all significant predictors. The number of embryos per transfer was not a significant predictor ($P = 0.198$). When culturing embryos in 5% O_2, the odds of a resulting live birth was 1.85 times higher [95% confidence interval (CI): 1.06–3.23] than when culturing in 21% O_2.

No differences were found between the low-O_2 and high-O_2 treatment groups in normal fertilization rate (1129/1491,

Table III. Implantation, clinical pregnancy and live birth outcomes following transfer on Day 3 for the atmospheric-O_2 and low-O_2 treatment groups.

Parameter	21% Oxygen	5% Oxygen	*P*-value[a]	Difference (95% CI)
Implantation rate	20/87 (23.0%)	12/54 (22.2%)	0.730	0.8% (−14.0–14.1)
Live-birth implantation rate	13/87 (14.9%)	11/54 (20.4%)	0.634	5.5% (−7.0–19.3)
Clinical pregnancy rate	11/33 (33.3%)	8/22 (36.4%)	0.916	3.1% (−20.8–27.8)
Live birth rate	7/33 (21.2%)	8/22 (36.4%)	0.241	15.2% (−8.3–38.4)

[a]General linear models for proportions, controlling for the covariates: patient age, day of transfer and the number of embryos per transfer.
CI, confidence interval.

Table IV. Implantation, clinical pregnancy and live birth outcomes following transfer on Day 5 for the atmospheric-O_2 and low-O_2 treatment groups.

Parameter	21% Oxygen	5% Oxygen	*P*-value[a]	Difference (95% CI)
Implantation rate	75/180 (41.7%)	110/193 (57.0%)	<0.001	15.3% (5.2–25.0)
Live-birth implantation rate	69/180 (38.3%)	95/193 (49.2%)	0.005	10.9% (0.8–20.6)
Clinical pregnancy rate	45/82 (54.9%)	66/93 (71.0%)	0.011	16.1% (1.8–29.6)
Live birth rate	42/82 (51.2%)	58/93 (62.4%)	0.087	11.2% (−3.5–25.2)

[a]General linear models for proportions, controlling for the covariates: patient age, day of transfer and the number of embryos per transfer.

Table V. Overall implantation, clinical pregnancy and live birth outcomes for the atmospheric-O_2 and low-O_2 treatment groups, regardless of the day of transfer.

Parameter	21% Oxygen	5% Oxygen	*P*-value[a]	Difference (95% CI)
Implantation rate	95/267 (35.6%)	122/247 (49.4%)	0.003	13.8% (5.3–22.1)
Live-birth implantation rate	82/267 (30.7%)	106/247 (42.9%)	0.005	12.2% (3.9–20.3)
Clinical pregnancy rate	56/115 (48.7%)	74/115 (64.3%)	0.027	15.6% (2.8–27.8)
Live birth rate	49/115 (42.6%)	66/115 (57.4%)	0.043	14.8% (1.9–27.0)

[a]General linear models for proportions, controlling for the covariates: patient age, day of transfer and the number of embryos per transfer.

75.7% and 1090/1455, 74.9%; respectively) or cleavage rate (1115/1129, 98.8% and 1070/1090, 98.2%; respectively). More embryos ($P < 0.04$) were transferred in the high-O_2 treatment group (2.32 per patient) than for the low-O_2 treatment group (2.17). Overall, 55 patients had embryos transferred on Day 2–3 (55/230, 23.9%) and 175 patients had transfers on Day 5 (175/230, 76.1%). The high-O_2 treatment group had 33/115 (29%) of transfers conducted on Day 2–3 and the low-O_2 treatment group had 22/115 (19%) of patients with transfers on Day 2–3 which was not different. Only one 39-year-old patient in the low-O_2 treatment group had no blastocysts to transfer on Day 5. All 175 other patients designated for Day 5 transfers on Day 2 or on Day 3 had blastocysts for transfer. Among patients from the low-O_2 treatment group, 29/115 (25.2%) had blastocysts cryopreserved, compared with 23/115 (20%) patients from the high-O_2 treatment group which was not significantly different. Also, no significant difference was found in the number of blastocysts cryopreserved per patient with 98 blastocysts cryopreserved (98/115, 0.85 blastocysts/patient) for patients in the low-O_2 treatment group and 84 blastocysts cryopreserved (84/115, 0.73 blastocysts/patient) for patients in the high-O_2 treatment group.

