

Article

Influence of atmospheric versus reduced oxygen concentration on development of human blastocysts *in vitro*: a prospective study on sibling oocytes



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Borut Kovačič started his career in 1988 when the IVF Laboratory of the Department of Reproductive Medicine and Gynecologic Endocrinology at Maribor Teaching Hospital was opened. Today he is the embryologist in charge. Under the supervision of Professor Veljko Vlaisavljevič he has been involved in several projects on male infertility treatment, natural cycles in human IVF and blastocyst culture and cryopreservation. In 1999, Borut Kovačič was awarded a PhD by the University of Ljubljana, Slovenia; his thesis covered the molecular aspect of fertilization failures after ICSI.

Abstract

Numerous studies show the beneficial effect of reduced oxygen on the culture of animal embryos *in vitro*. However, few similar studies have been carried out in humans, and the conclusions from these were contradictory. Using sibling human oocytes, a prospective study was carried out to analyse the effect of 5 and 20% oxygen on prolonged development of embryos. The outcomes measured were fertilization rate and proportion of morphologically optimal embryos, blastocysts and optimal blastocysts developing on day 5. The results were analysed separately for the group of IVF ($n = 988$ oocytes) and ICSI ($n = 928$ oocytes) cycles. It was found that low oxygen did not influence fertilization, but in comparison with 20% oxygen, it resulted in a significantly higher proportion of embryos being optimal on day 3 after IVF (59 versus 43.2%; $P < 0.001$) as well as after ICSI cycles (51.2 versus 28.5%; $P < 0.001$). In both methods, the lower oxygen concentration improved the blastulation rate (73.2 versus 63.1%; $P < 0.05$ and 67.4 versus 54.7%; $P < 0.001$) and increased the proportion of embryos reaching the stage of expanded blastocyst with normal inner cell mass on day 5 (31.1 versus 14.6%; $P < 0.001$ and 18.9 versus 11.4%; $P < 0.01$). The ratio of successful embryo development to optimal blastocyst stage on day 5 of culture, calculated for two oxygen concentrations, was 2.1 for IVF and 1.7 for ICSI, in favour of lower oxygen tension.

Keywords: blastocyst, embryo development, oxygen concentration, randomized study, sibling oocytes

Introduction

Embryonic metabolism promotes a balance between the useful and harmful effect of oxygen. Oxygen is consumed in oxidative phosphorylation, and the consumption rate depends on substrates that are available, on the oxygen concentration and on the integrity of mitochondrial enzyme complexes that produce and channel high-energy electrons. However, non-integrity of mitochondrial membrane complexes can cause the transfer of electrons to other molecules where reactive oxygen species (ROS) are generated, such as the highly reducing superoxide anion radical (O_2^-), hydrogen peroxide

(H_2O_2) and highly toxic oxidizing hydroxyl radical (OH^\bullet) (Trounce, 2000; Guerin *et al.*, 2001), causing various degrees of damage (Shigenaga *et al.*, 1994).

Despite some endogenous protective mechanisms in the oocytes and embryos, which involve the activity of superoxide dismutases, catalases and peroxidases (El Mouatassim *et al.*, 1999), the oxygen free radicals mostly affect mitochondrial DNA, proteins and lipids as well as the cytoplasm, where they disturb the ratio of glutathione to glutathione disulphide

(Orrenius *et al.*, 1992) and aggregate cytoskeleton components and endoplasmic reticulum condensates (Tarin, 1996). Oxidative stress also induces DNA fragmentation. All of these injuries can cause embryonic fragmentation (Noda *et al.*, 1994; Yang *et al.*, 1998), apoptosis, retarded or arrested development (Johnson and Nasr-Esfahani, 1994), or they may later influence the vitality of pregnancy (Catt and Henman, 2000).

