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Effect of oxygen concentration on human in-vitro fertilization and embryo culture*

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In this prospective randomized study on 1380 consecutive in-vitro fertilization (IVF) treatments, the results were compared of culture of human oocytes and embryos for the first 2 or 3 days of development in microdroplets of medium under oil using a gas phase containing either atmospheric (~20%) or reduced (5%) O_2 concentrations. No significant differences were found between the two groups cultured under either 5% or 20% O_2 in rates of fertilization (60 versus 61%, respectively), embryo development at day 2 or 3, pregnancy (26.6 versus 25.4%, respectively), and implantation (13.4 versus 14.0%, respectively). Culture of surplus embryos under 5% O_2 resulted in a significantly higher mean incidence of blastocyst formation per cycle as compared to the 20% O_2 group (25.8 ± 2.0 versus 20.4 ± 1.9, respectively). The mean number of cells of embryos classified as blastocysts by microscopic observation of a blastocoel was significantly higher in the 5% O_2 group as compared to the 20% O_2 group, both in blastocysts fixed on day 5 (39.8 ± 1.7 versus 31.9 ± 1.9, respectively), as well as those fixed on day 6 (45.6 ± 2.6 versus 33.7 ± 3.4, respectively). This difference was due to the fact that significantly more blastocysts of the 20% O_2 group had an abnormal low cell number of < 25 as compared to the 5% O_2 group, both in blastocysts fixed on day 5 (39 versus 22%, respectively), as well as those fixed on day 6 (43 versus 22%, respectively). To conclude, although culture under 5% O_2 leads to slightly improved preimplantation embryonic viability, this effect is either too marginal to result in higher pregnancy rates, or low O_2 concentrations exert an effect during the later stages of preimplantation development only.

Key words: embryo culture/oxygen/preimplantation development

Introduction

Mammalian embryos can be cultured successfully using a gas phase containing either atmospheric (~20%) or reduced (5%) O_2 concentrations. For human in-vitro fertilization (IVF) treatments, both O_2 concentrations are widely used and similar success rates have been reported. In a previous study, we evaluated the effect of both of these O_2 concentrations on IVF and embryo culture in the human and found no beneficial effect of culturing under 5% O_2 as compared to culturing under atmospheric O_2 concentrations (Dumoulin *et al*., 1995). It has been demonstrated, however, that embryos from several species (mouse, sheep, goat, cattle) show improved development when cultured under reduced O_2 tension (Bavister, 1995).

The use of small, static droplets of medium covered with an oil overlay has been speculated to result in marginally hypoxic culture conditions at lower O_2 concentrations (Byatt-Smith *et al*., 1991). Furthermore, it has been shown in several animal studies that different culture systems may require different O_2 concentrations for optimal results (Fukui *et al*., 1991; Voelkel and Hu, 1992; Ali *et al*., 1993). Therefore, the results from our previous study, in which human embryos were cultured in 1 ml of medium without an oil overlay, may not be applicable to a culture system of droplets of medium under oil. In the present study, we compared the results of human IVF and embryo culture under both of these gas phases, using 20 µl droplets under oil.

Materials and methods

Patients

During a period of $3\frac{1}{2}$ years, 1380 consecutive IVF and intracytoplasmic sperm injection (ICSI) treatment cycles were included in this study. Oocytes and embryos were alternately allocated per set of two treatment cycles to fertilization and culture either under ambient (~20%) or reduced (5%) O_2. During the last $2\frac{1}{2}$ years of the present study, a second study was performed, in which oocytes and embryos were alternately allocated to the use of either of two culture media: either IVF-50™ or human tubal fluid (HTF). By this allocation procedure, a random distribution of treatments over the different culture techniques was ensured and the two studies were independent. The results of this second study will be published separately.

ICSI treatment was performed in cases of male subfertility (621 cycles), and when no fertilization had occurred in two previous IVF cycles (93 cycles). Male subfertility was defined as a progressive motile sperm concentration of $<3\times10^6$/ml in combination with <5% morphologically normal spermatozoa, evaluated using strict criteria (Enginsu *et al*., 1992). For all other indications (666 cycles), a conventional in-vitro insemination procedure with 50 000 motile spermatozoa/ml was used.

