Comparison of 2, 5, and 20 % O₂ on the development of post-thaw human embryos

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Abstract

Purpose The objective of this study is to investigate the effect of 2, 5, and 20 % O₂ on post-thaw day 3 human embryo culture until blastocyst stage.

Methods One hundred fifty-five day 3 human embryos were used. One hundred twenty out of 155 embryos were recovered after thawing. Surviving embryos were distributed into 2, 5, or 20 % O₂ groups and cultured for 2.5 days. At the end of culture, blastocyst formation was assessed, and then, embryos were collected for RT-qPCR or immunofluorescence analysis. **Results** Using visible blastocoel to define blastocyst formation, 58.7 % (27/46) of surviving day 3 embryos formed blastocyst at 2 % O₂, 63.6 % (28/44) at 5 % O₂, and 66.7 % (20/30) at 20 % O₂. The difference in blastocyst formation rates was not significant. Average blastocyst cell number was 119.44 ± 11.64 at 2 % O₂, 142.55 ± 22.47 at 5 % O₂, and 97.29 ± 14.87 at 20 % O₂. Average apoptotic rate was

4.7 % ± 0.4 % for blastocyst formed at 2 % O₂, 3.5 % ± 0.7 % at 5 % O₂, and 5.8 % ± 1.1 % at 20 % O₂. Apoptosis rate was significantly lower for blastocysts formed at 5 % O₂ ($p < 0.05$). Compared with gene expression levels at 5 % O₂, which were arbitrarily set as “1,” 20 % O₂ is associated with significantly higher expression of *BAX* (2.14 ± 0.47), *G6PD* (2.92 ± 1.06), *MnSOD* (2.87 ± 0.88), and *HSP70.1* (8.68 ± 4.19). For all genes tested, no significant differences were found between 2 and 5 % O₂.

Conclusion The result suggests that development of cryopreserved human embryos from day 3 to blastocyst stage benefits from culture at 5 % O₂.

Keywords Human embryo · Oxygen · Stress · PCR · Apoptosis

Capsule The result suggests that development of cryopreserved human embryos from day 3 to blastocyst stage benefits from culture at 5 % O₂.

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Introduction

Optimizing O₂ level for human IVF culture

Compared with 20 % O₂, 5 % O₂ has been reported to improve embryo development for multiple species, reviewed in [1]. For human embryos, studies on the effect of O₂ during cleavage stage development showed controversial results. Some work demonstrated advantage of 5 % O₂ [2, 3], while others reported no difference in clinical outcome between 5 and 20 % O₂ [4–6]. It is suggested that 5 % O₂ is more likely to exert a beneficial effect on post-compaction stage of preimplantation development [6]. When 5 % O₂ was applied from the day of oocyte retrieval to blastocyst stage, improved embryo development and pregnancy outcome were demonstrated in multiple prospective randomized studies [7, 8]. The recent Cochrane review also showed that 5 % O₂ is related to increased live birth rate [9]. Overall, current evidence supports the use of 5 % O₂ in

human embryo development, especially at post-compaction stage or from fertilization throughout blastocyst stage.

The effect of O₂ below 5 % on embryo development in animal models

The advantage of 5 % over 20 % O₂ on animal embryo development has also been well documented [10, 11]. However, studies on the effect of O₂ lower than 5 % in embryo development are scarce, with results being inconclusive and species dependent. O₂ at 2.5–5 % was found to be optimal for in vitro culture of 1–8 cell stage mouse embryos retrieved from pregnant female to blastocyst stage [12]. But when 2 % O₂ was applied only from morula to blastocyst stage in the in vitro culture of mouse embryo, increased embryo resorption and decreased fetal weight were reported at day 18 of pregnancy [13]. For bovine embryos, starting from insemination to morula or blastocyst stage, 5 % O₂ appears to be superior to 2–2.5 % or higher O₂ in advancing blastocyst cell number, though developmental rate to morula and blastocyst stage was similar among different O₂ levels [14, 15]. However, when 2 % O₂ was applied to bovine embryo culture only from day 5 to 7, no difference was found in blastocyst formation rate or total cell number compared with 7 or 20 % O₂ [16], and another study reported 2 % O₂ is optimal for bovine embryo development at this stage [17]. For rabbit embryos, culture at 1 and 5 % O₂ from one cell stage to blastocyst produced similar rate of hatching blastocyst and comparable blastocyst cell number, and both O₂ levels were superior to 20 % O₂ [18]. Thus, the effects of O₂ on embryo development are species specific, and information gained from animal studies does not necessarily inform optimization of O₂ for human IVF. Moreover, O₂ effects are stage specific, and each stage may require a different O₂ optimum. It is possible that the beneficial effects of O₂ at one stage may be confounded by the harmful effects of the same O₂ at another stage, so there is a need to optimize each embryonic stage separately.

Effects of O₂ on embryo development are not always reflected in the rate of morula or blastocyst formation, which can be similar; while at the same time blastocyst quality measures, including cell counts, expression of molecular markers, or in vivo developmental outcomes after implantation were different at various O₂ levels [13–15]. Thus, the effect of O₂ on embryo development cannot be fully appreciated by morphological standards alone. Additional criteria are needed. Re-implantation, post-implantation development, and birth data would be the best standards. But when it comes to human embryos, this is not always practical or possible, especially when the benefit or harm from certain intervention is uncertain. The use of molecular markers, cell growth, and apoptotic rates etc. ex vivo methods is necessary to obtain a better understanding of O₂-dependent effects on embryo development.

