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Beneficial effect of reduced oxygen concentration with transfer of blastocysts in IVF patients older than 40 years old

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ABSTRACT

The aim of the present study was to determine the impact of oxygen concentration on implantation, pregnancy and delivery rates in IVF patients older than 40 year old with transfer of blastocysts. Included were 558 women aged 23-45 years old undergoing IVF/ICSI procedures whose embryos were cultured at blastocyst stage under two different oxygen environments (a bi-gas system: 5.6% CO_2 in air and a tri-gas system: 5.6% CO_2, 5% de O_2 and 89.4% N_2). The main outcome measures of this study are implantation, pregnancy and delivery rates. Implantation, pregnancy and delivery rates are found to be reduced in women older than 40 years old. The implantation and pregnancy rates are significantly higher in women older than 40 years old from the 5% of O_2 group, in comparison to the 20% group (25.00% versus 2.70% and 41.38% versus 5.56%; P < 0.05). The deliveries rates were 13.79% and 5.56% in the 5% and 20% oxygen groups respectively (P: NS). The birthweight was similar in both study groups (P: NS). Gestational age was significantly longer in women from the 5% of O_2 group, in comparison to the 20% (36.87 versus 35.87 weeks, P < 0.05). Results indicated that the embryonic culture with 5% of oxygen and transfer of blastocysts in women older than 40 years old improve the results in the *in Vitro* fertilization/intracytoplasmic injection procedures (IVF/ICSI).

Keywords: ART; Blastocyst; IVF; ICSI; Oxygen

1. INTRODUCTION

Embryos from several mammal species, including human, were exposed in vivo to low oxygen concentrations, ranging from 2 to 8% observed in atmospheric air [1-3]. This probably corresponds to an adaptation mechanism, as it is proven that higher oxygen concentrations may be harmful to the embryo [4] by generating reactive oxygen species (ROS) [5-7].

In Vitro fertilization studies (IVF) in mice [8,9]; cattle [5]; sheep [10]; rabbits [11]; hamsters [12]; rats [13]; cows [14] and pigs [15] have demonstrated that when cultured in oxygen concentrations of 5% present a higher viability and a better development to the blastocyst stage.

However, a pioneer study in human embryos showed that cultures *in Vitro* in atmospheric concentration (20%) or reduced (5%) resulted in similar fecundation and preimplantational embryo development processes [16]. Therefore, in several laboratories of assisted reproduction, the culture of human embryos using oxygen concentration of 20% [17] is now a common practice.

Furthermore, a study in which the effect of oxygen over the 2nd and 3rd day of human embryo development was evaluated was unable to find any differences in the pregnancy and implantation rates when 5% or 20% of O_2 was used [17]. This absence of differences in the results obtained might be because the beneficial effect of low O_2 concentrations happen during the late stages of preimplantational embryo development (day 4-6) [18]; however, the addition of antioxidants to the culture media had as a result better rates of implantation and pregnancy when embryos cultured in 5% of O_2 are transferred in the 2nd and 3rd day [5].

The importance of the transfer in blastocyst stage and the concentration of oxygen have been recently recognized [19,20] These studies reported significant increases in the pregnancy and implantation rates when transfers were done in blastocyst stage and when the cultures were made with 5% of O_2 compared to the culture effect in conditions of 20% of O_2.

Physiologically, the uterus provides a nutritional environment different from than in the fallopian tubes. Therefore, embryo transfer in the cleavage stage would

cause homeostatic stress of the embryo and a reduction in its implantatory potential [21]. Consequently, the transfer in blastocyst stage would allow a better synchronization with rhythm of uterine contractions and the embryo [22,23].

However, there are contradictory results in studies where the human embryos cultured in reduced (5%) or atmospheric oxygen concentrations (20%) are compared. Thus, no improvements in terms of pregnancy and implantation rates were observed if the transfer was performed in the 3rd day of the development in terms of pregnancy and implantation rates [17,20,24]. Similar results have been observed if transfer was done into blastocyst stage (day 5) [24-27].