The stimulation protocol used has been described previously (Land

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et al., 1996). In summary, the gonadotrophin-releasing hormone (GnRH) agonist nafarelin (Synarel; Searle BV, Maarssen, The Netherlands) was used in combination with human menopausal gonadotrophin (HMG, Pergonal; Serono, Amsterdam, The Netherlands; or Humegon, Organon, Oss, The Netherlands) to stimulate multiple follicular development. Follicle growth was monitored by ultrasound and 5000 IU of human chorionic gonadotrophin (HCG, Pregnyl; Organon) was given as soon as the dominant follicle was judged to be mature (>18 mm), to induce final follicular and oocyte maturation. Ultrasound-guided oocyte retrieval was performed 34–35 h after HCG administration. Insemination or ICSI was performed ~5 h after oocyte retrieval.

Culture procedures

Oocytes and embryos were cultured in 50 and 20 µl droplets, respectively, under mineral oil (Sigma, cat. no. M-8410). HTF medium was 'in-house' prepared according to Quinn *et al*. (1985), and supplemented with 8% (v/v) of a pasteurized human plasma protein solution (PPS) obtained from the Central Laboratory of the Blood Transfusion Service (Amsterdam, The Netherlands) as described by Huisman *et al*. (1992). IVF-50™ is a ready-to-use commercially available medium (Scandinavian IVF Science AB, Göteborg, Sweden). The incubator used was a Napco double-chamber 7300 model with separate gas controlling systems for both chambers (Boom BV, Meppel, The Netherlands). In one chamber, an atmosphere of 5% CO_2 in air (~20% O2) was used, while in the other chamber a gas mixture of 5% CO_2, 5% O_2 and 90% N_2 was used. The CO_2 and O_2 concentrations of both incubator chambers were regularly checked using a Servomex-570A Oxygen Analyzer (Servomex, Zoetermeer, The Netherlands) and a Normocap 200 CO_2-Analyzer (Datex Medical Electronics, Hoevelaken, The Netherlands), and, if necessary, the incubator chambers were recalibrated. After incubation for 18–20 h, the oocytes were checked for the presence of pronuclei as proof of fertilization, washed once and, after transfer to fresh medium, cultured for another day. At the second and third day after oocyte recovery, the developmental stage and morphological aspect of all embryos were assessed under an inverted microscope at ×200 magnification according to the criteria of Bolton *et al*. (1989). For each embryo, an embryo score was calculated by multiplying the morphological grade by the number of blastomeres (Steer *et al*., 1992). For each treatment cycle, the score of all embryos was averaged to obtain a mean embryo score (MES) (Steer *et al*., 1992). Embryo transfer was routinely performed on day 2 after ovum retrieval, or, in a minority of the cases, on day 3 for reasons of convenience to avoid transfers on Sundays. If available, two or three embryos, depending on the developmental stage and morphological appearance of the embryos, as well as on the age of the patient, were transferred. After transfer, any supernumerary embryos were cultured until the third day after ovum retrieval. Cryopreservation of supernumerary embryos was performed on the morning of the third day after insemination if one or more embryos had reached the 8-cell stage, and if they were of good morphological quality (grades 3 and 4; Bolton *et al*., 1989).

Culture of human surplus embryos

If cryopreservation was deemed unfeasible, surplus embryos were used in one of the studies running in our centre. These studies have been approved by the local Ethics Committee. To avoid selection bias, surplus embryos of all treatment cycles during prearranged periods were used in only one study. In the present study, surplus embryos were left in their original culture medium for another 2 or 3 days. Developmental stages were recorded at each day of in-vitro development. On the morning of day 5 after ovum retrieval, surplus embryos that cavitated to form blastocyst-like structures (defined as a rim of cells surrounding a large cavity of extracellular fluid accumulated within the embryo) were fixed and stained with 4′,6-diamidino-2-phenylindole (DAPI) as described earlier (Coonen *et al*., 1994), provided that the patients had given consent. The number of nuclei stained with DAPI was taken as the number of cells of the embryo. All other embryos, including those that had only just started to form a small blastocoelic cavity, were cultured for another day and were subsequently fixed on day 6 when they had developed to the full blastocyst stage.