Several lines of evidence suggest that O₂ less than 5 % may be more beneficial for human embryo development compared

with 5 % O₂, which are enumerated below. Improvement of IVF has long relied on a “back to nature” approach [19]. Human uterine luminal O₂ level was reported to be ~2 % throughout the menstrual cycle [20], although a later work showed some variation in O₂ level ranging from 0.8 to 5.7 % with an average of 2.5 % [21]. It is likely that human embryos are exposed to O₂ below 5 % near implantation into the uterus and in vitro culture of embryos can be done at O₂ below 5 %, especially at later developmental stage when the embryo has already reached uterus, which approximates the post-compaction stage [22]. Thus, in our study, the comparison on the different effect of O₂ in human embryo development was done at post-compaction stages.

The objective of this study is to investigate the effect of 2, 5, and 20 % O₂ on the development of cryopreserved day 3 embryos after thawing to blastocyst stage. The outcome measures include blastocyst formation rate, total cell number of blastocyst, apoptosis rate, and messenger RNA (mRNA) expression of molecular markers indicating oxidative stress and adaptive response, apoptosis, glucose uptake, and cell connection. Representative mRNA markers in these areas have been commonly used to reflect stresses embryos were subjected to or embryo quality. Examples are mRNAs of apoptosis marker pro-apoptotic bcl-2-like protein 4 (*BAX*) [23], heat shock protein (*HSP*) 70.1 [24, 25] and anti-oxidative pentose phosphate pathway enzyme glucose-6-phosphate dehydrogenase (*G6PD*) [26], and anti-oxidant superoxide dismutase 2/mitochondrial (*MnSOD*) [27]. Glucose transport *GLUT1* is normally required for embryogenesis, and it is often measured when comparing different culture medium composition [25, 28]. Cell connexin protein *CX43* is a gap junction protein, and it has been reported to be higher in in vivo derived embryos [29].

Materials and methods

Embryo source Patients seeking IVF treatment at the First Affiliated Hospital of Sun Yat-Sen University who have healthy live birth donated their day 3 cryopreserved embryos for research. Written informed consent was obtained from all participants before the usage of their embryos. The institutional review board (IRB) of the First Affiliated Hospital of Sun Yat-Sen University approved the study. The informed consent forms and IRB approval are available on request.

Thawing of day 3 embryos One hundred fifty-five day 3 embryos developed at 20 % O₂ were utilized in this study. Slow programmable freezing was used for the cryopreservation of these embryos [30]. The freezing standard for day 3 embryos was cell number ≥ 4 , all blastomere with equal size or the difference in cell diameter < 20 %, and fragmentation < 20 %. Embryos were thawed at room temperature. Straws containing embryos were removed from liquid nitrogen and exposed to air

for 30 s, then in 30 °C water bath for 30 s. Embryos were then put into the following solutions in sequence: 0.5 M sucrose for 10 min, 0.35 M sucrose for 5 min (1:1 mixture of 0.5 M and 0.2 M sucrose), and 0.2 M sucrose for 5 min. After that, embryos were transferred into HEPES solution and assessed for viability before they were put into three different O₂ levels for blastocyst culture. The embryo thawing kit was purchased from SAGE In Vitro Fertilization (ART-8016, CT).

Distribution of embryos Embryos from women of similar age (± 2 years) and causes of infertility (male or female causes) were treated as a pool, and each straw containing cryopreserved embryo in the pool was assigned a number. A random number was generated among the number of straws. Thawing of embryos started from that number and the distribution into 2, 5 and 20 % O₂ group was done by embryologists in a cyclical manner. The first thawed straw of embryos was distributed to 2 %. The straw number immediately following the first straw was distributed 5 % and the next to 20 % O₂, with subsequent allocations following the same order. Both CO₂ incubator and triple gas incubator used were same size incubator from ASTEC co. LTD, Model No: APC-30D and SMA-30DR, Japan. Surviving embryos after thawing were defined as having no less than four intact blastomeres, no more than 50 % fragmentation, and blastomere size (cell diameter) less than 50 % difference [31]. Media used for cleavage embryo culture before cryopreservation, and post-thaw culture were from Vitrolife G-series culture media (Vitrolife, Sweden), G-1 PLUS, and G-2 PLUS, respectively. Surviving embryos were cultured for another 2.5 days without change in media. At the end of blastocyst culture, blastocyst formation and blastocyst grade were recorded. Blastocyst grading system proposed by [32] was adopted to assess blastocyst quality. Blastocyst formation was determined if there was presence of visible blastocoel. Good-quality blastocysts were defined as expanded or further developed blastocysts with inner cell mass at grade A (tightly packed, with many cells) or grade B (loosely grouped, with several cells), and trophectoderm at grade A (many cells forming a cohesive epithelium) or grade B (few cells forming a loose epithelium).

Gene expression analysis The technique for gene expression in a small amount of starting materials has been described [33]. It frequently involves pre-amplification of the starting complementary DNA (cDNA) for subsequent analysis. The efficacy and fidelity of the pre-amplification method we used here has been demonstrated elsewhere [34], and it has been used in mouse embryo study [35]. The accuracy and sensitivity of doing RT-qPCR on crude cell lysate in situations where a limited number of cells are available for RNA purification has been demonstrated by Van Peer et al. [36]. The method reported by Van Peer et al. was adopted here because single blastocyst was used for gene expression analysis.