The maternal age is an important factor to be taken into account in studies on. In fact, there has been a decrease in the women's fertility from 35 years old [28,29], being this reduction significant from the 40 year olds and over, in women attending a processes of assisted reproduction [30,31].

The Latin American Registry of Assisted Reproduction (REDLARA) reported in 2006 a clinical pregnancy rate of 39.6% in patients ≤ 34 years old, 32.8% in patients from 35 to 39 years old and 18.6% in women ≥ 40 years old respectively [32]. In older women there is commonly a reduction in the ovarian follicular reserve and a greater prevalence of chromosomal alterations in the oocyte, which lead to a significant reduction in the implantation rates [33,34] and high rates of miscarriages [35,36].

In the studies comparing the effect of different oxygen concentrations in the embryo cultures, it has not been taken into consideration the maternal age impact when blastocysts are transferred in the programs of assisted reproduction [19,25,27,37].

In a recent publication Kovačič *et al*. [26] did not find improvements in the implantation rates in older women over 40 years of age whose embryos were cultured with oxygen at 5% as compared cultures at 20% of O_2 and embryo transfer in the 3rd day. It is possible that effects of low oxygen concentration may be observed if embryos are transferred in the 5th or 6th days.

We hypothesize that reducing the percentage of oxygen to 5% in the embryo culture systems would have a much more beneficial effect than the usage of oxygen at 20%.

The objective of this study was to evaluate in an IVF/ICSI program, the relationship between the pregnancy and the implantation rates with maternal age whose embryos were cultured in 5% of O_2, compared to those women whose embryos were cultured at 20% of O_2. In addition, the results of pregnancies were assessed.

2. MATERIALS AND METHODS

2.1. Patients

This is a retrospective non randomized study based on secondary analysis of data obtained from 558 cycles of IVF and ICSI at the Laboratories of Assisted Reproduction of Pranor Group (Lima, Peru) between January 2007 and June 2009. This study was approved by the Institutional Review Board (IRB) at the Concebir Clinic (Lima, Peru).

The study group were those gametes and embryos cultured at 37˚C in an atmosphere of 5.6% CO_2, 5% of O_2 and 89.4% N_2 (341 cycles); and a control group of those gametes and embryos cultured at 37˚C and an atmosphere of 5.6% CO_2 in air (20% O_2) (217 cycles). The same kind of incubators (Thermo Scientific, USA) was used for the bi-gas and tri-gas systems.

2.2. Ovarian Stimulation and Oocyte Collection

The patients were submitted to a controlled ovarian stimulation with Leuprolide Acetate (Lupron®, Abbott Laboratories) or Ganirelix (Orgalutran®, Organon) in combination with Recombinant FSH (Puregon®, Organon Laboratories) or HMG (Humegon®, Organon Laboratories) according to the established protocols. The follicular growth was monitored by ultrasound and the ovulation was induced by applying Human Chorionic Gonadotropin (hCG) (Ovidrel® 250 ug, Serono Laboratories or Pregnyl® 10,000 UI, Organon Laboratories). The follicular aspiration was made 34 to 36 hours after giving the hCG. The insemination or ICSI procedure was made-5 hours after the oocyte recovery.

2.3. Semen Samples

The semen samples were obtained by masturbation of every patient's male in aseptic conditions. After the liquefaction process, the motile spermatozoa were recovered from the seminal plasma by centrifugation through Isolate gradients of 45% and 95% (Irvine Scientific, USA) for 10 minutes at 300 × g. the recovered spermatozoa were washed in Sperm Washing Media (Irvine Scientific, USA). In oligospermic samples the spermatozoa were washed in Sperm Washing Media and then placed in 10 µL drops of HTF-Hepes + 10% SSS for the ICSI.

2.4. Fertilization and Embryo Culture

In each one of the evaluated groups, the embryo culture media and mineral oil were prepared and used according to the specifications of the company. The CO_2 concentration in the incubators was of 5.6% and resulting pH

was approximately 7.30 in all the culture media.