High oxygen tension in the gas atmosphere generates more oxygen free radicals in culture systems (Goto *et al.*, 1993). Although direct measurements of oxygen tension in the oviduct and uterus of different species showed that it varies between 2 and 8% (Fischer and Bavister, 1993), most IVF laboratories use atmospheric oxygen concentration (ca. 20%) and 5–6% CO₂ in air as the standard atmosphere for embryo culture.

Several studies have shown a beneficial effect of reduced oxygen concentration on embryo development in mice (Pabon *et al.*, 1989; Umaoka *et al.*, 1992), hamsters (McKiernan and Bavister, 1990), rats (Kishi *et al.*, 1991), rabbits (Li and Foote, 1993), cows (Fukui *et al.*, 1991), sheep (Thompson *et al.*, 1990), goats (Batt *et al.*, 1991) and pigs (Kitagawa *et al.*, 2004). On the other hand, some studies show that different oxygen tensions have no effect on cat and mouse embryos *in vitro* (Johnston *et al.*, 1991; Nasr-Esfahani *et al.*, 1992; Ali *et al.*, 1993).

There are surprisingly few studies assessing the effect of oxygen tension on human embryo development *in vitro*, and nearly all of these analysed day 2 and day 3 embryos only (Dumoulin *et al.*, 1995; Catt and Henman, 2000; Bahçeci *et al.*, 2005). Prolonged culture of embryos under various oxygen concentrations was investigated using only surplus non-transferable and non-freezable embryos (Dumoulin *et al.*, 1999). Due to the contradictory conclusions from existing studies so far on human embryos, it is not clear whether the use of reduced oxygen tension in routine human IVF is reasonable. The aim of the present study was to clarify this issue.

The primary end-point of this prospective non-blind trial on sibling oocytes, randomized by alternation, was to answer whether a lower oxygen tension had any effect on blastulation of embryos. Some additional analyses were also done in order to assess the influence of different oxygen tensions on fertilization rate, embryo morphology and cleavage rate. The results were analysed separately for IVF and ICSI cycles.

Materials and methods

Study design

The trial was constructed considering the revised CONSORT statement for reporting randomized trials (Altman *et al.*, 2001).

This prospective randomized non-blind study was carried out using sibling oocytes from routine consecutive stimulated IVF and ICSI cycles in the IVF laboratory of the University Clinical Centre, Maribor.

Ovarian stimulation

The women were synchronized with oral contraceptives and then started the long stimulation protocol with triptorelin (Decapeptyl 0.1 mg) or goserelin (Zoladex 3.6 mg; Zeneca, Cheshire, UK). Pure FSH (Metrodin-HP; Serono, Geneva, Switzerland) or recombinant FSH (Gonal F; Serono) was administered for ovarian stimulation. Ovulation was induced in all women by self-administration of human chorionic gonadotrophin (HCG) (Profasi; Serono, Auborne, Switzerland) in doses of 10,000 IU subcutaneously. Follicular puncture followed 36–37 h later. The cumulus–oocyte complexes (COC) were collected in Flushing medium (Medicult), and prepared for insemination either by IVF or ICSI, depending on the indications. The oocytes were enrolled in the study after the number of mature oocytes was checked.

Eligibility criteria and randomization method

In the IVF group, all COC from hormonally stimulated cycles in which more than five expanded COC were obtained and which were proposed for day 5 transfer were included. The oocytes were excluded if: (i) the COC were not expanded and immature oocytes were observed; (ii) the cumulus cells were luteinized; (iii) the patient decided on short-term culture; and (iv) the woman's age was over 40.

In the ICSI group, all metaphase II (MII) oocytes from the ICSI cycles having more than five MII oocytes that had undergone a normal ICSI procedure, were included. The following were not included: (i) in-vitro matured oocytes; (ii) atretic or vacuolated oocytes; (iii) oocytes from cycles foreseen for short-term embryo culture; and (iv) oocytes from women aged over 40.