After observation, blastocyst scores were recorded, and embryos of similar quality in the three O₂ groups were used for gene expression analysis. Eight blastocysts from 2 % O₂, eight from 5 % O₂, and six from 20 % O₂ were used for this purpose. Blastocysts were put into 0.5 % (w/v) pronase (10165921001, Roche) for 3–5 min to remove zona pellucida, washed in PBS, and transferred into 0.5 ml microcentrifuge tubes individually. RNA extraction and gene expression analysis were done according to manufacturer's protocol. Briefly, 25 μ l lysis buffer with DNase I was added to each blastocyst and incubated for 5 min, followed by stop solution for 2 min at room temperature. Twenty microliter lysate was used for each 50 μ l reverse transcription (RT) reaction. 12.5 μ l cDNA from RT was used for pre-amplification PCR (4384267, ABI). Reaction condition for pre-amplification PCR was 95 °C 10 min, then 10 cycles of 95 °C 15 s followed by 60 °C 4 min. Pre-amplification products were diluted five times in 1 $\times$ TE buffer (93302, Sigma) for further use. RT and pre-amplification PCR were carried out by GeneAmp PCR System 9700 (ABI).

Expressions of target genes *BAX*, *GLUT1*, *CX43*, *MnSOD*, *G6PD*, and *HAP70.1* and reference genes *GAPDH*, *YWHAZ*, and *SDHA* were determined by real time PCR in 7500 Real Time PCR System (ABI) using TaqMan Gene Expression Assays 4331182 [Hs00414514_m1/BAX/, Hs00892681_m1/GLUT1/, Hs00167309_m1/MnSOD/, Hs00166169_m1/G6PD/, Hs04187663_g1/HSP70.1/, Hs02786624_g1/GAPDH/, Hs03044281_g1/YWHAZ/, Hs00417200_m1/SDHA/], and 4448892 [Hs04259536_g1/CX43/]. The real time PCR was carried out in a final volume of 20 μ l containing 5 μ l of diluted cDNA. Cycling program was set at initial hold at 95 °C for 10 min, followed by 40 cycles of denaturation at 95 °C for 15 s, annealing and extension at 60 °C for 1 min. Other than the specified reagents, all reagents used were included in Taqman Gene Expression Cells-To-CT Kit (4399002, ABI). Fold changes in gene expression were determined using 2- $\Delta\Delta$ Ct method [37], and all mRNA expression values were normalized to the geometric average of three reference genes.

Immunostaining Nineteen blastocysts from 2 % O₂ (one hatched blastocyst was lost during handling), 20 from 5 % O₂, and 14 from 20 % O₂ were used for immunostaining. They were fixed in 4 % paraformaldehyde (Sojubio, China) for 30 min followed by 0.5 % (v/v) Triton X-100 1 h. The embryos were then washed 3 $\times$ in 0.5 % (w/v) PBS-BSA (Sigma) 10 min. Blocking was done for 45 min with 10 % FBS (Invitrogen). All the previous procedures were carried out at room temperature. 1:50 rabbit monoclonal anti-human active caspase 3 (700182, Molecular Probes) was incubated at 4 °C overnight. After 3 $\times$ 10 min wash in 0.1 % (v/v) PBST, the embryos were incubated with 1:1000 anti-rabbit second antibody (A10040, Molecular Probes) at room temperature for 1 h. After counterstaining with DAPI (Cytocell, UK) for 15 min, the

embryos were micrographed using a fluorescence microscope (Olympus BX61, Japan). Total cell number was counted based on DAPI staining. Apoptotic cells were indicated by active caspase 3 staining [38]. Apoptosis rate was calculated as apoptotic cell number divided by the total cell number.

Statistics

Distribution and frequency data were analyzed using the heterogeneity chi-square test. Gene expression levels were compared by one-way ANOVA followed by Dunnett's post hoc test with the 5 % O₂ group as reference. Data for G6PD was square root transformed to meet the normality assumption for ANOVA. Cell number and apoptosis rates were logarithmic transformed to satisfy normality assumption and analyzed using one-way ANOVA followed by Dunnett's post hoc test. Values are presented as means ± SEM.

Results

One hundred fifty five day 3 embryos donated by 21 couples were included in the study. One hundred twenty embryos survived the thawing procedure, with recovery rate 77.4 %. Forty-six, 44, and 30 of surviving embryos were distributed into 2, 5, and 20 % O₂ group, respectively and cultured for 2.5 days. The reasons for sub-fertility in these couples were sperm dysfunction 38.1 % (8/21), tubal disease 57.1 % (12/21), and unexplained infertility 4.8 % (1/21). Two patients with tubal disease had concomitant polycystic ovarian syndrome (PCOS), and one case was combined with endometriosis. One couple had a combination of male infertility and endometriosis. Mean (± standard deviation [SD]) age for the female donors was 32.62 ± 3.48 years. Age was classified into <30, 30–35, and >35 groups. There was no significant difference in age distribution for the embryos assigned to the three O₂ groups (Table 1). Cell number of the embryos at thaw was also grouped into three categories namely 4 cells, 5–7 cells, and 8 or more cells. There was no significant difference in embryo cell number distribution among the three O₂ groups (Table 2). The rates of blastocyst formation and good-quality blastocysts based on Gardner blastocyst grading system

Table 1 Maternal age distribution of embryos among three O₂ levels

Maternal age (year)	O ₂ levels		
	2 %	5 %	20 %
<30	13.0 % (6/46)	20.5 % (9/44)	16.7 % (5/30)
30–35	65.2 % (30/46)	50.0 % (22/44)	56.7 % (17/30)
>35	21.7 % (10/46)	29.5 % (13/44)	26.7 % (8/30)

Table 2 Cell number distribution of post-thaw human embryos among three O₂ levels

Cell number	O ₂ levels		
	2 %	5 %	20 %
4 cells	10.9 % (5/46)	9.1 % (4/44)	6.7 % (2/30)
5–7 cells	60.9 % (28/46)	63.6 % (28/44)	63.3 % (19/30)
≥8 cells	28.3 % (13/46)	27.3 % (12/44)	30.0 % (9/30)

(“Material and methods”) were not statistically different among three O₂ groups (Table 3).

