J Assist Reprod Genet (2015) 32:77–82
DOI 10.1007/s10815-014-0368-z

EMBRYO BIOLOGY

Can we use incubators with atmospheric oxygen tension in the first phase of in vitro fertilization? A retrospective analysis

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Received: 8 August 2014 / Accepted: 8 October 2014 / Published online: 22 October 2014

Abstract
Purpose Aim of the present study was to compare two culture strategies used in our routine in vitro fertilization program.
Methods This is a retrospective analysis. Two culture systems were used in parallel and analysed retrospectively: 1) Use of atmospheric oxygen tension (~20 %) until insemination followed by use of low (~5 %) oxygen concentration; 2) Exclusive use of low oxygen concentration. Main outcome was the utilization rate defined as the number of transferred+ vitrified embryos per inseminated oocytes. Secondary outcomes were clinical pregnancy and live birth rates.
Results A total of 402 in vitro fertilization cycles were analyzed. Demographic and clinical data of patients belonging to the two culture systems were not significantly different. Utilization rate, cumulative clinical pregnancy rate and cumulative live birth rate per cycle was similar using two different oxygen concentration compared to exclusive use of low oxygen tension (37 % versus 39 %; 30 % versus 30 %; 23 % versus 28 %, respectively).
Conclusions The use of a culture system with atmospheric oxygen tension from recovery of oocytes until insemination followed by culture in low oxygen gives results similar to exclusive use of low oxygen concentration.

Keywords Embryo culture · Low oxygen tension · Incubators · IVF

Capsule The use of a culture system with atmospheric oxygen tension from recovery of oocytes until insemination followed by culture in low oxygen gives results similar to exclusive use of low oxygen concentration in terms of embryo development and pregnancy rate.

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Introduction

A damaging effect of oxygen (O_2) at atmospheric tension on embryo development has been documented in several species including humans [1,2]. In particular, in humans, a beneficial effect of lowering atmospheric O_2 tension to 5 % has been observed both for embryo quality and pregnancy rates, mainly in trials in which embryos were transferred at the blastocyst stage [3–5]. Although ideal culture conditions have not been established yet, most laboratories are discarding old generation incubators in order to control O_2 concentration in the culture system. Due to high costs for replacement of instruments, it is likely that this policy leads to a tendency to overstock available low oxygen incubators with a potential rebound effect for culture conditions and micro-environment maintenance caused by recurrent opening/closing of the door [6]. In our laboratory, old generation incubators can be replaced slowly over time. In the meantime we prefer to keep using them at least in the first phase of in vitro fertilization in order to avoid excessive stocking of available O_2-controlled incubators.

The accumulation of reactive oxygen species (ROS) in the cytoplasm is probably the mechanism through which high O_2 concentration reduces developmental ability of embryos during in vitro culture [7]. Meuter et al. [8] showed that in in-vitro cultured murine blastocysts markers of senescence may be induced through oxidative stress and suboptimal culture conditions; moreover, culture in 5 % oxygen reduced markers of senescence to levels similar to that seen with in vivo embryos. It is known that ROS can damage cell membranes and DNA and might play a role in apoptosis [9].

The gas atmosphere composition is a critical factor for the regulation of the pH in the medium and in the cultured cells [10]. In vivo, the oocyte is likely protected by surrounding cumulus cells which form a matrix that helps maintaining homeostasis and provides protection from alterations in

intracellular pH. Disruption of intracellular pH in mammalian embryos results in impaired development and metabolism in culture [11,12]. Therefore, intracellular pH must be finely regulated to allow normal development and cumulus cells play a significant protective role to the oocyte that is lost when oocytes are denuded and placed in culture [13].

In our hypothesis the beneficial effects of lowering O_2 tension are not crucial when oocytes are enclosed in their cumulus cells. This is the reason why we decided to use controlled but also atmospheric O_2 tension until decumulation of oocytes. Aim of the present study was to compare these two culture strategies that were used in parallel in our routine IVF/ICSI program.

Materials and methods

Patients who underwent IVF-ICSI between May 2012 and October 2012 at the Infertility Unit of the Fondazione Ca' Granda Ospedale Maggiore, were retrospectively reviewed.

All women undergoing IVF/ICSI cycles routinely provide informed consent for their clinical data and anonymous records to be used for research purposes in general. Local Institutional Review Board approvals for the use of clinical data for research studies were obtained.