The criteria for COC and MII oocyte enrolment into the study were the same as the criteria used routinely to evaluate oocyte acceptance for clinical use. In the IVF group, the expansion of COC and checked oocyte maturity were assessed by observing the cumulus in a flattened drop of medium. COC with clearly visible immature oocytes and luteinized cumulus were excluded. Other COC were randomly allocated to two culture dishes.

Expanded COC from the IVF group ($n = 785$ oocytes) were randomized by alternation (similarly expanded cumulus: one here, one there) and allocated for culture either at low ($n = 388$ oocytes) or atmospheric ($n = 397$ oocytes) oxygen tension for the next 5 days. Immediately after the oocytes were allocated, the COC were inseminated with 150,000–200,000 motile spermatozoa.

In ICSI cycles, the oocytes were first denuded and inseminated by ICSI procedure, so that injected MII oocytes ($n = 924$ oocytes) were randomly allocated to low ($n = 462$ oocytes) or to atmospheric oxygen tension ($n = 462$ oocytes). The sibling oocytes were always injected by the same embryologist.

Oocyte and embryo culture

Oocytes and embryos were cultured in drops of BlastAssist medium (Medicult, Jyllinge, Denmark) under equilibrated mineral oil in four-well dishes. The oocytes and embryos were incubated in the same type of incubators (CB 150; Binder, Tuttlingen, Germany) at 95% relative humidity, either at standard gas atmosphere of 6% CO₂ in air (approx. 20% O₂) or at 5% O₂, 6% CO₂ and 89% N₂. The gas concentrations were measured each day before the incubator doors were opened, using an independent gas meter.

Assessment of embryo morphology

Since particular interest was focused on embryo morphology, all embryos were evaluated for cell number and percentage of fragmentation by three experienced embryologists; non-significant interobserver variations in morphology assessment had been established in previous tests.

The embryologists assessing the embryos were not blinded to group assignment. Embryos were observed every 24–25 h at a magnification of $\times 400$ and the pictures were projected on a big screen.

The criteria for assessing an embryo as morphologically optimal are as follows: (i) day 2 optimal embryos had more than two blastomeres and less than 20% of fragments; (ii) day 3 optimal embryos were those with more than six blastomeres and less than 20% of fragments; and (iii) day 4 optimal embryos were compact morulae.

Blastocysts were evaluated twice on day 5, first in the morning and again 3 h later, using a previously described scoring system (Kovačič *et al.*, 2004), based on implantation capability decreasing from score B1 toward B8. According to this scoring system, the optimal day 5 embryo was a blastocyst with an expanded blastocoele, a cohesive trophectoderm, oval and compact inner cell mass, and no excluded blastomeres or fragments from the formation of the blastocyst.

The clinical results (pregnancy and implantation rates) from a sibling oocyte study were not the main outcome measures because the cycles were not randomized, and the groups are therefore not comparable. Nevertheless, they are summarized to show the trends.

Statistical methods

The experimental layout involved a total of 160 patients, 85 of whom received IVF treatment and the remaining 75 received ICSI. The oocytes from each patient were divided numerically into two groups and these groups were randomly allocated to one of the two oxygen treatments (5 and 20%). The design therefore corresponded to a split-plot arrangement with type of assisted reproduction (IVF, ICSI) as the main-plot treatments and the oxygen content (5, 20%) as the sub-plot treatments.

As many of the variables of interest were proportions, logistic regression analysis was used, but although the variables were, therefore, analysed on the logistic scale, all results will

be presented on the original scale of proportions. Although this type of hierarchical structure, with an unequal degree of replication, results in a rather awkward analysis for logistic transforms, the algorithm GENSTAT was able to cope with, and carry out the appropriate calculations.

Standard analysis of variance was also employed, and individual comparisons of proportions were carried out using Fisher's exact test.

The difference was considered statistically significant if P was ≤ 0.05 .

Results

The study material was collected during a period of 6 months. The oocytes for the study were recruited from among 1081 COC from 92 patients in the IVF group and from among 1070 MII oocytes from 94 patients in the ICSI group.

