The effect of two distinct levels of oxygen concentration on embryo development in a sibling oocyte study

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Abstract

Purpose This prospective randomized study used sibling oocytes of 258 women with ≥ 8 oocytes to compare the effect of 5 % O_2 versus 20 % O_2 concentrations on embryo development and clinical outcome.

Methods Oocytes of each case were divided between incubators with either 5 % or 20 % O_2 concentration. Outcome measures were fertilization, cleavage, embryo quality, blastocyst formation, and implantation, pregnancy and live birth rates.

Results Fertilization and cleavage rates were similar in both groups. The 5 % O_2 group had significantly more blastomeres ($P < 0.05$) and more top-quality embryos on day 3 ($P < 0.02$), as well as significantly more available embryos for transfer (31.6 % vs. 23.1 % for the 20 % O_2 group; $P < 0.0001$). There were significantly more cycles with good embryos in the 5 % group (76/258) than in the 20 % group (38/258) ($P < 0.0001$). Implantation and pregnancy rates were significantly higher for 5 % O_2 embryos ($P < 0.03$ and $P < 0.05$, respectively). Live birth rates per embryo transfer were 34.2 % and 15.8 %, respectively, $P < 0.05$.

Conclusions Implantation, pregnancy and live birth rates are higher, and more good quality embryos are available for transfer and freezing with reduced rather than with atmospheric oxygen concentrations during embryo incubation.

Capsule Implantation, pregnancy and live birth rate are higher, and more good quality embryos are available for transfer and freezing with reduced rather than with atmospheric oxygen concentrations during embryo incubation.

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Introduction

Embryo development depends on a variety of factors, including culture media, incubation volume, embryo density and reduced oxygen tension [9, 11]. O_2 concentration has a crucial effect on human embryo development. The main gas phases utilized in IVF laboratories are 5 %/6 % carbon dioxide (CO_2) in air (20 % O_2) or 5 %/6 % CO_2 , 5 % oxygen (O_2), and 90 % nitrogen (N_2). Although oxygen tension in the oviduct and uterus of most mammalian species has been found to range from 2 % to 8 % [8, 28], many laboratories are still using atmospheric oxygen concentrations (20 %) for the culture of human embryos. Elevated and non-physiological O_2 concentrations could create unfavorable conditions in the substrate to produce free oxygen radicals that cause oxidative stress, which may be involved in the etiology of defective embryo development with higher rates of fragmentation [2, 13]. Reactive oxygen species (ROS) may originate from embryo metabolism and/or embryo surroundings [12].

The results of oxygen concentration studies on human embryos have been controversial. No significant differences were found in fertilization, cleavage, pregnancy and implantation rates during the culture of embryos up to day 2 or day 3 when using O_2 concentrations of 5 % or 20 % [7]. On the other hand, significantly more surplus embryos reached the blastocyst stage when cultured under 5 % O_2 compared to 20 % O_2 concentration and had more cells when fixed on day 5 as well as on day 6 [6]. A better blastocyst outcome and a significant improvement in pregnancy and birth rates were found with low oxygen concentrations by Waldenström et al. [27], but their study was not conducted on sibling oocytes. Kea et al. [15] found that different oxygen concentrations did not significantly

affect fertilization rate, blastocyst formation and quality or pregnancy rate, but that there was a significant difference in the mean embryo score on day 3, in favor of the reduced oxygen concentration.

Another study showed that a lower oxygen concentration improved the blastulation rate and increased the proportion of embryos reaching the stage of expanded blastocyst with a normal inner cell mass on day 5 [17]. The same authors later reported that although the ongoing pregnancies and implantations were similar in the two oxygen concentration groups, the cumulative pregnancy rate (of both fresh and frozen thawed embryos) was significantly higher in the 5 % O₂ group [16]. Ciray et al.'s study [5] on sibling oocytes concluded that 5 % O₂ significantly improved the total blastocyst yield as well as the quality of day 3 and day 5 embryos. Finally, Meintjes et al.'s [19] study showed an overall increase in live births when embryos were cultured in reduced oxygen concentrations. A recent meta-analysis on four studies with a relatively low methodological quality showed a beneficial effect of culturing in low oxygen concentrations for live birth rates (OR 1.39; 95 % CI 1.11 to 1.76; $P=0.005$; $I(2) = 0\%$). This would mean that a typical clinic could improve a 30 % live birth rate using atmospheric oxygen concentration to somewhere between 32 % and 43 % by using a low oxygen concentration [3]. These studies, however, analyzed certain characteristics of embryo development or, alternatively, only compared the clinical outcome, i.e., implantation, pregnancy and live birth rates. Moreover, some did not compare exclusively between sibling oocytes. In contrast, the present investigation was aimed solely at sibling oocytes, and it monitored all the developmental stages, from zygote until blastocyst formation, and the clinical outcome after incubation under each of the two oxygen concentrations.