To test for the O₂ level that best supports embryonic development from day 3 to blastocyst stage, we first assayed the total number of cells and the apoptotic rate of blastocysts after 2.5 days in culture. Average blastocyst cell number was 119.44 ± 11.64 at 2 % O₂, 142.55 ± 22.47 at 5 % O₂, and 97.29 ± 14.87 at 20 % O₂. Average apoptotic rate was 4.7 % ± 0.4 % at 2 %, 3.5 % ± 0.7 % at 5 % O₂, and 5.8 % ± 1.1 % at 20 % O₂. The highest cell number and the lowest apoptotic rate were observed in blastocysts cultured at 5 % O₂ (Fig. 1a, b). There was significantly higher rate of apoptosis at 20 % O₂ ($p < 0.05$, ANOVA). Intermediate in both cell numbers and apoptotic rate was embryos cultured at 2 % O₂. Average cell number was not significantly different between 2 and 5 % O₂, but rate of apoptosis was higher in 2 % O₂ ($p < 0.05$, ANOVA and Dunnett's post hoc test). Representative human embryos cultured at 2 %, and 20 % O₂ were presented in Fig. 2, which showed the assay for apoptosis and cell number by using cleaved caspase 3 and DAPI immunofluorescent staining.

We next measured mRNA markers of apoptosis (*BAX*), oxidative stress and adaptive response (*MnSOD*, *G6PD*, *HSP70.1*), glucose transport (*GLUT1*), and cell connection (*CX43*). Real time qPCR was used to assay embryos at the end of 2.5 day culture. Compared with gene expression levels at 5 % O₂, which are arbitrarily set at “1”, the fold change (mean ± SE) of gene expression levels for *BAX* was 2.14 ± 0.47 at 20 % O₂ and 1.20 ± 0.26 at 2 % O₂; for *GLUT1* was 2.23 ± 1.32 at 20 % O₂ and 1.06 ± 0.35 at 2 % O₂; for *CX43* was 0.86 ± 0.15 at 20 % O₂ and 0.88 ± 0.04 at 2 % O₂; for *MnSOD* was 2.87 ± 0.88 at 20 % O₂ and 1.21 ± 0.31 at 2 % O₂; for *G6PD* was 2.92 ± 1.06 at 20 % O₂ and 1.25 ± 0.24 at

Table 3 Rate of blastocyst development at different O₂ levels

Embryo development	O ₂ levels		
	2 %	5 %	20 %
Blastocyst formation	58.7 % (27/46)	63.6 % (28/44)	66.7 % (20/30)
Good-quality blastocysts	23.9 % (11/46)	22.7 % (10/44)	20.0 % (6/30)

Fig. 1 Blastocyst cell numbers were not significantly different among the three O₂ groups. Apoptosis rate was significantly lower in 5 % O₂ compared with 2 and 20 % O₂. **a** Embryos were cultured from thawed day 3 embryos for another 2.5 days to blastocyst stage. This figure shows average blastocyst cell number in each group. **b** Apoptosis was significantly lower at 5 % O₂ ($p < 0.05$), but no difference was found between 2 and 20 % O₂. Original data was log transformed, and the comparison among three groups was done by one-way ANOVA followed by Dunnett's post hoc test. Distinct letters *a* and *b* on top of the histogram bars indicate a significant difference between the comparison groups ($p < 0.05$). Data is presented with average $\pm$ SEM

2 % O₂, and for *HSP70.1* the fold change was 8.68 ± 4.19 at 20 % O₂, and 1.34 ± 0.33 at 2 % O₂.

Of six markers assayed, four were significantly higher at 20 % O₂ compared with 5 or 2 % O₂, namely *BAX*, *MnSOD*, *G6PD*, and *HSP70.1* ($p < 0.5$, ANOVA followed by Dunnett's post hoc test) (Fig. 3). Two markers, gap junction protein *CX43* and *GLUT1* were not significantly different for embryos cultured at all three O₂ levels, and all markers were not significantly different in embryo cultured at 5 and 2 % O₂.

Discussion

Summary of findings

The study here indicates that culture of cryopreserved human embryos from day 3 to blastocyst stage has highest growth, lowest apoptosis, lowest oxidative, and heat shock stress at 5 % O₂. The opposite happened at 20 % O₂. Our results are in agreement with past studies on the effect of 20 or 5 % O₂ in human IVF [9] and support the current use of 5 % O₂.