The flow of enrolled, excluded and included oocytes as well as allocated oocytes, oocytes not completing the study and those that were analysed is presented in the flow diagram (Figure 1).

The criteria for exclusion were not very rigorous, since in routine IVF/ICSI programmes as many COC and oocytes as possible are inseminated or injected. Twenty-eight oocytes with clearly visible immature COC and 13 atretic MII oocytes with large vacuoles, dark cytoplasm, fragments in the subzonal space were excluded, as were those having irregular shape. The study also excluded 40 oocytes from cycles that were planned for IVF, but in which the patients chose to share the insemination between IVF and ICSI. In nine ICSI cycles ($n = 65$ oocytes excluded), short-term embryo culture was arranged due to previous blastulation failure in extended embryo culture.

Finally, 1013 COC from 88 patients in the IVF group and 992 MII oocytes from 85 ICSI patients were allocated for culture at 5 and 20% oxygen respectively.

After allocation, 25 COC and 68 MII oocytes were excluded, since the patients later decided on day 3 embryo transfer.

The main analysis included 988 COC from 85 patients of the IVF group and 924 MII oocytes from 75 patients of the ICSI group.

The women from IVF cycles were aged 31.6 (24–39). Their mean number of previous IVF attempts was 0.8 (0–6). The indications for IVF were: male factor in 3.5%, female factor in 74%, combined factor in 9.6% and unknown factor in 13%. After ovarian stimulation with a mean number of 25.3 gonadotrophin ampoules and oocyte retrieval, a mean number of 11.6 ± 4.8 cumulus–oocyte complexes was obtained.

The mean age of women in ICSI cycles was 32.6 (20–43), mean number of previous cycles was 1.2 (0–13) and the indications for ICSI were: male infertility in 44%, female infertility in 8%, combined factor in 46.7% and unknown infertility in 1.3%.