Our primary aim was to evaluate the effect of the two different O₂ concentrations on the embryo development of sibling oocytes in terms of fertilization, cleavage, embryo quality and blastocyst formation. Secondary outcomes included implantation, ongoing pregnancy and live births.

Materials and methods

Study design

This prospective randomized study was approved by our Institutional Review Board and was conducted at a University Hospital IVF Center from October 2010 till December 2011.

The participants included 258 women who underwent intracytoplasmic sperm injection (ICSI) treatment with at least eight aspirated oocytes. Exclusion criteria were: the use of testicular sperm, cryopreservation of all embryos, complete fertilization failure, PGD/PGS cases and severe ovarian hyperstimulation syndrome (OHSS).

Ovarian stimulation

Controlled ovarian hyperstimulation was performed according to the standard long and antagonist protocols: the long gonadotropin-releasing hormone agonist [GnRHa, Decapeptyl® (Ferring Pharmaceuticals, Malmo, Sweden) 0.1 mg daily injection] starting in the mid-luteal phase using mainly human menopausal gonadotrophin (Menogon®; Ferring Pharmaceuticals, Malmo, Sweden); the antagonist protocol, Cetorelix acetate 0.25 mg/day (Cetrotide; Asta Medica AG, Frankfurt, Germany) was administered subcutaneously when the leading follicle measured ≥ 12 mm in diameter. Human chorionic gonadotrophin (hCG) (Ovitrelle 250 µg) was injected subcutaneously when at least one follicle measured ≥ 18 mm.

Oocyte and embryo culture

The retrieved oocytes were denuded and then injected according to the standard ICSI procedure. After ICSI, the oocytes were randomly divided into two groups: one-half of the oocytes were incubated in an incubator containing 5 % O₂ and the other half were incubated under 20 % O₂. The temperature in both incubators was 37 °C. The culture dishes had been equilibrated overnight in the incubators with their respective gas mixture prior to egg retrieval. The oocytes were cultured in 25 µl culture-media droplets under oil (Irvine Scientific, Santa Ana, California). Each injected oocyte, embryo and blastocyst was placed in a single droplet. Culture medium G1 (Vitrolife, Sweden) was used for the first 3 days of culture after which the embryos were transferred to culture medium G2 (Vitrolife) until they reached the blastocyst stage. The oocytes of each group were placed in a NuAire incubator (Model NU-4950-E, NuAire, USA) equipped with HEPA filters. One of these two NuAire incubators was exposed to air (20 % O₂), and the other was inflated with nitrogen gas to create 5 % O₂ partial pressure. The CO₂, temperature and O₂ concentrations of both incubator chambers were checked daily before the incubator doors were opened.

Fertilization and embryo evaluation

Evaluations of fertilization and cleavage were performed at 18–20 h, day 2 and day 3 following the ICSI procedure, and blastocyst development on day 5 and day 6. Normal fertilization was defined as two clearly visible pronuclei. Cleavage rate was defined as the total number of cleaved embryos from the total number of fertilized oocytes. Embryo quality was scored according to the presence of equal-sized blastomeres with no fragmentation (grade 1), <20 % fragmentation (grade 2), 20 %–50 % fragmentation (grade 3), and >50 % fragmentation (grade 4) [22]. The grade was reduced by one-half when embryos had unequal-sized blastomeres. A top-quality embryo was defined as an 8-cell embryo grade 1 on

day 3. The best qualified embryos were selected for transfer. Blastocyst characteristics were scored on a scale of 1–3: zona pellucida (ZP) thickness (1 = thin, 2 = medium, and 3 = thick), inner cell mass (ICM; 1 = good, 2 = intermediate, and 3 = inferior), and trophectoderm appearance (1 = good, 2 = intermediate, and 3 = inferior). Blastocysts that fulfilled the criteria for cryopreservation had a ZP score of 1, an ICM score of 1 or 2, and a trophectoderm score of 1 or 2.