The aspired oocytes were washed in a HTF-Hepes medium (IVFonline, Guelph, ON, Canada) supplemented with 10% vol/vol of SSS (Irvine Scientific, USA) and cultured in a 200 µL drop of HTF medium + 10% SSS under mineral oil at 37˚C for 5 hours before the insemination or ICSI procedure.

The insemination was made with 50,000-100,000 motile spermatozoa in 200 µL drop of HTF medium + 10% SSS, where from 1 to 5 oocytes were placed. In the cases of ICSI, the oocytes in metaphase II were injected in every patient by using methods previously described (38). After the insemination or ICSI in 0 day, all the oocytes were cultured up to the evaluation of the fertilization at 37˚C.

The fertilization was evaluated 16-18 hours post insemination or ICSI by the presence of two pronuclei and two polar bodies (day 1). The zygotes with two pronuclei were cultured individually, under mineral oil, in 10 µL drops of Global medium (IVFonline, Guelph, ON, Canada) supplemented with 10% vol/vol of SSS (Irvine Scientific, USA) from day 1 to day 3. On the 3rd day, the embryos were changed to 10 µL drops of fresh Global medium + 10% SSS and cultured 2 or 3 days more up to the transfer day in blastocyst stage. Therefore, the transfer was made in 5 or 6 days.

2.5. Embryo Transfer

The embryos were transferred in blastocyst stage, being the average of 1.96 and 2 embryos transferred in the group of 5% and 20% of O_2 respectively ($P < 0.05$ among the evaluated groups). In the 5% of O_2 group, 16 patients received 1 embryo, 324 received 2 embryos and 1 patient received 3 embryos. In the 20% of O_2 group, 4 patients received 1 embryo, 209 patients received 2 embryos and 4 patients received 3 embryos (**Table 1**).

The embryos that were not transferred were cryopreserved or eliminated according to their morphology. The embryo transfer was made with a Frydman Ultrasoft catheter (CCD Laboratoire, Paris, France) that was previously washed with a culture medium. The catheter was completely filled with culture medium and the embryos filled in the last 10 µL of the catheter medium. All the transfers were made according to the methods previously described by Mansour [39].

The biochemical pregnancy was determined approximately 12 to 14 days after the embryo transfer by measuring the Human Chorionic Gonadotropin beta subunit (hCG-b) in blood. The clinical pregnancy was determined by the presence of the gestational sac and the heart beat which were evaluated by ultrasound at the 21st and 28th days post transfer respectively.

2.6. Statistical Analysis

Data were statistically analyzed using the χ^2 test and Student's t-test as appropriate and differences were considered to be significant at $P < 0.05$. All statistical analysis was carried out using the statistic package Stata 10 (StataCorp, College Station, TX).

In this study, the cycles were organized in 3 segments according to the age of the patient: < 35 years old, 35-39 years old and ≥ 40 years old. The normal fertilization rate was calculated from the number of zygotes with two pronuclei of IVF and ICSI divided by the number of mature oocytes inseminated by 100. The rate of implantation was calculated dividing the number of gestational sacs observed by ultrasound at the 21st day post transfer divided by the total number of embryos transferred by 100. The rate of clinic pregnancy was calculated from the number of patients with at least one gestational sac divided by the total embryo transfers by 100. The abortion rate was defined as the number of pregnancies with total loss of the gestational sacs before the 20 weeks of gestation between the numbers of pregnancies by 100.

3. RESULTS

A total of 558 cycles in which the embryos were cultured under two different O_2 environments were evaluated; embryos of 341 and 217 cycles were cultured in 5% and 20% of O_2 respectively. The age of the patients was similar in both evaluated groups (P: NS). The fertilization rate was similar in the 5% of O_2 group versus the 20% in each age group evaluated in this study (**Table 2**).

Table 1. Characteristics of the two study groups whose embryos were cultured in 5% or 20% of O_2.