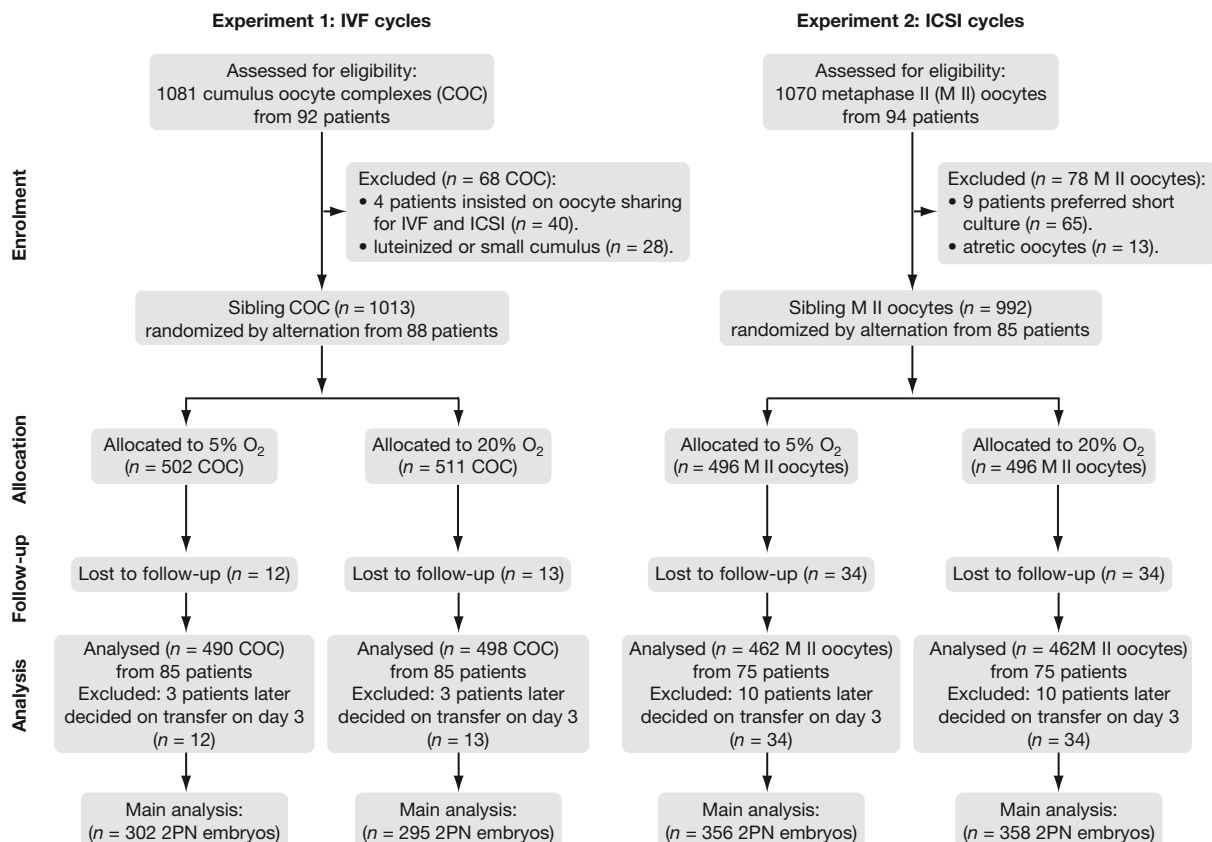


Figure 1. Flow diagrams of the trials (experiment 1: IVF cycles, experiment 2: ICSI cycles), comparing the effect of low and atmospheric oxygen tension on the development of embryos through the entire preimplantation period.

Insemination of COC by conventional IVF resulted in the fertilization of 62.4% (306/490) of COC and development of 302 embryos in low oxygen, and 60.2% fertilization (300/498) and 295 embryos developed in atmospheric oxygen respectively.

The percentages of fertilized MII oocytes after ICSI were 77.5% (356 embryos) in the low and 77.7% (358 embryos) in the high oxygen group.

The summary of primary and secondary outcome results and the comparison between the groups are presented in **Table 1** and in **Table 2**.

There was no difference in the mean proportion of fertilized oocytes between the two oxygen concentrations in IVF and ICSI cycles. However, the low oxygen treatment was generally superior to the 20% oxygen treatment in the sense of better embryo development in the entire preimplantation period from day 2 to day 5, in IVF as well as in ICSI cycles. In both methods

(IVF and ICSI), the lower oxygen tension much improved the blastulation rate (73.2 versus 63.1%; $P < 0.05$ and 67.4 versus 54.7%; $P < 0.001$) and increased the proportion of embryos reaching the stage of expanded blastocyst with normal inner cell mass on day 5 (31.1 versus 14.6%; $P < 0.001$ and 18.9 versus 11.4%; $P < 0.01$).

The ratio of successful embryo development to the optimal blastocyst on day 5 of culture, calculated for the two oxygen concentrations, was 2.1 for IVF and 1.7 for ICSI, in favour of low oxygen.

The pregnancy and implantation rates from the transfer of IVF and ICSI blastocysts from 5% oxygen only, from 20% oxygen only, and from both oxygen levels are presented in **Table 3**.

The P -value for pooled comparison of pregnancies for oxygen levels is fairly close (0.082) to the 5% value, in the sense that the lower level gives the higher pregnancy rate (60 versus 40%).

Table 1. Estimated mean proportions (and the corresponding standard errors) of optimal embryos developed in IVF and ICSI cycles at different oxygen tensions.