Embryo transfer and cryopreservation

The transfer of the embryos to the uterus was performed on day 3 after retrieval. The number of transferred embryos was determined by the guidelines of the Israeli Ministry of Health, i.e., two embryos in each of the first three IVF treatment cycles and three embryos in each additional trial. The best 8-cell embryos were chosen for transfer, preferably from the same O₂ concentration group, if available. The transferred embryos from cycles in which equally good quality embryos were found in both O₂ concentrations were randomized into two groups according to their origin by means of a random number generator program: embryos came from cycles in which all transferred embryos originated from the 5 % O₂ concentration and from cycles in which all transferred embryos originated from the 20 % O₂ concentration. If there were too few good quality embryos for transfer in one group, the best embryos were chosen from both groups and the data of these cycles were grouped in the “mixed group”. Only the remaining best quality embryos were frozen. The surplus (intermediate and poor quality) embryos were cultured further for blastocyst formation. Blastocysts were evaluated on day 5 and day 6 after retrieval, and those with good morphology were cryopreserved.

Implantations and pregnancies

The luteal phase was supported by progestative vaginal suppositories 600 mg/day (Utrogestan, Besins, Paris, France). The first β hCG test was performed 14 days after transfer. Pregnancy was determined solely by the documentation of a visible sac with a fetal heart beat on vaginal sonography in the 6th gestational week. The implantation rate was calculated as the total number of gestational sacs divided by the total number of transferred embryos. The live birth rate was defined as the number of patients with a live birth of at least one live neonate, divided by the total number of patients undergoing embryo transfer. The details of the newborns were collected from the delivery rooms database and by contacting the mothers 3 months after giving birth.

Statistical analysis

The results of numerical measurements are presented using means and SDs, while the results of categorical measurements

are presented using percentages. In order to examine the differences in the results between the two treatment types, 5 % vs. 20 % oxygen, we used the following statistical methods: (1) for the numerical measurements we used the *t*-test for independent groups or the *t*-test for paired groups depending on the study design; (2) for the categorical measurements we used the Chi-square test or Fisher’s exact test. In order to control for the effect of possible confounders, such as mother age and others, we also used the ANOVA with repeated measurements multivariate model, which is part of the Generalized Linear Models (GLM). All analyses were done using SPSS statistical software ver. 18 © IBM.

Results

The general characteristics of the 258 women are listed in Table 1. A total of 3,638 mature (metaphase II) oocytes were retrieved, of which 1,833 were incubated under 5 % O₂ (mean number of oocytes per patient 6.96 ± 2.63) and their 1,805 sibling oocytes under 20 % O₂ concentration (6.86 ± 2.41 per patient respectively).

Table 2 displays the ICSI outcome and embryo development in the two O₂ concentration groups. The fertilization and cleavage rates were similar for both groups. However, the cleavage rhythm (i.e., the number of cells on day 3) and the number of top-quality embryos per patient were significantly higher under 5 % O₂ than with 20 % O₂ concentrations ($P < 0.05$ and $P < 0.02$, respectively). The number of frozen embryos, the blastulation rates and the blastocysts suitable for freezing were comparable.

No significant differences were observed in the blastulation or freezing percentages between both groups.

Of all the retrieved oocytes, the number of embryos used for transfer and for freezing per patient was significantly higher in the low oxygen concentration group compared with the atmospheric one, i.e., 2.57 ± 1.578 and 2.10 ± 1.770 , respectively ($P < 0.01$).

In order to be able to conclude which O₂ concentration had a better influence on clinical outcome (i.e., implantation, pregnancy and live birth rates), we separately analyzed the cycles in which transferred embryos originated from the same O₂ concentration group. In this way, we could be sure which embryo implanted (Table 3). Table 3 shows the clinical outcome of the two treatment arms.

From these 258 cycles, following our criteria of embryos suitable for transfer, in 76 cycles we transferred only embryos originating from 5 % O₂ and in 38 cycles all transferred embryos originating from 20 % O₂. The remaining cycles were embryos transferred from mixed O₂ concentrations. Significantly more cycles with good embryos were available in the low O₂ concentration group than in the higher one (76/258 versus 38/258; $P < 0.0001$).

Table 1 General characteristics of the 258 patients in the study

Mean age (y)	30.8±4.63 (range 20–43 years)
Missed abortions in the past	7/258 (2.7 %)
Previous IVF treatment cycles (none of them resulted in a pregnancy)	4.6±3.28
Basal FSH level	5.8±1.80 IU/L.
Gonadotrophin amount used (IU)	2771.6±1393.66 IU
Indication for IVF\ICSI:	
Male factor (cycles)	219 (84.9 %),
Tubal factor combined with male subfertility (cycles)	48 (18.6 %)
Endometriosis, PCO and unexplained factors with poor fertilization in previous IVF treatment (cycles)	52 (20.2 %)

ICSI intracytoplasmic sperm injection; PCO ovarian syndrome

Implantation, pregnancy and live birth rates were significantly higher in the low O₂ concentration compared to the higher one (22.1 % vs. 10.3 %, $P<0.03$; 38.2 % vs. 18.4 %, $P<0.05$; 34.2 % vs. 15.8 %, $P<0.05$ respectively).