Table I. Characteristics of the two study groups cultured under either 5% or 20% O_2

Gas phase	5% O_2	20% O_2
Number of cycles	690	690
Number of patients	400	402
Age (years)[a]	33.0 ± 0.1	33.0 ± 0.1
Indication[b]		
Tubal infertility	101 (25)	84 (21)
Idiopathic infertility	89 (22)	96 (24)
Male subfertility	193 (48)	197 (49)
Other	17 (4)	25 (6)
First attempts[c]	352 (51)	338 (49)
Duration of infertility[a]	5.4 ± 0.1	5.5 ± 0.1
Type of infertility[b]		
Primary	272 (68)	285 (71)
Secondary	128 (32)	117 (29)
Standard IVF[c]	324 (47)	342 (50)
ICSI	366 (53)	348 (50)
Culture medium[c]		
IVF-50™	262 (38)	260 (38)
HTF	428 (62)	430 (62)
Day of embryo transfer[d]		
Day 2	519 (80)	513 (81)
Day 3	130 (20)	121 (19)

[a]Mean ± SEM with regard to the female partner at the time of ovum retrieval.
[b]Values in parentheses are percentages of total number of patients.
[c]Values in parentheses are percentages of total number of treatment cycles.
[d]Values in parentheses are percentages of total number of embryo transfers.

Analysis of results

Data were analysed by χ^2-test or unpaired Student's *t*-test, where appropriate.

Results

The patient's characteristics and other factors are summarized in Table I. No significant differences for any of the parameters studied were found between the two groups.

In Tables II and III, the results in terms of fertilization, embryo development, implantation rates and pregnancy rates of the culture under either 5% or 20% O_2 are summarized. None of the investigated parameters was significantly different between the two groups.

In 501 of the 1380 cycles, at least one surplus embryo was cultured to day 5 or 6. In the other 889 cycles, either no fertilization took place, available embryos were all transferred, surplus embryos were cryopreserved, or they were used in another study. In Table IV, development of surplus embryos to the blastocyst stage is summarized. The mean incidence of blastocyst formation per cycle in which surplus embryos were

Table II. Summary of results of the culture under either 5% or 20% O_2: fertilization and embryo development at day 2 and day 3 of in-vitro development

Gas phase	5% O_2	20% O_2[a]
Cycles	690	690
No. of oocytes per cycle[b]	10.0 ± 0.2	9.6 ± 0.2
Fertilization rate per cycle[b,c]	0.60 ± 0.01	0.61 ± 0.01
Embryonic development at day 2		
Cleavage to ⩾4-cell stage[b,d]	49 ± 2	45 ± 2
Morphologically normal embryos[b,e]	56 ± 1	56 ± 2
Embryo score per cycle (MES)[b]	8.3 ± 0.2	8.1 ± 0.1
Embryonic development at day 3[f]		
Cleavage to ⩾6-cell stage[b,g]	42 ± 3	36 ± 3
Morphologically normal embryos[b,h]	52 ± 3	56 ± 4
Embryo score per cycle (MES)[b]	15.4 ± 0.6	14.7 ± 0.6

[a]No significant differences were noted between the two groups.
[b]Results are expressed as means ± SEM.
[c]Percentage of number fertilized oocytes/total number inseminated oocytes per cycle.
[d]Percentage of embryos developed to the 4-cell stage or more at 39–42 h after insemination or injection per cycle.
[e]Percentage of embryos of good morphological quality (grades 3 and 4; Bolton *et al*., 1989) at 39–42 h after insemination or injection per cycle.
[f]Only cycles of which the embryo transfer was performed on day 3 (i.e. all embryos were cultured until day 3) were taken into account.
[g]Percentage of embryos developed to the 6-cell stage or more at 63–66 h after insemination or injection per cycle.
[h]Percentage of embryos of good morphological quality (grades 3 and 4; Bolton *et al*., 1989) at 63–66 h after insemination or injection per cycle.