	IVF			ICSI		
	5% O ₂	20% O ₂	Success ratio	5% O ₂	20% O ₂	Success ratio
Normal fertilization	0.622 ± 0.013	0.605 ± 0.019	—	0.773 ± 0.018	0.779 ± 0.018	—
Day 2 optimal embryos	0.701 ± 0.027	0.570 ± 0.029**	1.2	0.688 ± 0.027	0.519 ± 0.028***	1.3
Day 3 optimal embryos	0.590 ± 0.024	0.432 ± 0.025***	1.4	0.512 ± 0.027	0.285 ± 0.025***	1.8
Day 4 compact morulae	0.377 ± 0.021	0.360 ± 0.021	—	0.386 ± 0.023	0.272 ± 0.022***	1.4
Day 5 blastocysts/morulae	0.732 ± 0.027	0.631 ± 0.029*	1.2	0.674 ± 0.025	0.547 ± 0.027***	1.2
Day 5 optimal blastocysts	0.311 ± 0.020	0.146 ± 0.017***	2.1	0.189 ± 0.018	0.114 ± 0.015**	1.7
Mean blastocyst score ^a	3.38 ± 0.19	4.42 ± 0.20***	—	4.04 ± 0.23	4.19 ± 0.21	—
Most frequent blastocyst score (mode) ^a	1	3	—	1	3	—

The definitions of optimal embryos and blastocysts are explained in the text.

^aBlastocyst scoring system by Kovačič *et al.* (2004), based on implantation capability decreasing from score B1 toward B8.

The variables analysed by logistic regression appear as row headings. The indicators of statistical significance relate to the two oxygen levels: **P* < 0.05;

P* < 0.01; *P* < 0.001.

Table 2. Summarized results for the oxygen treatments over the two assisted reproduction methods (IVF and ICSI). The values are the mean proportions (and the standard errors) of optimal embryos per each day of culturing *in vitro*.

	Oxygen treatment		Success ratio
	5% O ₂	20% O ₂	
Normal fertilization	0.710 ± 0.015	0.703 ± 0.015	—
Day 2 optimal embryos	0.694 ± 0.019	0.542 ± 0.020***	1.3
Day 3 optimal embryos	0.548 ± 0.018	0.352 ± 0.018***	1.6
Day 4 compact morulae	0.382 ± 0.016	0.312 ± 0.015**	1.2
Day 5 blastocysts/morulae	0.701 ± 0.018	0.585 ± 0.020***	1.2
Day 5 optimal blastocysts	0.244 ± 0.014	0.129 ± 0.011***	1.9

The definitions of optimal embryos and blastocysts are explained in the text.

The variables analysed by logistic regression appear as row headings. The indicators of statistical significance relate to the two oxygen levels.

P* < 0.05; *P* < 0.01; ****P* < 0.001.

Table 3. Clinical results of IVF and ICSI cycles after transfer of blastocysts chosen only from low oxygen or only from high oxygen, and transfer of blastocysts chosen from low and high oxygen.

	Transfers of blastocysts		
	Only from 5% O ₂	Only from 20% O ₂	Mixed
IVF cycles (<i>n</i> = 85)			
No. of transfers ^c	38	16	20
Transferred blastocysts	58	23	40
Mean blastocysts per transfer	1.5 ± 0.5	1.4 ± 0.5	2 ± 0
Ongoing pregnancies	26 (68.4)	6 (37.5)	16 (80)
Implantations	34 (58.6)	8 (34.8)	19 (47.5)
ICSI cycles (<i>n</i> = 75)			
No. of transfers ^c	32	14	23
Transferred blastocysts	45	24	46
Mean blastocysts per transfer	1.4 ± 0.5	1.7 ± 0.5	2 ± 0
Ongoing pregnancies	16 (50)	6 (42.9)	14 (60.9)
Implantations	18 (40)	10 (37.5)	18 (39.1)
Overall			
Pregnancy rate	42/70 (60) ^a	12/30 (40) ^a	30/43 (69.8)
Implantation rate	52/103 (50.5) ^b	18/47 (38.3) ^b	37/86 (43)

Values in parentheses are percentages.

^aFisher's exact test (*P* = 0.082).

^bFisher's exact test (*P* = 0.217).

^cThere are 11 IVF cycles and six ICSI cycles without transfer due to: total fertilization failure six, all embryos arrested 7, prevention of ovarian hyperstimulation syndrome four.

