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Pericellular oxygen concentration of cultured primary human trophoblasts

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A R T I C L E I N F O

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Introduction: Oxygen is pivotal in placental development and function. *In vitro* culture of human trophoblasts provides a useful model to study this phenomenon, but a hotly debated issue is whether or not the oxygen tension of the culture conditions mimics *in vivo* conditions. We tested the hypothesis that ambient oxygen tensions in culture reflect the pericellular oxygen levels.
Methods: We used a microelectrode oxygen sensor to measure the concentration of dissolved oxygen in the culture medium equilibrated with 21%, 8% or <0.5% oxygen.
Results: The concentration of oxygen in medium without cells resembled that in the ambient atmosphere. The oxygen concentration present in medium bathing trophoblasts was remarkably dependent on the depth within the medium where sampling occurred, and the oxygen concentration within the overlying atmosphere was not reflected in medium immediately adjacent to the cells. Indeed, the pericellular oxygen concentration was in a range that most would consider severe hypoxia, at ≤0.6% oxygen or about 4.6 mm Hg, when the overlying atmosphere was 21% oxygen.
Conclusions: We conclude that culture conditions of 21% oxygen are unable to replicate the pO_2 of 40–60 mm Hg commonly attributed to the maternal blood in the intervillous space in the second and third trimesters of pregnancy. We further surmise that oxygen atmospheres in culture conditions between 0.5% and 21% provide different oxygen fluxes in the immediate pericellular environment yet can still yield insights into the responses of human trophoblast to different oxygen conditions.
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1. Introduction

Oxygen is the Janus gas [1], and the two-faced effect of this necessary but potentially damaging gas is especially important in human placental development. Low oxygen tension at <20 mm Hg is a prerequisite for extra villous trophoblasts to optimally invade decidua and establish the early placenta [2]. Oxygen tension rises at 10–12 weeks' gestation to offer an estimated 40–60 mm Hg pO_2 in the intervillous space where maternal blood bathes villous trophoblasts [3–5]. These critical variations in oxygen tensions *in vivo* have created a hotly debated issue in placental biology: what oxygen conditions should be used for *in vitro* experiments to best mimic conditions *in vivo*? Reviewers of papers and attendees at placental-focused conferences have strong opinions about this question, yet little data exist to support or refute the different perspectives. We do not resolve this controversy here. Instead we test the hypothesis that ambient atmospheric oxygen tensions reflect pericellular oxygen levels experienced by cultured trophoblasts. We used a microelectrode to directly measure pericellular oxygen concentrations in the culture medium of primary human trophoblasts. We discovered that this hypothesis is simplistic and wrong.

2. Methods

2.1. Cell culture

This study was approved by the Institutional Review Board of Washington University in St Louis. Primary human cytotrophoblasts were isolated from term placentas, from uncomplicated pregnancies delivered by scheduled cesarean sections, by the trypsin-deoxyribonuclease-Dispase/Percoll method as described previously [6]. Three million cells were plated at a density of 3.4×10^5 per cm^2 with 2 ml Dulbecco's modified Eagle's medium (DMEM, Sigma, St. Louis, MO) containing 10% fetal bovine serum (Sigma), 20 mM HEPES (pH 7.4, Sigma), 100 U/ml penicillin, 100 μg/ml streptomycin, and 0.25 mg/ml Fungizone (all from the Washington University Tissue Culture Support Center), at 37 °C in 5% CO_2-air atmosphere with 21% O_2 (standard conditions), in a 35 mm culture dish (total culture area: 8.8 cm^2; total volume of dish: 9.3 ml; total height of 2 ml medium: 1.8–1.9 mm. Nunc, Rochester, NY). After allowing cells to attach for 4 h, the dishes were washed thoroughly three times with PBS to eliminate nonattached cells and villous fragments and then replenished with 2 ml of 37 °C pre-warmed, atmospheric-air-equilibrated culture medium and used for dissolved oxygen concentration measurements.

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2.2. Incubators and hypoxia chambers

Two Forma Scientific anaerobic chambers at 37 °C, one set at 5% CO_2/8% O_2/87% N_2 (oxygen sensor reading 8% O_2), and the other set at 5% CO_2/10% H_2/85% N_2 (oxygen sensor reading <0.5% O_2), were used to equilibrate culture medium to make "8%" and "<0.5%" media, respectively. A Forma Scientific water-jacketed incubator (Forma Scientific, USA) that provides standard culture conditions (21% O_2) was used to equilibrate standard culture medium and for normal culture of primary human trophoblasts.

2.2.1. Calibration of the oxygen sensor

A micro dissolved-O_2 electrode (DO-166MT-1, LAZAR research laboratories, Los Angeles, CA), which has a 3 mm wide tip, was used to measure the dissolved oxygen concentration in the culture medium. The concentration was recorded every second with the ArrowDO sampler program provided with the electrode. The microelectrode oxygen sensor was calibrated according to the manufacturer's instructions to 21% O_2 in the incubator that provides standard culture conditions, and to an atmosphere of 0% O_2 in nitrogen gas.

2.2.2. Measurement of dissolved oxygen concentration in medium without cells

Culture dishes (35 mm, $n = 3$) containing 2 ml of fresh medium without cells were transferred to 21%, 8%, or <0.5% oxygen incubators for 24 h until measured. The microelectrode was clamped vertically to a Narishige micromanipulator (Tokyo, Japan), which allowed precise adjustment of the electrode height (Fig. 1A). The microelectrode was introduced through a 5 mm hole in the center of the dish cover (Fig. 1B), and placed as close to the bottom of the culture dish as feasible without damaging the tip; this position was designated as 0 mm. All experiments were conducted with the microelectrode and micromanipulator within the incubators. For the two anaerobic chambers (<0.5% and 8% oxygen), the height of the microelectrode in the medium was adjusted from outside the incubator through gloves attached to the front of the chambers. For the incubator that provided 21% oxygen, the height of the microelectrode was adjusted from inside the incubator after briefly opening the door. The dissolved O_2 concentration of medium was measured at heights noted in the text, with 0 mm being the pericellular location and 1.8 mm being at the top of the medium. A stable reading was obtained after continuous measurement for 5 min at each site.