Table III. Summary of results of the culture under either 5% or 20% O_2: pregnancy and implantation rates

Gas phase	5% O_2	20% O_2[a]
Cycles	690	690
Embryo transfers	649 (94.1)	634 (91.9)
Pregnancies (%)[b,c]	184 (26.7)	175 (25.4)
Ongoing (⩾12 weeks) pregnancies (%)[b,d]	155 (22.5)	147 (21.3)
Multiple pregnancies (%)[d,e]	41 (26.5)	50 (34.0)
Mean no. of embryos transferred per cycle (± SEM)	2.62 ± 0.04	2.55 ± 0.04
Total no. of transferred embryos	1762	1757
Implantation sites (%)[d,f]	236 (13.4)	246 (14.0)
Viable fetuses (⩾12 weeks) (%)[d,f]	196 (11.1)	204 (11.6)

[a]No significant differences were noted between the two groups.
[b]Values in parentheses are percentages of total number of treatment cycles.
[c]Positive urinary pregnancy test (sensitivity 50 IU/l HCG) at 16–18 days after ovum retrieval.
[d]As determined by ultrasound at 5 and ⩾12 weeks after ovum retrieval, respectively.
[e]Values in parentheses are percentages of total number of ongoing pregnancies.
[f]Values in parentheses are percentages of total number of embryos replaced.

cultured was significantly higher in the group cultured under 5% O_2. Of the embryos that developed a blastocoel, 236 of 310 (76%) embryos cultured under 5% O_2, and 157 of 231 (68%) embryos cultured under 20% O_2 were fixed. Reasons for not fixing were either that no consent was obtained from the patients, or embryos that were classified as early blastocysts on day 5 and subsequently cultured for another day, were found to be degenerated on day 6. The mean number of cells per blastocyst (as determined by the observation of a clear blastocoel under the microscope) was significantly higher in the group cultured under 5% O_2, both in blastocysts fixed on day 5, as well as those fixed on day 6. This difference was for the largest part due to the fact that significantly more blastocysts of the 20% O_2 group consisted of <25 cells.

Table IV. Development to the blastocyst stage of surplus embryos cultured under either 5% or 20% O_2

Gas phase	5% O_2	20% O_2
Cycles with at least one surplus embryo cultured	247	254
Total no. of surplus embryos cultured	1034	1006
Total no. of blastocysts observed on day 5 or 6[a]	310 (30.0)	231 (23.0)[h]
Incidence of blastocyst formation per cycle[b]	25.8 ± 2.0	20.4 ± 1.9[i]
Fixation of blastocysts on day 5		
Blastocysts fixed	112	103
Fixation successful[c]	103 (92)	89 (86)
Cells per blastocyst[d]	39.8 ± 1.7	31.9 ± 1.9[j]
Blastocysts consisting of at least 25 cells[e]	80 (78)	54 (61)[g]
Cells per confirmed blastocyst[f]	46.2 ± 1.6	42.2 ± 2.1
Fixation of blastocysts on day 6		
Blastocysts fixed	124	54
Fixation successful[c]	117 (94)	44 (81)
Cells per embryo[d]	45.6 ± 2.6	33.7 ± 3.4[j]
Blastocysts consisting of at least 25 cells[e]	91 (78)	25 (57)[g]
Cells per confirmed blastocyst[f]	54.9. ± 2.6	49.1 ± 4.6

[a]Values in parentheses are percentages of total number of surplus embryos cultured.
[b]Expressed as mean percentage ± SEM of number of embryos developing to the blastocyst stage on day 5 or 6/total number of surplus embryos cultured per cycle.
[c]Values in parentheses are percentages of total number of blastocysts fixed.
[d]Expressed as mean number ± SEM of cells per successfully fixed embryo.
[e]Values in parentheses are percentages of successfully fixed blastocysts.
[f]Only blastocysts with a minimum of 25 cells are taken into account.
[g,h,i,j]Significant difference when compared with culture under 5% O_2: χ^2-test ([g]$P < 0.05$; [h]$P < 0.01$), or unpaired Student's *t*-test ([i]$P < 0.05$; [j]$P < 0.001$).

Discussion

This prospective randomized study on 1380 consecutive IVF treatments shows that culture of human oocytes and embryos for the first 2 or 3 days of development under a gas phase of either 5% CO_2/95% air (20% O_2) or 5% CO_2/90% N_2/5% O_2 does not result in significant differences between the two groups, neither in pregnancy rates, nor in implantation rates. Rates of fertilization and cleavage of the embryos also did not differ significantly between the two groups. These results are comparable to those reported in an earlier study in which oocytes and embryos were cultured in 1 ml medium instead of in microdroplets under an oil overlay (Dumoulin *et al*., 1995).