Mathematical modeling of oxygen diffusion in static culture suggests that mouse embryos are likely to derive sufficient oxygen by diffusion alone, but human embryos may become marginally hypoxic when cultured at 5 % O₂ [39]. However, this model did not include the effect of convection in facilitating oxygen exchange. Another study by Baltz et al. indicates that in static culture, convection is faster than diffusion and would serve to mix the culture microdrops so that anoxia cannot develop [40]. Convection is driven by temperature difference, which is quite haphazard as it can be influenced by routine procedures such as opening the incubator door and embryo

Fig. 2 Representative blastocysts cultured at 2 or 20 % O₂ with nuclear and apoptosis staining. **a**, **d** Active caspase-3 staining of apoptosis for blastocysts cultured at 2 and 20 % O₂, respectively. **b**, **e** Same embryos as in **a** and **d**, respectively, showing DAPI staining of nucleus. **c**, **f** Merged image of **a** and **b** and **d** and **e**. Micron bar represents 25 μ m

Fig. 3 The mRNA expression level of six marker genes showed no significant difference between 2 and 5 % O₂. The mRNA levels of BAX, MnSOD, G6PD, and HSP70.1 were significantly higher in 20 % O₂ than in 5 % O₂. Data were analyzed using ANOVA followed by

Dunnett's post hoc test. The same pair of letters *a* and *a* indicates no significant difference between the comparison groups. Distinct letters *a* and *b* indicate a significant difference ($p < 0.05$). Data is presented with average $\pm$ SEM

observation etc. That is why it is not included in the modeling by Byatt-Smith et al. [39]. Also mathematical modeling of the balance between oxygen supply and oxygen consumption by cultured embryos is influenced by the estimation embryo oxygen consumption rate, which is not a constant and exhibits a non-linear relationship with oxygen concentration [41]. So it is not a replacement of directly studying the effect of O₂ on embryo development experimentally. Here, we reported the effect of 2 % O₂ on human post-compaction preimplantation embryonic development, which has not been mathematically modeled or experimentally studied before.

Interestingly, we found that except for a slightly higher apoptosis rate, 2 % O₂ was not significantly different from 5 % O₂ in other measures of embryo quality, in contrast to 20 % O₂. This indicates that using the current static culture system, optimal O₂ level for post-compaction stage human embryo development is less than 20 %. Five percent is the best O₂ level among the three tested in our culture condition. However, it does not rule out the possibility that optimal O₂ for human IVF is not within the tested three, and it may also be a range rather than a specific O₂ level. It is especially true when it comes to different culture medium formulas, as their varied compositions of antioxidants and free radical scavengers may alleviate the detrimental effect of less ideal O₂ in different degrees.

Discussion of markers

BAX was high only at 20 % O₂. *BAX* is a marker of apoptosis, and it was associated with poor human oocytes and embryos quality [23] where improper culture increased *BAX*. This indicates that 20 % O₂, but not 2 or 5 % O₂, increases apoptosis at the end of post-thaw culture. *MnSOD* is an antioxidant enzyme located in mitochondria, participating in the transformation of

toxic superoxide produced during mitochondrial respiration. Twenty percent O₂ has been shown to increase the expression of *MnSOD* [27]. The higher level of *MnSOD* in embryos cultured at 20 % O₂ suggests the existence of oxidative stress and corresponding increase in anti-oxidative response.

G6PD is a critical enzyme in the pentose phosphate shunt, which supplies NADPH reducing power for anti-oxidative response. Higher level of *G6PD* was observed in embryos cultured in vitro compared with in vivo and embryos cultured at higher O₂ level [26]. Similar to *MnSOD*, the biological significance of *G6PD* is to increase the antioxidant capacity of blastocysts cultured at 20 % O₂, which would cause more ROS production.

Heat shock proteins are molecular chaperones assisting the correct folding of cellular proteins [42]. *HSP 70.1* is one of the heat shock protein family members. It mediates cellular stress response and has been used to measure embryonic stress caused by suboptimal culture conditions [24]. The mRNA level of *HSP 70.1* was shown to be higher in in vitro derived embryos compared with in vivo generated counterparts [26]. The increase of *HSP70.1* in embryos cultured at 20 % rather than 2 or 5 % O₂ indicates that 20 % O₂ is a more stressful culture condition.

Glucose enters cell either by an active process via sodium-coupled glucose transporters or by facilitative glucose transporters. *GLUT1* is one of the 13 known members of the facilitative glucose transporter family [43]. It is expressed in human embryonic stem cells [44] as well as human oocyte and 2–12-cell stage embryos [45]. We did not find differences in *GLUT1* mRNA expression among the three O₂ groups. This result is surprising because *GLUT1* is a downstream target of hypoxia inducible factor (HIF) and lower O₂ has been reported to increase *GLUT1* mRNA expression in bovine and mouse blastocysts [16, 46]. We speculate that the composition of

trophectoderm and inner cell mass (ICM) in blastocyst formed at different O_2 , and the activation status of HIF may account for the lack of difference here. First of all, 2 % O_2 culture is related to lower percentage of cells allocated to trophectoderm [16], and there is more *GLUT1* expression in trophectoderm than in ICM in bovine blastocyst [47]. The lack of difference in *GLUT1* here may be the result of lesser percentage of trophectoderm cells at lower O_2 . Secondly, the primary goal of this study was to investigate the possibility of optimal O_2 below 5 %, so all the markers chosen were in one way or another reflecting embryo development and quality. We did not look at other HIF responsive genes to know exactly the activation status of HIF. Lastly, *GLUT1* is associated with embryo quality, even when O_2 is not a variable [25]. Since blastocysts of similar morphological appearance in the three O_2 groups were chosen for gene expression analysis, which may also contribute to the lack of difference in *GLUT1* expression.

CX43 is a gap junction protein used in cell communication. At blastocyst stage, co-expression of *CX26*, *CX45*, and *CX1* with *CX43* was found in human embryos [48]. There is redundant expression and functional compensation between connexins [49]. Transcription level of *CX43* is higher in in vivo produced embryos than in vitro ones [29]. We did not find any difference in *CX43* expression among three O_2 groups. It could either be that the gap junction function of blastocysts cultured at different O_2 levels is similar, or that *CX43* expression does not respond to O_2 levels.