2.2.3. Measurement of dissolved oxygen concentration in medium with cells

For measurement of pericellular oxygen concentrations, the microelectrode was placed at the bottom (cell surface) of the culture dish ($n = 4$, from same placenta) as described above, and the oxygen concentration was recorded for ≤5 h beginning immediately after replenishing the culture with fresh medium equilibrated with 21% oxygen.

For measurement of the oxygen concentration at different heights above cultured trophoblasts ($n = 3$, from the same placenta), primary trophoblasts were plated for 4 h, washed and the medium replenished. After an additional 5 h of culture, the microelectrode was then placed immediately above the cells on the bottom of the dish at 0 mm for the first measurement. The microelectrode was then raised in a stepwise manner, to the heights indicated in the figure for each subsequent recording. At each height, the dissolved oxygen concentration was continuously measured for at least 15 min before adjusting the tip to another height. Two controls were performed to ensure that the observed oxygen gradient was not due to cellular debris blocking the electrode tip at the bottom of the plate and slowly detaching as the tip was raised. In the first control, after being raised, the microelectrode was then lowered in a stepwise manner and the oxygen concentrations again measured, showing a stepwise decrease (data not shown). In the second control, the cultured trophoblasts ($n = 3$ from the same placenta) were plated, washed and replenished as described above. The cells were then treated with methanol at −20 °C for 10 min, washed with PBS and replenished at 37 °C with fresh medium equilibrated to the ambient air in the incubator that provides 21% oxygen. Five hours after the medium was replenished, the oxygen concentration at different heights of the medium was measured as described above.

2.3. Statistical analysis

Data are presented as mean ± SD and comparisons were by ANOVA with Bonferroni corrections. A $p < 0.05$ was significant.

3. Results

The microelectrode system set-up is shown in Fig. 1. We first determined the time required for the electrode to obtain a stable reading for the oxygen tension using culture medium equilibrated in ambient atmospheres of 21%, 8%, and <0.5% O_2. There was no significant difference in the time for equilibration among the three oxygen tensions (data not shown), with a stable reading consistently obtained by 5 min.

3.1. Medium oxygen levels in the absence of cells

We next compared the ambient oxygen concentration in the atmosphere of the incubator to the concentration of dissolved oxygen in medium without cells. Medium equilibrated with ambient atmospheres of 21%, 8%, and <0.5% O_2 for 24 h at 37 °C yielded dissolved O_2 concentrations of ∼19.3%, ∼7.9% and ∼0.5%, respectively (Fig. 2).

3.2. Pericellular oxygen concentrations in trophoblast cultures

We next measured the dissolved oxygen concentration in medium in four separate, near-confluent cultures of cells that were exposed to an atmosphere of 21% O_2 at 37 °C. Immediately after introduction of fresh medium equilibrated in 21% O_2, the dissolved pericellular oxygen concentration (near the bottom of the dish) was initially ∼20%. Between 11 and 75 min after introduction of the fresh medium, the pericellular oxygen level plummeted, eventually reaching a steady-state oxygen concentration between 0.1% and 0.6% (Fig. 3A).

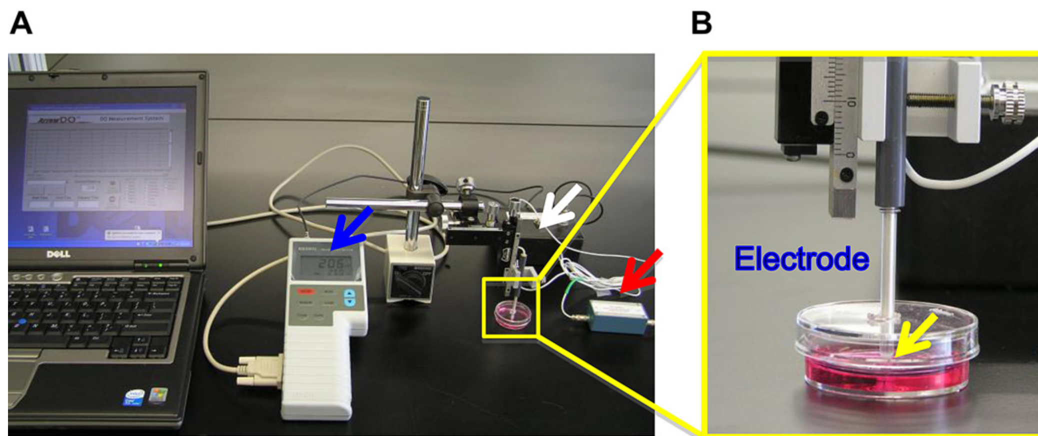

Fig. 1. Apparatus for measurement of oxygen concentration. A. Oxygen levels were measured using a DO-166MT-1 micro dissolved oxygen electrode. The oxygen microelectrode was held by a micromanipulator and inserted into the culture medium through a hole in the cover of a 35 mm culture dish. Blue arrow: pH/millivolt meter. White arrow: Attenuator. Red arrow: Amplifier. B. The enlarged area outlined in panel A. Yellow arrow: the tip of the microelectrode.

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