Women were treated and monitored as previously reported in details [14]. The regimen to be used and the dose of gonadotrophins were determined on an individual basis. Treatment cycles were monitored by serial transvaginal ultrasound and blood hormonal assessments. Cycles were cancelled in the case of arrest/absence of follicular growth. Human chorionic gonadotrophin (hCG) was administered when at least one follicle had a mean diameter of 18 mm and oocytes were retrieved after about 36 h. Oocyte–cumulus complexes were washed in flushing medium and transferred to IVF medium (Quinn's Advantage Protein Plus Fertilization Medium; Sage, Trumbull, CT, USA). Subsequent incubation was performed in either one of two culture systems:

- Group 20 % O_2+5 % O_2: use of uncontrolled oxygen tension (~20 % O_2) until insemination on Day 0 for ICSI cycles or until decumulation on Day 1 for standard IVF, followed by use of low oxygen concentration (5 % O_2) until embryo transfer;
- Group 5 % O_2: exclusive use of 5 % O_2 from ovum pick-up to embryo transfer.

The two culture systems were used in parallel in the ordinary clinical activity in order to avoid overstocking of incubators. CO_2 was set at 6 %. In fact, the day before oocyte aspiration (Day -1), cycles were alternately allocated to one of the two culture systems. Front-load incubators with controlled (n=6, MCO-5ACL, SANYO Electric Co., Ltd., internal volume 49 l) or atmospheric tension of oxygen (n=5, Hera-Cell, Heraeus, internal volume 150 l) were used.

Insemination with standard IVF or ICSI was performed after an incubation of 3±0.5 h in one of the two culture systems; cumulus cells were completely removed from all oocytes of ICSI cycles by pipetting them through 140/170 μm internal diameter pipettes (Flexipet; Cook, Bloomington, IN, USA) after a brief exposure to 40 IU/ml of hyaluronidase (Sage) in IVF medium (Sage). ICSI procedure was performed immediately and inseminated oocytes were cultured in cleavage medium (Sage). Standard IVF was performed by adding 25.000–50.000 spermatozoa to up to 4 cumulus-oocyte complexes in 1 ml of IVF medium and incubating in the original conditions for 16–18 h when cumulus cells were removed and fertilization was checked.

Zygotes were cultured in 20 μl of fresh cleavage medium in 5 % O_2 and 6 % CO_2 until embryo transfer.

Embryo transfer (ET) was performed on Day 2, 3 or 5 after oocyte collection, according to the number and quality of available embryos. On day 3, embryos were moved into fresh 20 μl drop media and supernumerary embryos were cultured to the blastocyst stage in 5 % O_2 and 6 % CO_2 to be vitrified on Day 5 or Day 6 according to the Cryotop method [15].

An embryo was considered to be of 'top quality' once it showed a stage-appropriate number of evenly sized blastomeres, complete absence of multinucleation and less than 10 % fragmentation. 'Top quality' blastocyst was defined as advanced blastocyst (full blastocyst and expanded blastocyst) with an inner cell mass scored 1 or 2 and multicellular trophectoderm scored 1 or 2 according to the Istanbul Consensus [16]. Clinical pregnancy rate (CPR) was defined as ultrasonographic demonstration of an intrauterine gestational sac with a vital embryo 4–5 weeks after embryo transfer per cycle. An active follow up of the pregnancy course was systematically performed in the study unit. The live birth rate (LBR) refers to the birth of at least one viable child.

Cycles in which insemination was not performed and thawing cycles were excluded.

Utilization rate, defined as the number of transferred+vitrified embryos/ fresh inseminated oocytes, was the main outcome measure and was used to make sample size calculation. Secondary outcomes were clinical pregnancy rate (CPR) and live birth rate (LBR). On the basis of the centre's experience, the mean number of inseminated oocytes was 5.5 and the standard utilization rate with 5 % O_2 culture system was 42 %; the study was designed to highlight a 15 % relative decrease of this value. A minimum of 1000 fresh oocytes were required to be inseminated per group (type 1 and type 2 errors set at 5 and 20 %, respectively) and assuming that 20 % of recruited patients would be subsequently unhelpful for comparison (following cycle/insemination cancellation), it was calculated that the number of patients to be enrolled was about

500 women (about 400 women per data analysis), corresponding to 6 month of clinical activity.

Data analysis was performed using Statistics Package for Social Sciences version 18.0 (PASW Statistics 18.0, Chicago, Illinois). Continuous variables were analyzed using the Student's *t*-test to compare two means (normal data distribution). Categorical variables were analyzed using the Chi-square test. A logistic regression model was used to adjust clinical pregnancy and live birth rates for baseline variables found to differ between the study groups or known to be influencing the outcomes (IVF/ICSI, female age, number of retrieved oocytes, total FSH, ET stage). Results are expressed as mean±standard deviation (SD), Odds Ratio (OR) and adjusted OR (aOR) with 95 % confidence intervals (95%CI). A binomial distribution model was used to calculate the 95%CI of proportions. A p value <0.05 was considered statistically significant.