Discussion

Due to better possibilities for blastocyst selection for embryo transfer, prolonged embryo culture *in vitro* is an assisted reproduction method increasingly applied in IVF programmes. Nevertheless, its use constantly gives rise to scruples about whether the embryos in 5-day culture *in vitro* have a sufficient supply of all the necessary substrates. Some studies argue that in-vivo-developed blastocysts of some primates are morphologically different, that they have a smaller blastocoele and a larger ICM as well as a larger number of cells compared with those cultivated *in vitro* (Bavister, 2004). With constant improvements in culture media, both the morphology of preimplantation embryos and the blastulation rate are also slowly improving. The present study proves that embryonic development to the blastocyst stage can also be significantly improved by modifying the atmosphere in which the embryos are cultivated for 5 days. A blastulation rate exceeding 67% can thus be reached.

Decreasing the oxygen concentration did not improve the fertilization rate in the study. Instead, the lower oxygen tension resulted in the development of a larger number of morphologically optimal embryos in IVF and ICSI cycles. In contrast to all studies carried out so far, the beneficial effect of low oxygen was already evident on day 2, since a higher mean proportion of morphologically good four-cell embryos developed at low rather than at high oxygen tension. This trend persisted over the next 3 days. At lower oxygen concentrations,

a higher mean proportion of fast-cleaving and non-fragmented embryos was observed on day 3. Over 50% of all embryos were morphologically normal, with 7 or more blastomeres. Such an improvement in the morphology of early embryos is extremely important with respect to a possible improvement in clinical results. In the study by Check *et al.* (2007), day 3 embryos with six to eight blastomeres had up to six times greater chance of implantation than embryos with fewer cells.

In four previous similar studies on the effect of different oxygen tensions on early day 3 human embryos, some authors reached the same and others opposite conclusions. Neither Dumoulin *et al.* (1995) nor Catt and Henman (2000) observed any beneficial effect of lower oxygen concentrations on early embryos. However, Bahceci *et al.* (2005) and Kea *et al.* (2007) reported a higher mean embryo score on day 3 using 5% oxygen in comparison with atmospheric oxygen concentration.

Only one study deals with the effect of oxygen on prolonged cultivation of human embryos to the blastocyst stage. Dumoulin *et al.* (1999) published a study similar to the present one, but they used extended culture to day 5 only for morphologically poor embryos that were unsuitable for cryopreservation and not clinically used. Their report was the first to mention a better blastulation rate (30 versus 23%). However, the authors did not assess the effect of oxygen tension on blastocyst morphology and ICM. Noda *et al.*, who used 5% oxygen for prolonged cultivation of embryos in a non-comparative study, reported a high blastulation rate of 58.5% (Noda *et al.*, 1994).

Using the lower oxygen concentration, the blastulation rate in this study improved by around 10% in IVF as well as in ICSI cycles, reaching 67.4% in ICSI and 73.2% in IVF cycles.

It is also important to note an improvement in blastocyst quality at 5% oxygen in IVF and ICSI cycles. At low oxygen concentrations, as many as 24.4% of all embryos developed to optimal type B1 on day 5, with expanded blastocoele, cohesive trophoctoderm, oval ICM and no excluded blastomeres from blastocyst formation.

In IVF cycles, the proportion of optimal blastocysts on day 5 (31.1%) exceeded the number of arrested embryos. Better blastocyst quality at low oxygen was also noted in ICSI cycles. This was not detected in comparing the mean blastocyst scores between two oxygen concentration groups, but was evident with the significantly higher proportion of optimal blastocysts in the low than in the high oxygen group. This type of blastocyst (B1) is also the type most frequently found in the low oxygen group, while B3 type (compact morula or early blastocyst) prevailed in the atmospheric oxygen group. The time difference in the development from early to expanded blastocyst amounts to only a few hours, while compact morulae require an additional day of culture to develop an expanded blastocoele. It is known that the implantation potential of morulae or early blastocysts that are transferred to the uterus on day 5 is lower in comparison with expanded blastocysts, resulting in births at approximately half the rate of optimal expanded blastocysts (Kovačič *et al.*, 2004). If these embryos were cultured to day 6, a smaller ICM and poorer clinical results could be expected (Shapiro *et al.*, 2001).