Our study here is unique in showing 2 % O_2 is not significantly different from 5 % O_2 for most outcomes measured. It suggests the need of O_2 in human post-compact stage embryo culture is relatively low. The same is true for rat embryos, for which oxidative phosphorylation is not required for blastocyst formation [50]. Mouse trophoblast stem cells (TSCs) are derived from the polar trophectoderm (pTE) of blastocyst. TSCs have highest potency and growth, lowest differentiation at 2 % O_2 [51]. And cultured TSCs have relatively low mitochondrial activity and active aerobic glycolysis [52] compare with later in vitro differentiated lineages. Compared with TE, ICM of mouse blastocyst have even lower oxygen consumption [53]. Human embryonic stem cells (hESC) derived from blastocyst ICM proliferated well at 3–5 % O_2 as they did at 21 % O_2 , and growth was only slightly reduced at 1 % O_2 . Potency and the ability to form embryoid were enhanced by hypoxic culture [54]. Taken together, these data indicate a preference for 2–5 % O_2 in TSC and ESC culture, which argues for the potential application of less 5 % O_2 in post-compact embryo development, since TSC and ESC are essentially the trophectoderm and ICM component of blastocyst.

Limitations and future studies

All the donated day 3 human embryos used in this study were cultured at 20 % O_2 before cryopreservation. It is possible that

20 % O_2 culture during the first 3 days and cryopreservation itself may have an adverse effect on subsequently embryo development after thawing, even though this effect is equal in three O_2 groups. Since there are only a few studies investigating the effect of O_2 below 5 % on animal embryo development and none on human, it is unethical to apply any O_2 below 5 % in human IVF before having a good understanding of its role on embryo quality and development. That necessitates the use of spare embryos donated by patients. And we can only use in vitro indicators of embryo quality such as apoptosis and mRNA expression of stress-related genes instead of re-implantation and observing long term development. Compared with the studies on the effect of 5 versus 20 % O_2 in human embryo development, which can be carried out in clinical setting without sacrificing the experimental embryos, we are limited by a relatively small sample size. Despite that, our result agrees that 5 % is optimal for post-thaw cleavage stage human embryo culture among the three O_2 groups tested. And interestingly, we found that 2 % O_2 is not as detrimental as 20 % O_2 .

Future studies need to have a larger sample size, and it would be informative to look at the molecular mechanisms mediating O_2 effect, such as oxygen consumption rate, glucose and amino acids transport as well as the action of HIF. O_2 level during cleavage stage embryo culture should also be taken into consideration, preferably at 5 % O_2 [55]. Furthermore, we think that unlike the progression from 20 to 5 % O_2 , the possible progression from 5 % O_2 to a new optimum at <5 % O_2 may require microfluidics to supply constant nutrition and provide waste removal [56]. Studies on mouse TSCs showed that at the growth-optimizing 2 % O_2 , the consumption of nutrients and accumulation of acidic metabolites is much faster, requiring frequent medium change [52]. It is also important to establish toxicity below the optimum as for mouse TSCs; stress increases and growth rates decreases rapidly below the O_2 optimum [51].

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Compliance with ethical standards Written informed consent was obtained from all participants before the usage of their embryos. The institutional review board (IRB) of the First Affiliated Hospital of Sun Yat-Sen University approved the study.

Conflict of interest The authors declare that they have no conflict of interest.