Results

A total of 701 IVF/ICSI cycles were performed during the study period. Two hundred ninety-nine cycles were excluded for the following reasons: thawing cycles (n=175), cancelled cycles (n=35), insemination not performed (n=79) and exclusive use of normal atmospheric tension of O_2 (n=10) leaving 402 cycles for data analysis.

Table 1 gives detailed information on patients' characteristics of the two groups analysed. Baseline characteristics and indications to in vitro fertilization were comparable. Characteristics and outcomes of the IVF-ICSI cycles are summarized in Table 2. Utilization rate and the main embryological variables were similar between the two groups.

In analyses of subgroups, utilization rate was similar between cases cultured in 20 % O_2+5 % O_2 and controls cultured in 5 % O_2 either in patients treated with standard IVF (38 and 37 %, respectively, p=0.78) or with ICSI (37 and 40 %, respectively, p=0.39), in patients undergoing embryo transfer at the cleavage stage (43 and 41 %, respectively, p=0.40) or at the blastocyst stage (32 and 37 %, respectively, p=0.21).

Regarding the secondary outcomes, after including thawing cycles from patients who did not perform fresh embryo or had supernumerary cryopreserved embryos, OR and aOR for cumulative CPR were 0.99 (0.65–1.53) and 0.97 (0.62–1.53), respectively. Odds Ratio and aOR for cumulative LBR was not significantly different between "5 % O_2 Group" and "20 % O_2+5 % O_2 Group": OR and aOR were 1.25 (0.80–1.97) and 1.22 (0.76–1.97), respectively.

A subgroup analysis was performed in order to compare IVF and ICSI in the "20 % O_2+5 % O_2 Group"; in fact, oocytes treated by conventional IVF were exposed to atmospheric oxygen for ~20 h compared to ~3 h of the ICSI-treated oocytes. Utilization rate was 38 % (n=193/505) compared to 37 % (n=234/633) in IVF and ICSI subgroups, respectively (p=0.67). Cumulative CPR was comparable between IVF and ICSI being 31 % (n=30/97) and 29 % (n=34/117), respectively (p=0.77); cumulative LBR was 22 % (n=21/97) and 25 % (n=29/117), respectively (p=0.60). Pregnancies ending with a live birth were 70 % (n=21/30) in IVF group and 85 % (n=29/34) in ICSI group, respectively (p=0.23).

Table 1 Baseline characteristics of women belonging to the two groups

Characteristics	Group 20 % O_2+5 % O_2 n=214	Group 5 % O_2 n=188	p
Age (years)	36.2±4.3	36.3±4.0	0.76
Primary infertility	133 (62 %)	128 (68 %)	0.22
Stimulation protocol			0.28
Long protocol	81 (38 %)	58 (31 %)	
GnRH antagonist	64 (30 %)	64 (34 %)	
Flare up	62 (29 %)	64 (34 %)	
Other	7 (3 %)	2 (1 %)	
Number of previous IVF-ICSI cycle			0.14
0	75 (35 %)	81 (43 %)	
1–2	118 (55 %)	94 (50 %)	
3 or more	21 (10 %)	13 (7 %)	
Indication			0.42
Unexplained / reduced ovarian reserve	82 (38 %)	80 (43 %)	
Male factor	67 (31 %)	60 (32 %)	
Tubal Factor / endometriosis	46 (20 %)	30 (16 %)	
Anovulatory	4 (2 %)	1 (1 %)	
Mixed	15 (7 %)	17 (9 %)	

Data are reported as mean±standard deviation or number (Percentage)

Discussion

Considering physiological tension of O_2 across the reproductive tract [17], human gametes and embryos should be kept in a reduced oxygen atmosphere from the time of their collection to the end of the culture performing all procedures (i.e., oocyte retrieval, insemination, embryo evaluation…) in a O_2 controlled atmosphere. Unfortunately, this is not applicable in most IVF laboratories and intermediate options have been proposed, such as lowering O_2 tension at least during the time that oocytes/embryos remain inside incubators. However, embryologists are aware that current IVF protocols are far from resembling the physiologic environment of the female reproductive tract.

Many studies have been performed to analyze whether reduced O_2 tension in embryo culture may somehow improve embryological and clinical outcomes. Results obtained are largely inconsistent. Some papers highlighted benefits in both embryo quality and clinical outcome [4,18,19]; others reported an improvement in clinical outcomes even though embryo quality was not affected [3,9,20–22]; lastly, some authors observed an improvements in embryo quality without an increase in overall pregnancy results [23–26].