	5% O_2	**20% O_2**
Cycles	341	217
Age (y)		
Range	25-45	23-45
Mean ± SE	34.47 ± 0.20	34.33 ± 0.26
Indication[a]		
Tubal Factor	37 (11)	16 (7)
Other female	161 (47)	95 (44)
Male Factor	48 (14)	29 (13)
Multiple Factor	88 (26)	72 (33)
Unexplained	7 (2)	5 (3)
Procedure Class [a]		
Standard IVF	194 (57)	96 (44)
ICSI	147 (43)	121 (56)

Data are Mean ± Standard Error; [a]Values in parentheses are percentages of the total number of patients; P: NS.

Table 2. Implantation rate, pregnancy rate, abortion rate and birth rate by age group in cycles with embryos cultured in atmosphere of 5% and 20% of oxygen.

	< 35 years		35-39 years		≥ 40 years	
	5% O_2	**20% O_2**	**5% O_2**	**20% O_2**	**5% O_2**	**20% O_2**
Cycles	169	108	143	91	29	18
Fertilization rate (%)	83.03	79.78	84.04	78.16	83.52	81.60
Transferred embryos	1.98 ± 0.01	1.99 ± 0.02	1.93 ± 0.02[b]	2.00 ± 0.02	1.93 ± 0.05[a]	2.06 ± 0.01
Implantation rate (%)	34.63	35.81	26.09	26.92	25.00[a]	2.70
Clinical Pregnancy rate (%)	50.89	51.85	42.66	42.86	41.38[a]	5.56
Abortion rate (%)	5.92	7.41	7.69	10.99	17.24[c]	00.00
Birth per transfer rate (%)	42.59	44.44	32.12	31.87	13.79	5.56[d]
Ongoing pregnancy	7	0	6	0	3	0

Data are Mean ± Standard Error; [a]P < 0.05 compared to the average in patients ≥ 40 years old from the 20% O_2 group; [b]P < 0.05 compared to the average in patients of 35-39 years old from the 20% O_2 group; [c]P < 0.05 compared to the average in patients of < 35 years old from the 5% O_2 group; [d]P < 0.05 compared to the average in patients of < 35 years old from the 5% and 20% O_2 group.

The patients ≥ 40 years old from 5% group of O_2 had implantation and pregnancy rates significantly higher compared to those patients from 20% of O_2 group (P < 0.05). Furthermore, these patients older than 40 years old from the 5% of O_2 group received a significantly less number of embryos transferred, compared to the patients from 20% group (P < 0.05). The group of patients < 35 years old and of 35-39 years old had similar implantation and pregnancy rates (P: NS).

Women ≥ 40 years old from 5% of O_2 group had a higher abortion rate compared to women < 35 years old from the same study group (P < 0.05). However, in the group of patients whose embryos were cultured in 20% of O_2, the older women (≥ 40 years old) had a lower delivery rates in comparison to women < 35 years old in both evaluated groups (5% and 20% of O_2) (P < 0.05). In women ≥ 40 years old from 20% of O_2 group there was only 1 pregnancy out of 18 transfers, which resulted in a healthy born baby. In the 5% of O_2 group there were 12 pregnancies from which 5 women had an abortion before the 20th week of gestation, 4 had a normal delivery and 3 pregnancies have a normal development. The delivery rate was similar in women older than 40 years of age in the 5% of O_2 group compared to the 20% of O_2 group (13.79% vs. 5.56%; P > 0.05).

The pregnancy rates according to the kind of procedure of IVF or ICSI were similar among both procedures in the evaluated groups (5% and 20% of O_2) in women < 35 years old, 35-39 years old and older than 40 years old. Less pregnancies were achieved in women ≥ 40 years old when their embryos were cultured in a 20% of O_2 atmosphere, independently from the kind of procedure of IVF or ICSI, in comparison to the group of women < 35 years old and of 35-39 years old (P < 0.05) (data not shown).