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References

- Bavister B. Oxygen concentration and preimplantation development. *Reprod Biomed Online*. 2004;9(5):484–6.
- Kirkegaard K, Hindkjaer JJ, Ingerslev HJ. Effect of oxygen concentration on human embryo development evaluated by time-lapse monitoring. *Fertil Steril*. 2013;99(3):738–44.e4.
- Kasterstein E, Strassburger D, Komarovskiy D, Bern O, Komsky A, Raziel A, et al. The effect of two distinct levels of oxygen concentration on embryo development in a sibling oocyte study. *J Assist Reprod Genet*. 2013;30(8):1073–9.
- De Los Santos MJ, Gamiz P, Albert C, Galan A, Vilorio T, Perez S, et al. Reduced oxygen tension improves embryo quality but not clinical pregnancy rates: a randomized clinical study into ovum donation cycles. *Fertil Steril*. 2013;100(2):402–7.
- Bahceci M, Ciray HN, Karagenc L, Ulug U, Bener F. Effect of oxygen concentration during the incubation of embryos of women undergoing ICSI and embryo transfer: a prospective randomized study. *Reprod Biomed Online*. 2005;11(4):438–43.
- Dumoulin JCM, Meijers CJJ, Bras M, Coonen E, Geraedts JPM, Evers JLH. Effect of oxygen concentration on human in-vitro fertilization and embryo culture. *Hum Reprod*. 1999;14(2):465–9.
- Kovacic B, Vlasisavljevic V. Influence of atmospheric versus reduced oxygen concentration on development of human blastocysts in vitro: a prospective study on sibling oocytes. *Reprod Biomed Online*. 2008;17(2):229–36.
- Meintjes M, Chantilis SJ, Douglas JD, Rodriguez AJ, Guerami AR, Bookout DM, et al. A controlled randomized trial evaluating the effect of lowered incubator oxygen tension on live births in a predominantly blastocyst transfer program dagger. *Hum Reprod*. 2009;24(2):300–7.
- Bontekoe S, Mantikou E, van Wely M, Seshadri S, Repping S, Mastenbroek S. Low oxygen concentrations for embryo culture in assisted reproductive technologies. *Cochrane Database Syst Rev*. 2012 (7). doi:10.1002/14651858.Cd008950.Pub2.
- Thompson JG, Simpson AC, Pugh PA, Donnelly PE, Tervit HR. Effect of oxygen concentration on in-vitro development of preimplantation sheep and cattle embryos. *J Reprod Fertil*. 1990;89(2):573–8.
- Berthelot F, Terqui M. Effects of oxygen, CO₂/pH and medium on the in vitro development of individually cultured porcine one- and two-cell embryos. *Reprod Nutr Dev*. 1996;36(3):241–51.
- Quinn P, Harlow GM. The effect of oxygen on the development of preimplantation mouse embryos in vitro. *J Exp Zool*. 1978;206(1):73–80.
- Feil D, Lane M, Roberts CT, Kelley RL, Edwards LJ, Thompson JG, et al. Effect of culturing mouse embryos under different oxygen concentrations on subsequent fetal and placental development. *J Physiol*. 2006;572(1):87–96.
- Takahashi Y, Hishinuma M, Matsui M, Tanaka H, Kanagawa H. Development of in vitro matured/fertilized bovine embryos in a chemically defined medium: influence of oxygen concentration in the gas atmosphere. *J Vet Med Sci*. 1996;58(9):897–902.
- Yuan YQ, Van Soom A, Coopman FO, Mintiens K, Boerjan ML, Van Zeveren A, et al. Influence of oxygen tension on apoptosis and hatching in bovine embryos cultured in vitro. *Theriogenology*. 2003;59(7):1585–96.
- Harvey AJ, Kind KL, Pantaleon M, Armstrong DT, Thompson JG. Oxygen-regulated gene expression in bovine blastocysts. *Biol Reprod*. 2004;71(4):1108–19.
- Thompson JG, McNaughton C, Gasparini B, McGowan LT, Tervit HR. Effect of inhibitors and uncouplers of oxidative phosphorylation during compaction and blastulation of bovine embryos cultured in vitro. *J Reprod Fertil*. 2000;118(1):47–55.
- Li J, Foote RH. Culture of rabbit zygotes into blastocysts in protein-free medium with one to twenty per cent oxygen. *J Reprod Fertil*. 1993;98(1):163–7.
- Leese HJ. Human embryo culture: back to nature. *J Assist Reprod Genet*. 1998;15(8):466–8.
- Yedwab GA, Paz G, Homonnai TZ, David MP, Kraicer PF. The temperature, pH, and partial pressure of oxygen in the cervix and uterus of women and uterus of rats during the cycle. *Fertil Steril*. 1976;27(3):304–9.
- Ottosen LD, Hindkjaer J, Husth M, Petersen DE, Kirk J, Ingerslev HJ. Observations on intrauterine oxygen tension measured by fibre-optic microsensors. *Reprod Biomed Online*. 2006;13(3):380–5.
- Diaz S, Ortiz ME, Croxatto HB. Studies on the duration of ovum transport by the human oviduct. III. Time interval between the luteinizing hormone peak and recovery of ova by transcervical flushing of the uterus in normal women. *Am J Obstet Gynecol*. 1980;137(1):116–21.
- Liu HC, He ZY, Mele CA, Veeck LL, Davis O, Rosenwaks Z. Expression of apoptosis-related genes in human oocytes and embryos. *J Assist Reprod Genet*. 2000;17(9):521–33.
- Wrenzycki C, Herrmann D, Carnwath JW, Niemann H. Alterations in the relative abundance of gene transcripts in preimplantation bovine embryos cultured in medium supplemented with either serum or PVA. *Mol Reprod Dev*. 1999;53(1):8–18.
- Niemann H, Wrenzycki C. Alterations of expression of developmentally important genes in preimplantation bovine embryos by in vitro culture conditions: implications for subsequent development. *Theriogenology*. 2000;53(1):21–34.
- Balasubramanian S, Son WJ, Kumar BM, Ock SA, Yoo JG, Im GS, et al. Expression pattern of oxygen and stress-responsive gene transcripts at various developmental stages of in vitro and in vivo preimplantation bovine embryos. *Theriogenology*. 2007;68(2):265–75.
- Rizos D, Gutierrez-Adan A, Moreira P, O'Meara C, Fair T, Evans AC, et al. Species-related differences in blastocyst quality are associated with differences in relative mRNA transcription. *Mol Reprod Dev*. 2004;69(4):381–6.
- Wrenzycki C, Herrmann D, Keskintepe L, Martins Jr A, Sirisathien S, Brackett B, et al. Effects of culture system and protein supplementation on mRNA expression in pre-implantation bovine embryos. *Hum Reprod*. 2001;16(5):893–901.
- Rizos D, Lonergan P, Boland MP, Arroyo-Garcia R, Pintado B, de la Fuente J, et al. Analysis of differential messenger RNA expression between bovine blastocysts produced in different culture systems: implications for blastocyst quality. *Biol Reprod*. 2002;66(3):589–95.
- Edgar DH, Bourne H, Speirs AL, McBain JC. A quantitative analysis of the impact of cryopreservation on the implantation potential of human early cleavage stage embryos. *Hum Reprod*. 2000;15(1):175–9.
- Cutting R, Morroll D, Roberts SA, Pickering S, Rutherford A, Bfs, et al. Elective single embryo transfer: guidelines for practice British Fertility Society and Association of Clinical Embryologists. *Hum Fertil (Camb)*. 2008;11(3):131–46.
- Gardner DK, Schoolcraft WB. A randomized trial of blastocyst culture and transfer in in-vitro fertilization: reply. *Hum Reprod*. 1999;14(6):1663A.
- Abruzzese RV, Fekete RA. Single cell gene expression analysis of pluripotent stem cells. *Methods Mol Biol*. 2013;997:217–24.
- Li J, Smyth P, Cahill S, Denning K, Flavin R, Aherne S, et al. Improved RNA quality and TaqMan pre-amplification method