Our results suggest that in IVF-cultures a similar proportion of viable embryos and good quality embryos can be obtained with a combined use of atmospheric and controlled oxygen or with exclusive use of low O_2 tension. With a 80 % study power, our final sample size allowed to highlight a minimum absolute difference of 6 and 8 % in utilization rate and top quality embryo rates between groups, respectively. The present study is probably the first reporting such a combined strategy of culture conditions therefore we can not compare our results with previous ones. It is worth underlying some of its limitations. First, this is a retrospective study and, although patients were not preferentially allocated to their culture system, we can not exclude the effect of unforeseen biases. Second, available incubators differ for several

Table 2 IVF-ICSI cycle characteristics

	Group 20 % O_2+5 % O_2 n=214	Group 5 % O_2 n=188	p
Number of oocytes	7.6±4.7	7.6±4.3	0.88
Number of suitable oocytes	5.8±3.7	5.7±3.4	0.76
Number of inseminated oocytes	5.3±3.0	5.3±2.8	0.95
Number of zygotes	3.8±2.5	3.9±2.3	0.87
Number of embryos[a]	3.7±2.4	3.6±2.3	0.78
Percentage of top quality embryos[a]	363/788 (46 %)	308/680 (45 %)	0.79
Percentage of cryopreserved embryos	95/788 (12 %)	74/680 (11 %)	0.51
Number of transferred embryos (fresh cycle)			0.21
0	23 (11 %)	10 (5 %)	
1	67 (31 %)	60 (32 %)	
2–3	124 (58 %)	118 (63 %)	
Utilization rate[b]	425/1138 (37 %)	389/1003 (39 %)	0.52
Technique used			0.012
IVF	97 (45 %)	62 (33 %)	
ICSI	117 (55 %)	126 (67 %)	
Cancelled embryo transfer			
OHSS (embryo freezing)	9 (4 %)	2 (1 %)	0.07
No viable embryos	14 (7 %)	8 (4 %)	0.38
Embryo Transfer stage			0.11
Cleavage stage (Day 2–3)	152 (80 %)	153 (86 %)	
Blastocyst stage (Day 5)	39 (20 %)	25 (14 %)	
Cumulative CPR	64/214 (30 %)	56/188 (30 %)	0.98
Cumulative IR	80/345 (23 %)	64/327 (20 %)	0.26
Cumulative LBR	50/214 (23 %)	52/188 (28 %)	0.32
Live births/transferred embryos	65/345 (19 %)	58/327 (18 %)	0.77

[a] with regard to the day of embryo transfer

[b] transferred+cryopreserved embryos / inseminated oocytes

Data are reported as mean±standard deviation or percentage

CPR Clinical pregnancy rate, *IR* Implantation rate, *LBR* Live birth rate

characteristics in addition to O_2 control. In particular our O_2 controlled incubators have a lower internal volume (~50 l) compared to conventional ones (~150 l); however this particular aspect should favour the culture system with exclusive use of 5 % O_2 as smaller incubators have a faster environment recovery ability. Last, due to sample size, our study can not take into account the most important outcome which is the cumulative live birth rate per cycle.

In the organization of an IVF laboratory, according to our findings, available incubators without oxygen control can be used—at least until insemination - in order to allocate culture dishes evenly and to avoid overstocking of low oxygen incubators. This is consistent with the evidence that cumulus cells can protect oocytes from micro-environment modification or sub-optimal culture conditions. However, given current evidences [5,27], we do believe that incubators without oxygen control should be replaced if possible; if not, they can be probably used until cumulus cells removal without a relevant reduction in both quality and quantity of produced embryos.

We also reported clinical pregnancy, implantation rate and live birth rates even though the study was not designed for this aim. While clinical pregnancy and implantation rates were similar between groups, a tendency for a higher delivery rate with exclusive use of low oxygen was observed. Therefore it can be speculated that the use 20 % O_2 after retrieval has the potential to reduce LBR and, consistently with the time of incubation in atmospheric O_2, this aspect is emphasised by the fact that in the "20 % O_2+5 % O_2 Group" a smaller - but not significant - percentage of pregnancies ending with a live birth was observed in IVF compared to ICSI. However, our sample size does not allow to analyse this aspect deeply and we believe that it warrants further investigation.

It has been provided evidence that brief co-incubation (1–3 h) of sperm and oocytes may improve the ongoing pregnancy and clinical pregnancy rates for infertile women undergoing IVF cycles [28]; this evidence, together with our findings, suggests the possibility of moving inseminated cumulus-oocyte complexes to the reduced O_2 at that point.

The present work supports the hypothesis that a culture system with oxygen at atmospheric concentration in the very first phase of oocyte culture can perform similarly to low oxygen tension, both in cleavage stage and blastocysts transfer programs. We hope that our findings can stimulate further research in IVF to shed light on the effects of oxygen tension through specific phases of in vitro culture of human gametes and embryos.

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