The results for the general linear models for proportions, controlling for day of transfer, patient age and the number of embryos per transfer are presented in Tables I–V. The low-O_2 treatment group appeared to consistently result in improved clinical outcomes for all age groups tested (Tables I and II). A significant improvement in the implantation rate was specifically observed in the ≤34-year-old age group (62/138, 44.9% versus 72/125, 57.6%; difference of 12.7% with 95% CI: 0.6–24.2, $P = 0.039$) as well as an increase in the live-birth implantation rate for the 38–40-year-old age group (3/47, 6.4% versus 13/37, 35.1%; difference of 28.8% with 95% CI: 11.6–45.4, $P = 0.01$) (Table I). The lack of significance in the other age groups or in all parameters within a specific age group were due, in part, to the lower numbers of patients represented in these subgroups. However, the overall results from this study across all patient age groups showed a significant improvement in the implantation rate, live-birth implantation rate, clinical pregnancies as well as live births when culturing gametes and embryos in 5% O_2 (Tables I, II and V). For example, the overall live birth rate increased from 49/115 (42.6%) to 66/115 (57.4%) which differed by 14.8% (95% CI, 1.9–27.0) (Table V). The number of patients needed with embryos to be cultured at a 5% O_2 concentration to gain one additional live birth was 6.8 (number needed to treat: 1/0.148, 95% CI 3.5–50).

When transferring embryos on Day 2–3, neither the implantation rate nor the live-birth implantation rate differed between the two treatment groups (Table III). Similarly, neither the clinical pregnancy rate nor the live birth rate was different when comparing the low-O_2 and high-O_2 treatment group if the embryos were transferred on Day 2–3 (Table III). In contrast, the implantation rate, the live-birth implantation rate and the clinical pregnancy rate were significantly improved in patients with embryos from the low-O_2 treatment group when transferring blastocysts on Day 5 (Table IV).

The birth weights of babies born from embryos cultured in the low-O_2 treatment group were not different from those of babies born from the high-O_2 treatment group when comparing singleton ($n = 26$, 3361 ± 685 g and $n = 19$, 3555 ± 339 g, mean $\pm$ SD; respectively) and twin births ($n = 80$, 2384 ± 405 g and $n = 54$, 2271 ± 709 g, mean $\pm$ SD; respectively). No triplets were born in the low-O_2 treatment group and three sets of triplets were born in the high-O_2 treatment group.

Discussion

Results from this study clearly demonstrate the beneficial effect of culturing human embryos in a lowered O_2 concentration and confirm the findings of numerous animal studies reporting the same. With a difference of 14.8% in the live birth rate between the low-O_2 and high-O_2 treatment groups, the embryos of only 7 patients have to be cultured in a reduced O_2 culture environment to result in one additional live birth or, alternatively, the low-O_2 treatment group, involving 115 patients in this study, gained 17 additional live births.

These results contrast with those of previous studies in the human that found either no benefit of culturing human embryos at lower O_2 concentrations (Dumoulin *et al.*, 1995) or some improvement in laboratory parameters only, without significantly affecting clinical outcomes (Noda *et al.*, 1994; Dumoulin *et al.*, 1999). It was reported that the implantation rate was increased from 10 to 14% and the pregnancy rate from 19 to 32% when culturing human embryos in MINC (Cook IVF) incubators in an atmosphere of 6% CO_2, 5% O_2 and 89% N_2 instead of in standard water-jacketed incubators in an atmosphere of 5% CO_2 in air (Catt and Henman, 2000). We know that the incubator type can have a profound effect on temperature and gas-phase recovery dynamics with significant effects on human blastocyst quality (Fujiwara *et al.*, 2007). These results reported by Catt and Henman appear to be seriously confounded by the difference in incubator type and CO_2 concentration between the two treatment groups. Other potentially confounding variables in their study include the non-simultaneous time period of treatment application, physician effect and various patient factors. The improvements in reported clinical outcomes may not have been due to the different O_2 concentrations at all. The controlled circumstances in which the present study was conducted is likely to have been crucial in differentiating the effect of incubator O_2 concentration from the many typical confounding factors found in other similar studies in the human.