- (PreAmp) to enhance expression analysis from formalin fixed paraffin embedded (FFPE) materials. *BMC Biotechnol.* 2008;8:10.
35. Jedrusik A, Parfitt DE, Guo G, Skamagki M, Grabarek JB, Johnson MH, et al. Role of Cdx2 and cell polarity in cell allocation and specification of trophectoderm and inner cell mass in the mouse embryo. *Genes Dev.* 2008;22(19):2692–706.
 36. Van Peer G, Mestdagh P, Vandesompele J. Accurate RT-qPCR gene expression analysis on cell culture lysates. *Sci Rep.* 2012;2:222.
 37. Duran EM, Shapshak P, Worley J, Minagar A, Ziegler F, Haliko S, et al. Presenilin-1 detection in brain neurons and FOXp3 in peripheral blood mononuclear cells: normalizer gene selection for real time reverse transcriptase PCR using the deltadeltaCt method. *Front Biosci.* 2005;10:2955–65.
 38. Pfister C, Pfrommer H, Tatagiba MS, Roser F. Detection and quantification of farnesol-induced apoptosis in difficult primary cell cultures by TaqMan protein assay. *Apoptosis.* 2013;18(4):452–66.
 39. Byattsmith JG, Leese HJ, Gosden RG. An investigation by mathematical modeling of whether mouse and human preimplantation embryos in static culture can satisfy their demands for oxygen by diffusion. *Hum Reprod.* 1991;6(1):52–7.
 40. Baltz JM, Biggers JD. Oxygen transport to embryos in microdrop cultures. *Mol Reprod Dev.* 1991;28(4):351–5.
 41. Clark AR, Stokes YM, Lane M, Thompson JG. Mathematical modelling of oxygen concentration in bovine and murine cumulus-oocyte complexes. *Reproduction.* 2006;131(6):999–1006.
 42. Saibil HR. Biochemistry. Machinery to reverse irreversible aggregates. *Science.* 2013;339(6123):1040–1.
 43. Riley JK, Moley KH. Glucose utilization and the PI3-K pathway: mechanisms for cell survival in preimplantation embryos. *Reproduction.* 2006;131(5):823–35.
 44. Heilig C, Brosius F, Siu B, Concepcion L, Mortensen R, Heilig K, et al. Implications of glucose transporter protein type 1 (GLUT1)-haplo deficiency in embryonic stem cells for their survival in response to hypoxic stress. *Am J Pathol.* 2003;163(5):1873–85.
 45. Dan-Goor M, Sasson S, Davarashvili A, Almagor M. Expression of glucose transporter and glucose uptake in human oocytes and preimplantation embryos. *Hum Reprod.* 1997;12(11):2508–10.
 46. Kind KL, Collett RA, Harvey AJ, Thompson JG. Oxygen-regulated expression of GLUT-1, GLUT-3, and VEGF in the mouse blastocyst. *Mol Reprod Dev.* 2005;70(1):37–44.
 47. Wrenzycki C, Herrmann D, Niemann H. Timing of blastocyst expansion affects spatial messenger RNA expression patterns of genes in bovine blastocysts produced in vitro. *Biol Reprod.* 2003;68(6):2073–80.
 48. Bloor DJ, Wilson Y, Kibschull M, Traub O, Leese HJ, Winterhager E, et al. Expression of connexins in human preimplantation embryos in vitro. *Reprod Biol Endocrinol.* 2004;2:25.
 49. Houghton FD. Role of gap junctions during early embryo development. *Reproduction.* 2005;129(2):129–35.
 50. Brison DR, Leese HJ. Blastocoel cavity formation by preimplantation rat embryos in the presence of cyanide and other inhibitors of oxidative phosphorylation. *J Reprod Fertil.* 1994;101(2):305–9.
 51. Zhou S, Xie Y, Puschek EE, Rappolee DA. Oxygen levels that optimize TSC culture are identified by maximizing growth rates and minimizing stress. *Placenta.* 2011;32(6):475–81.
 52. Xie Y, Zhou S, Jiang Z, Dai J, Puschek EE, Lee I, et al. Hypoxic stress induces, but cannot sustain trophoblast stem cell differentiation to labyrinthine placenta due to mitochondrial insufficiency. *Stem Cell Res.* 2014;13(3 Pt A):478–91.
 53. Houghton FD. Energy metabolism of the inner cell mass and trophectoderm of the mouse blastocyst. *Differentiation.* 2006;74(1):11–8.
 54. Ezashi T, Das P, Roberts RM. Low O₂ tensions and the prevention of differentiation of hES cells. *Proc Natl Acad Sci U S A.* 2005;102(13):4783–8.
 55. Wale PL, Gardner DK. Time-lapse analysis of mouse embryo development in oxygen gradients. *Reprod Biomed Online.* 2010;21(3):402–10.
 56. Heo YS, Cabrera LM, Bormann CL, Shah CT, Takayama S, Smith GD. Dynamic microfunnel culture enhances mouse embryo development and pregnancy rates. *Hum Reprod.* 2010;25(3):613–22.