The percentage of embryos that reached the blastocyst stage in relation to the total number of fertilized oocytes is show in **Table 3**. There were no differences in the embryonic blastulation rate among patients from the 5% and 20% of O_2 group; these percentages were equally similar in relation to the age of the patients. Furthermore, there was no difference in the pregnancy rates according to the kind of controlled ovarian stimulation in both evaluated groups in this study (**Table 4**).

The data about the gestational age at delivery and birth weight from both groups evaluated in this study is shown in **Table 5**. There were 87 deliveries in the 5% of O_2 group and 78 deliveries in the 20% of O_2 group. However, it was only possible to register information from 39 and 60 in each group respectively. Gestational age was higher in women whose embryos were cultured in reduced concentrations of oxygen, in comparison to those women whose embryos were cultured in atmospheric concentrations of oxygen (P < 0.05) (**Table 5**).

Table 3. Blastulation rate according to age in both study groups.

	5% O_2	**20% O_2**	**P**
Cycles	341	217	
No. of embryos (2PN)	2281	1612	
< 35 years old	39.82%	37.59%	0.487
35-39 years old	36.11%	39.69%	0.348
≥ 40 years old	34.01%	31.37%	0.756
Total	37.97%	37.97%	1.000

P: NS

Table 4. Pregnancy rate according to the protocol of ovarian stimulation with agonist or antagonist from the GnRH (GnRHa –GnRHant) and the recombinant FSH (rFSH) or human menopausal gonadotropin (HMG) in the study groups.

Stimulation Protocol	5% O_2	20% O_2
GnRHa + rFSH	60.00%	36.84%
GnRHa + HMG	50.00%	40.00%
GnRHa + rFSH + HMG	45.45%	53.33%
GnRHant + rFSH	50.43%	41.67%
GnRHant + HMG	55.00%	50.00%
GnRHant + rFSH + HMG	48.21%	60.87%
rFSH	27.27%	43.75%
HMG	57.14%	40.00%
rFSH + HMG	35.71%	38.89%

P: NS

Table 5. Gestational age and birth weight in the study groups.

	5% O_2	20% O_2	P
Biochemical pregnancy	5	4	
Clinical pregnancy	159	96	
Total delivery	87	78	
Deliveries with recorded data	39	60	< 0.05
Gestational age (weeks) (Mean ± SE)[a]	36.87 ± 0.30	35.87 ± 0.37	
Bithweight of newborns (gr.) (Mean ± SE)[a]	2816.26 ± 92.88	2752.96 ± 69.05	NS

[a]Data are Mean ± Standard Error

4. DISCUSSION

Although human embryos can develop successfully in atmospheric concentrations of oxygen (20%), some authors have suggested that low oxygen concentrations (5%) resemble the physiological conditions of the uterus effectively, and thereby improve the quality, viability and embryo morphology [40,41].

An important result of this study was to find that culturing embryos at reduced concentrations of O_2 (5%) is beneficial to patients ≥ 40 years old who perform assisted reproductive procedures with their own oocyte; these are the ones who achieve significantly higher implantation and pregnancy rates compared to those patients of similar age whose embryos were cultured under atmospheric oxygen concentrations (20%) (25.00% versus 2.70%; 41.38% versus 5.56%, respectively, P < 0.05). The implantation and pregnancy rates observed in women > 40 years old were similar to those seen in women < 35 years old and 35-39 years old (P:NS).

Meintjes *et al*. (20) embryos cultured in a 5% O_2 environment consistently resulted in higher rates of live birth implantation and live births when compared with rates among women whose embryos were cultured in an atmospheric O_2 environment.

Furthermore, Nanassy *et al*. [27] cultured human embryos under oxygen atmospheric conditions (20%) until the 3rd day and then cultured the embryos in 5% and 20% of O_2 from the 3rd to the 5th day of development without finding beneficial effects of 5% of O_2 in the advanced stages of preimplantational embryo development. These results suggest that the beneficial effect of hypoxia on embryonic development would be along all stages of *in vitro* cultures even from the oocyte before fertilization up to the blastocyst stage [42], which had previously been observed in mice [8,43] cattle [44], rabbits [45] and pigs [46].