Table 4 shows the comparison of the results of the mixed group with the 5 % and 20 % O₂ groups. These data are presented in order to better understand the efficiency of the incubation of oocytes and embryos in low oxygen concentration.

Discussion

The possible contribution of low oxygen concentrations to the better development of embryos, blastocysts and fetuses had already been demonstrated in a mice model in the late seventies [14, 23]. Later studies on sheep and bovine animals [24–26] confirmed a positive impact of low oxygen levels on number of cells, morula and blastocyst formation. However, this improvement had no significant effect on pregnancy rates or on the viability of developing fetuses.

These data challenged embryologists working with human IVF technology to investigate the impact of low oxygen concentrations on human embryos. Dumoulin et al. [6, 7] showed that the 5 % concentration level had no advantage over the 20 % oxygen concentration level with respect to fertilization, cleavage, pregnancy and implantation rates, but that it did improve the blastulation rate and the mean number of cells in blastocysts on day 5 and day 6. Gardner and Lane [10] also showed a 62 % blastulation rate for the lower concentration compared with 30 % for the higher one. These three studies were performed with a single medium culture, and the introduction of sequential media during the past few years sparked a new interest in the influence of low oxygen concentration cultures on the quality of embryos and blastocysts. However, studies published between 2000 and 2005 showed conflicting results about the contribution of low oxygen concentrations. Several additional studies in humans have been published since then [1, 5, 17, 27]. All of them were prospective, but the randomization methods they used for examining the differences between the two oxygen concentrations from fertilization to ongoing pregnancies and live births are problematic. Randomization was either based on

Table 2 Intracytoplasmic sperm injection (ICSI) outcome and embryo development in the 5 % and 20 % O₂ concentration groups

	<i>n</i> =258 Women's age=30.84±4.63		
	5 % oxygen concentration	20 % oxygen concentration	<i>P</i> -value adjusted*
Mature oocytes (per patient)	6.96±2.626	6.86±2.413	0.876
Fertilized oocytes (per patient)	4.80±2.036	4.72±2.211	0.426
Cleaved embryos (per patient)	4.50±2.116	4.36±2.249	0.345
No. of blastomeres on day 3	6.69±1.321	6.21±1.229	0.046
Embryo morphology on day 3	2.07±0.515	2.10±0.483	0.918
Top quality embryos day 3 (per patient)	1.26±1.075	1.01±0.983	0.016
Embryos transferred (per patient)	1.80±1.249	1.27±1.171	0.00001
Embryos frozen (per patient)	0.94±1.389	0.86±1.428	0.229
Blastocysts developed from surplus embryos (per patient) ^a	0.65±0.962	0.65±0.978	0.289
Blastocysts suitable for freezing (per patient)	0.18±0.542	0.20±0.617	0.679

**P*-value adjusted to take into consideration the female age and the number of previous treatment cycles

^a The blastocysts designated for culture were from the surplus embryos (remaining after transfer and frozen embryos)

Table 3 Intracytoplasmic sperm injection (ICSI) outcome of the treatment cycles where all embryos transferred were from only 5 % or only 20 % O₂ concentration

	5 % oxygen concentration	20 % oxygen concentration	P-value
Patients	76	38	
Age	30.2±4.57	30.±3.91	0.606
Paternal age	32.9±5.26	33.1±6.02	0.856
No of previous treatment cycles	2.6±3.27	3.5±3.22	0.182
BMI	21.5±1.17	22.2±0.88	0.37
Basal FSH	5.5±1.38	5.8±1.77	0.394
No. of oocyte (per patient)	14.2±4.32	13.9±4.73	0.744
Fertilized oocytes (per patient)	9.9±3.78	9.6±4.23	0.737
No. of embryos (per patient)	9.4±3.69	9.2±4.24	0.733

day of admission to the IVF laboratory [1], age of the woman, infertility factor, amount of gonadotrophins, number of retrieved oocytes [17], or per patient [15, 19, 27]. They all saw higher implantation, pregnancy and birth rates with reduced O₂ concentrations. However, the study of Nanassy et al. [20] did not show any improvement in embryo development or higher implantation and pregnancy rates in a reduced oxygen concentration culture from day 3 until day 5.

The value of low oxygen concentrations in an IVF laboratory is a continuing question and many centers are still in doubt as to the real importance. Moreover, the high cost of installation of nitrous gas and the maintenance required to create low oxygen tension does not facilitate the decision to convert the oxygen concentration used for culture to 5 %.