It has been speculated that the use of small medium droplets under an oil phase could result in O_2 levels in the medium close to detrimentally low levels, especially when large numbers of embryos or other cells (e.g. cumulus cells) are cultured together (Baltz and Biggers, 1991; Byatt-Smith *et al*., 1991). From these studies employing mathematical models it was calculated that human embryos possibly could become marginally hypoxic at lower O_2 concentrations (Byatt-Smith *et al*., 1991). In our

study, however, no evidence was found of any adverse effect when embryos were cultured under oil at 5% O_2.

In the present study we show that culture under 5% O_2 results in significantly more human surplus embryos reaching the blastocyst stage, as well as a higher proportion of blastocysts consisting of a normal number of cells. In a study from Hardy *et al*. (1989) it was shown that the minimum number of cells in normally fertilized human blastocysts on day 5 of development was ~25 cells. The mean number of cells per blastocyst in their study was 58 on day 5, which is somewhat higher than in the present study (42–46 cells, Table IV). A possible reason for this discrepancy is the fact that all surplus embryos used in our study were of poor morphological quality. Another reason could be the fact that the culture media used in the present study are not as suitable as the recently developed sequential media to support the development of viable blastocysts *in vitro* (Gardner *et al*., 1998).

The use of a low O_2 concentration has been shown to be beneficial for embryo development *in vitro* to the blastocyst stage in various animal species, e.g. mouse (Quinn and Harlow, 1978; Pabon *et al*., 1989; Umaoka *et al*., 1992; Gardner and Lane, 1996); hamster (McKiernan and Bavister, 1990); rabbit (Li and Foote, 1993); rat (Kishi *et al*., 1991); pig (Berthelot and Terqui, 1996); sheep (Thompson *et al*., 1990); cow (Fukui *et al*., 1991; Liu and Foote, 1995), and goat (Batt *et al*., 1991). Also in the human, indirect evidence exists that a low O_2 tension of 5% appears to enhance the blastulation rate of surplus embryos (Noda *et al*., 1994). However, other studies have failed to demonstrate the beneficial effect of low O_2 on the development to the blastocyst stage in the mouse (Nasr-Esfahani *et al*., 1992; Ali *et al*., 1993), cat (Johnston *et al*., 1991), and sheep (Betterbed and Wright, 1985). Also it appears that different culture systems may require different O_2 concentrations for optimal results. When cow or sheep embryos are co-cultured with other cells, 20% O_2 is found to give better results as compared to 5% O_2 in several studies (Fukui *et al*., 1991; Voelkel and Hu, 1992; Watson *et al*., 1994).

Several other lines of evidence would suggest that low O_2 concentrations are more suitable culture conditions for preimplantation embryos. Embryos develop *in vivo* under low O_2 levels, as in the oviduct and uterus of various mammalian species O_2 concentrations have been reported to be ~11–60 mmHg, which corresponds to ~1.5–9% O_2 (Fischer and Bavister, 1993). Furthermore, a reduced O_2 concentration of 5% has been shown to result in a slightly decreased formation of reactive oxygen species in mouse embryos as compared to 20% O_2 (Goto *et al*., 1993). Reactive oxygen species have been implicated in the retardation of early embryo development *in vitro* (Johnson and Nasr-Esfahani, 1994; Tarín, 1996).

The majority of studies thus indicate that culture under low O_2 is beneficial for complete preimplantation embryonic development *in vitro*. However, little is known about the effect of a lower O_2 concentration during the first 2 or 3 days of in-vitro development on postimplantation embryonic development and pregnancy rates. The present study shows that, although culture under 5% O_2 indeed leads to slightly improved preimplantation embryonic viability in the human, this effect is either too marginal to result in higher pregnancy rates, or low O_2 concentrations exert an effect during the later stages of preimplantation development only.

To conclude, in a culture system of microdroplets of medium under oil, no beneficial effect on fertilization, embryonic development and pregnancy rates of culturing human oocytes and embryos for 2 or 3 days under 5% O_2 as compared to atmospheric O_2 concentrations was found. Only when embryos were cultured *in vitro* during the total preimplantation period, was a slight but significant improvement of development to the blastocyst stage found when 5% O_2 was used.

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