Lowering the oxygen concentration evidently promotes embryonic development in a larger number of embryos. This finding corresponds to the observations of Dumoulin *et al.*, who report that the blastocysts cultured at low oxygen had more cells than those cultured at 20% oxygen (Dumoulin *et al.*, 1999). The higher cleavage rate of embryos at low oxygen had a further advantage, in that the expansion allowed the ICM formation to be evaluated on day 5 in a number of embryos. In comparison with other morphologic characteristics of the blastocyst, ICM quality is most closely related to the probability of a live birth (Kovačič *et al.*, 2004).

Other authors who carried out studies on animal embryos also observed that more blastocysts with normal ICM develop at low than at atmospheric oxygen. They found that the blastocyst trophoctoderm was less sensitive to atmospheric oxygen than the ICM (Enders *et al.*, 1989; Karagenc *et al.*, 2004). At 20% oxygen, they observed an ICM with disorganized, vacuolated and apoptotic cells more often, and the ICM cells were fewer in number. Molecular studies that demonstrate oxygen-regulated genes to be important for metabolism in the ICM attempted to answer the question of why atmospheric oxygen impairs ICM (Pantaleon and Kaye, 1998; Kind *et al.*, 2005).

As regards developmental dynamics, the embryos from IVF as well as from ICSI cycles profited from the lowering of the oxygen concentration. In the IVF procedure there is a greater risk of ROS derived from spermatozoa and their debris jeopardizing the culture conditions, and these have an unfavourable effect on the oocytes during coincubation with spermatozoa (Quinn *et al.*, 1998). Therefore, the lowered oxygen concentration was

expected to improve embryo morphology mainly in IVF cycles. However, the lowering of oxygen concentration also improved embryo morphology after the ICSI procedure.

In conventional IVF, the presence of cumulus oophorus on day 1 of culture also contributes to the improvement of conditions, as it has a potential antioxidant activity and additionally helps to neutralize the ROS (Bedaiwy *et al.*, 2004). The embryos from ICSI, however, do not have such support and are, therefore, more sensitive to ROS than IVF embryos (Bedaiwy *et al.*, 2004; Ebner *et al.*, 2006). Thus, the minimization of the ROS effect in ICSI cycles probably depends more strongly on the atmospheric concentration of oxygen.

Better embryo morphology and higher blastulation rate attained by lowering oxygen concentration does not necessarily mean better clinical results, even though such a trend is indicated in **Table 3**. It is not possible to compare the clinical results in a sibling oocyte study. As the morphology of blastocysts that developed under 5% oxygen was better, and they were larger in number as compared with blastocysts from 20% oxygen, the embryos for transfer were more often chosen from the first group. Therefore, the comparison presented in **Table 3** is inconclusive. Nevertheless, the *P*-value for the pooled comparison for oxygen levels is fairly close to the 5% value, in the sense that the lower level gives the higher pregnancy rates.

In the final comparison of the effect of 5 and 20% oxygen, it can be concluded that the use of 5% oxygen in the incubator atmosphere contributed to faster development of human embryos *in vitro*, resulting in a larger number of good quality and clinically applicable embryos, in short-term as well as in prolonged culture. In the latter, the process of embryo cavitation was initiated earlier. The more frequent expansion of the blastocoele at 5% oxygen made it easier to evaluate the morphology of the ICM. When choosing the embryos for transfer to the uterus, it was more usual to select those embryos that were cultured at low oxygen.

As the regular evaluation of embryo morphology under the microscope and the frequent opening of the incubator result in fluctuating oxygen concentrations, perhaps a system should be developed that will maintain a constant atmosphere in the best possible way.

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