Studies in animal models have shown that culture in high oxygen tension conditions leads to over production of reactive oxygen species (ROS). The ROS have been demonstrated to cause possible damage to the cell membrane, DNA fragmentation in somatic cells and possibly play a part in the apoptosis process. In a mice model, Kwon et al. [18] found that the development of the embryos was significantly

increased with infrequent fragmentation with 5 % O₂ as compared with 20 % O₂ concentrations where the embryos had a developmental delay or block.

In a mice model, Orsi and Leese [21] showed that a 5 % O₂ concentration significantly contributed to enhancing embryo development and also increased cell numbers.

In the present study, we aimed to compare the outcome of injected sibling oocytes at atmospheric vs. low oxygen concentrations throughout the entire process of zygote and embryonal development in terms of fertilization, from embryo until blastocyst formation. We also evaluated the impact of oxygen concentration on implantation, pregnancy and live birth rates. For this purpose, we created stringent guidelines for choosing embryos for transfer to the uterus. These guidelines enabled us to determine that there was a comparative clinical benefit of culturing under low oxygen concentrations. It emerged that the fertilization and cleavage rates in our series were not influenced by the changes in oxygen concentration. This coincides with the findings of other studies [1, 4, 7, 15, 17, 27]. However, the number of blastomeres on day 3 was significantly higher in the current study when the 5 % O₂

Table 4 Clinical outcome of the treatment cycles where all embryos transferred were from only 5 % or only 20 % O₂ concentration

	5 % oxygen concentration (%)	20 % oxygen concentration (%)	P-value
Patients	76	38	
Embryos/transfers	2.3±0.53	2.3±0.52	0.800
Implantation	38/172 (22.1)	9/87 (10.3)	0.04
Pregnancy	29/76 (38.2)	7/38 (18.4)	0.025
Twins	9/29 (31.0)	1/7 (14.3)	0.645
Abortion	2/29 ^a (6.9)	1/7 (14.3)	0.4882
Ectopic pregnancies	1/29 (3.4)	0	0.8056
Vanished sacs	3/38 (7.9)	0	0.5557
Live birth	26/76 (34.2)	6/38 (15.8)	0.047
Still birth	0	0	
NND	0	0	
Weight	2827.1±925.40	2855.0±525.21	0.95
Fetal malformations	1	0	0

^a Induced abortion due to fetal malformations at 19th week of gestation

concentration was used compared with the 20 % concentration. Kovacic and Vlaisavljević [17] obtained the same results as ours for both day 3 embryos and blastocysts.

The current study showed significantly more day 3 top-quality embryos with the low oxygen concentration regimen. These data are consistent with those of Bahçeci et al. [1], Ciray et al. [5] and Kea et al. [15] who also found better quality embryos when using a lower (5 %) oxygen concentration. Since top-quality embryos were more abundant in our 5 % O₂ group, 76 transferred embryos came from this group, significantly more than the 38 transfers from the high oxygen concentration group. Significantly higher implantation, pregnancy and live births rates were also found in the lower oxygen group compared with the higher oxygen group. We are aware of the disadvantage of using sibling oocytes when comparing clinical outcome (i.e. implantation, pregnancy birth rates). Since we had to transfer embryos only from one oxygen concentration group (in order to know the origin of the implanted embryo), the compared groups became much smaller than the primary study group. Yet, it seems to us that in order to evaluate the effect of low oxygen concentrations in an IVF culture system, the method of using sibling oocytes is preferable.

Recently, a Cochrane review [3], based on three studies that are cited in the current study [16, 19, 27], found evidence of a beneficial effect of culturing in low oxygen concentrations for the live birth rate (OR 1.39; 95 % CI 1.11 to 1.76; $P=0.005$; I²=0 %); this would mean that a typical clinic could improve a 30 % live birth rate using atmospheric oxygen concentration to somewhere between 32 % and 43 % by using a low oxygen concentration.

These conclusions are based on only three studies, which the reviewers claim are of relatively low methodological value.

The contribution of our study on sibling oocytes is mainly the methodology where throughout the whole culture period there were equal culture conditions, and embryos of the same O₂ concentration were transferred into the uterus. The results gathered in this study, therefore, add additional information on the contribution of culturing in 5 % O₂ tension, which leads to a higher cell number in the third cleavage stage and eventually to more top quality embryos, and higher implantation, pregnancy and birth rates. All the data gathered using animal models and in recent studies justify, in our opinion, the use of low oxygen concentrations in the regular work of an IVF laboratory.

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