Embryo transfers in most human studies have been conducted on Day 3, not allowing for the lowered O_2 concentration to benefit blastocyst and ICM development *in vitro* following embryonic genome activation (Dumoulin *et al.*, 1995, 1999; Catt and Henman, 2000). Interestingly, implantations and clinical pregnancies were almost identical in our study as well, regardless of the O_2 concentration when considering Day 2–3 transfers only. When blastocyst transfers were evaluated in a small study of 22 patients, the clinical pregnancy rate appeared to have improved in a reduced O_2 environment, although the number of patients was too small to reach statistical significance (Kea *et al.*, 2007). A recent study, comparing low-O_2 and atmospheric-O_2 culture of blastocysts, yielded a better numerical blastocyst outcome and a 10% improvement in relative birth rate, following culture in a reduced oxygen environment (Waldenström *et al.*, 2008). The overall improvement in implantations and clinical pregnancies observed when embryos were cultured in a reduced O_2 environment in our study, were also exclusively due to the marked improvements seen following blastocyst transfers. Roughly, 76% of all transfers in this study were conducted on Day 5. The transfer of some embryos on Day 2–3 in this study may complicate the analyses and the interpretation of the results. However, the lack of any benefit seen when culturing in low-O_2 concentrations and transferring embryos on Day 2–3 in this study, compared with the significant improvements seen when transferring on Day 5, may partially explain why the beneficial effect of low-O_2 culture was not identified in earlier human studies in which transfers occurred on Day 2–3.

The higher number of embryos per transfer for patients in the high-O_2 treatment group were probably due to the relatively higher number of Day 2–3 transfers in this group (29 versus 19%). Typically, three embryos were transferred on Day 2–3, but only one or two embryos on Day 5, resulting in this higher average number of embryos per transfer observed for the high-O_2 treatment group. Based on the criteria for transferring embryos on Day 2–3 in this study, it can be assumed that the more frequent observation of lower numbers of appropriately advanced embryos on Day 2–3 by the embryologists resulted in this bias towards Day 2–3 embryo transfers in the high-O_2 treatment group.

Most studies evaluating the influence of incubator O_2 concentration in the human have focused on laboratory endpoints and some may include clinical pregnancies as the ultimate endpoint (Kea *et al.*, 2007). As demonstrated in various animal models, laboratory outcomes and even initial clinical outcomes may not accurately reflect the ability of embryos to result in live offspring (Karagenc *et al.*, 2004). We also found no change in fertilization rate or cleavage rate when culturing embryos in a reduced O_2 environment. Initial clinical outcomes (implantation rate and clinical pregnancy rate) were only affected by atmospheric O_2 concentrations when considering Day 5 transfers. It can be argued, therefore, that only the embryos of blastocyst-transfer patients should be cultured in 5% O_2 and that the change to the reduced O_2 environment could be delayed until the third day of culture. However, numerous animal studies have found that even a brief exposure of pronuclear or cleavage-stage embryos to atmospheric O_2 can be detrimental even though it may only be manifested at the morula or blastocyst stage (Pabon *et al.*, 1989; Karagenc *et al.*, 2004). When considering not only the initial clinical results in the present study but also the eventual live birth implantations and live births, it became apparent that even embryos transferred on Day 2–3 might have benefited from culture in reduced O_2 as more implantations were lost from the high-O_2 treatment group. Therefore, selective later-stage reduced O_2 culture may be an ill-advised approach since the damaging effects of atmospheric O_2 may already be present in early embryos but only manifested at later stages of development and expressed as altered metabolism or gene expression with reduced viability.

Indications were that more patients had some blastocysts to cryopreserve and that more blastocysts were frozen per patient

when culture took place in a reduced O_2 environment. However, with only a fraction of patients typically having embryos to cryopreserve, the number of patients enrolled in this study was not sufficient to achieve statistical significance for differences in cryopreservation rates between the two treatment groups. Future studies should consider not only the number of embryos cryopreserved following culture in reduced O_2 concentrations, but also the viability of the frozen–thawed blastocysts following their transfer to the uterus. No detrimental increase in birth weight when culturing in atmospheric O_2 concentrations could be demonstrated in this study as previously reported in calves (Iwata *et al*., 2000).

It has been reported that porcine embryos obtained from different sources (*in vitro*, *in vivo* or parthenogenic) differ in resilience when exposed to atmospheric O_2 concentrations (Booth *et al*., 2005). One may, therefore, speculate that the more robust embryos from younger patients or embryos derived from donor oocytes may not require the lowered O_2 tension. However, the consistent pattern of increased live births across all age groups in this study when culturing in a low-O_2 environment suggests that the embryos of all patients may benefit from a reduced O_2 environment. The overall increase in live births when culturing embryos in a reduced O_2 environment in this study, clearly justifies the additional effort and expense associated with low-O_2 culture. Even though extended culture of embryos to Day 5 may be responsible, in part, for the demonstration of the pronounced beneficial effect of lower O_2 concentrations on live births, human embryos should be cultured in a low-O_2 environment through all *in vitro* stages of development to ensure the highest live birth rate and most appropriate gene expression.

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