Culturing embryos in 20% of O_2, Karagenc [7] showed damage mainly in the embryonic inner cell mass (ICM). Similarly, Rhesus monkey embryo cultured *in vitro* in 20% of O_2 showed the ICM morphologically disorganized, diffuse, with few vacuolated cells, unlike the blastocysts with large and compact ICM cultured in low concentrations of O_2 [47].

Rho *et al*. [48] culturing bovine embryos has shown that low concentrations of oxygen produce higher rates of cleavage and blastocyst stages compared to embryos cultured in 20% of O_2. Also in mice there is a better development to blastocyst stage, bigger number and size of ICM, and gene expression profile similar to those observed in embryo *in vivo* [49].

The discrepancies between the data obtained in animals and humans could be explained based on the differences in the embryo physiology of each species and a variety of culture conditions and embryo transfer in the laboratory [47]. Beneficial effects of culture in 5% of O_2 have been demonstrated in animals where embryo transfers routinely occur in blastocyst stage [7,50].

Dumoulin *et al*. [18], Pabon *et al*. [8], Quinn and Harlow [43] suggested that the beneficial effect of O_2 in physiological concentrations should be observed in cultures extended to blastocyst, which are nowadays common in assisted reproduction laboratories.

Several studies report increases in pregnancy and implantation in blastocyst transfers compared to embryo transfers on the 3rd day [51] and others report significant increases only in implantation rates [52], which considering the results of this study, would have beneficial effects in those patients over 40 years old.

The culture up to the blastocyst would allow to choose

in a more "natural" way embryos with greater potential for development and implantation, however, this selection would also depend on the O_2 percentage in the systems in which the embryos are cultured [47] and the oocyte origin associated to the patient's age [53,54].

There are many authors who have showed the relationship between chromosomal abnormalities, maternal age and embryo morphology [35,55-59]. Munné *et al*. [54] performed genetic diagnosis for 9 chromosomes [13,15-18,21,22, X, Y) in > 6000 embryos and found that women < 35 years old with good quality embryos had 44% of euploid embryos and that this percentage decreased to 21% in patients ≥ 41 years old. Beside, in patients with poor morphology embryos only 30% and 12% were euploid embryos in the group of women > 35 years old and ≥ 41 years old respectively. It has also been shown that chromosomal abnormalities in human oocytes are common and that these aneuploidies are closely related to maternal age, exceeding 60% in women over 40 years old [60].

Since older women have a higher incidence of oocytes and embryos with aneuploidy [54,60] but with similar rates of embryonic blastulation than young women (≤ 35 years old, 35-39 years old; see **Table 3**) as it has been demonstrated in this study, we should expect similar pregnancy and implantation rates independently from the oxygen concentration under which cultures *in vitro* are performed, but in this study we found that low concentrations of oxygen (5%) are achieved significantly higher pregnancy and implantation rates in patients ≥ 40 years old, compared to the results in conditions of atmospheric concentrations of oxygen.

These results might be caused by high concentrations of oxygen that would affect the embryonic inner cell mass [7], an effect that would be even more noticeable because of the high incidence of aneuploidy observed in oocytes and embryos in older women.

In this study there were 87 births in the 5% of O_2 group and 78 births in the group of 20%, being able to obtain information of their results, in terms of gestational age and birthweight, in 39 and 60 cases respectively.

Within the data obtained, a significantly higher gestational age was observed in the group of patients whose embryos were cultured under 5% O_2 compared to the 20% of O_2 group (36.87 ± 0.30 vs. 35.87 ± 0.37; $P < 0.05$) which could be a consequence of preimplantational embryo development in more physiological concentrations of oxygen to which the embryos were subjected during *in vitro* culture, but we believe that further studies are needed to determinate the possible relationship between the culture in hypoxic conditions and obstetric characteristics of pregnancies.

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