Article

Duration and temperature of culture medium equilibration affect frequency of blastocyst development

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Abstract

Hamster 2-cell embryos were cultured in 50 µl drops of chemically defined medium (HECM-9) under oil in 60 mm Petri dishes. In the first experiment, the dishes were equilibrated with 5% O_2/10% CO_2/85% N_2 for 2 h either within sealed plastic bags or exposed directly to the same gas mixture in a tissue culture incubator. After culture of embryos for 48 h, there was no difference in development to the blastocyst stage. In the second experiment, the dishes were first equilibrated with 5% O_2/10% CO_2/ 85% N_2 within sealed plastic bags, (A) at 4°C overnight (16–18 h), or (B) at 37.5°C overnight or (C) at 37.5°C for 2 h. Dishes in treatment A were placed in the incubator at 37.5°C for 2 h next day just before use. Two-cell embryos from a superovulated, mated female were equally distributed among the three treatments, then the dishes were sealed in fresh bags containing the same gas mixture and incubated at 37.5°C for 48 h. This experiment was replicated 13 times with a total of 20 females and 268–275 embryos/treatment. There was no significant difference among the treatments for development to the (combined) morula/blastocyst stages. However, the percentage of blastocysts that developed in culture dishes that had been equilibrated overnight at 37.5°C (treatment B) was significantly lower [50 ± 14% (SEM)] than in treatments A and C, which were not different from one another (67 ± 11 and 60 ± 17% respectively). These results indicate that when culture medium is incubated at 37.5°C overnight, chemical deterioration occurs that is detrimental to embryo development, and that this can be avoided by equilibrating dishes at 4°C overnight, followed by a brief period at 37.5°C to warm the medium before inserting embryos. This finding may have clinical relevance for human embryo culture. The study also demonstrates the utility and advantages of the sealed bag system for embryo culture.

Keywords: *blastocysts, carbon dioxide, embryo culture, hamster, incubators, oxygen*

Introduction

Most research laboratories using animal embryos, and some human clinical laboratories, employ low (5–10%) O_2 concentrations for embryo culture. Usually, this is achieved by injecting N_2 into the incubator to displace ambient O_2. Equilibration for several hours is required to allow atmospheric O_2 (21%) saturating the culture medium to diffuse out through the oil overlay, until the desired O_2 tension is reached. Oxygen diffuses much more slowly than CO_2, and there is no simple way to monitor the rate of O_2 diffusion through culture media and oil, unlike CO_2 diffusion, which can be monitored visually using a pH indicator such as phenol red. Consequently, most laboratories, including ours, have assumed that the time needed for the medium to equilibrate with 5% CO_2, about 1–2 h, is also adequate for atmospheric O_2 to diffuse out of the medium and reach 5–10%. However, Lopes *et al*. (2003), using an O_2 microelectrode placed in the culture medium drops, showed that more than 9 h was needed for complete equilibration of medium with 5% O_2. The time

allowed for gas equilibration can easily be increased by placing culture drops in the incubator at 37°C overnight, which is common practice in many human IVF laboratories, but this introduces another problem. Lengthy equilibration at 37°C accelerates deterioration of culture media, degrading labile components such as pyruvate or some amino acids, including glutamine. This problem can be avoided by equilibrating culture media in a refrigerator at 4°C, allowing prolonged gas equilibration but at low temperature. After experimenting with devices such as modular (Billups–Rothenberg) chambers, which are large and cumbersome, the effectiveness of using small plastic bags that are filled with gas mixture then heat-sealed was examined.

The present study was carried out to examine (i) if incubating culture dishes in sealed plastic bags produces similar embryo development as in dishes exposed to a gas atmosphere inside a tissue culture incubator; (ii) if a short time (2 h) for gas (O_2, CO_2) equilibration of culture medium is sufficient for supporting embryo development *in vitro*, compared with overnight equilibration (16–18 h), and (iii) if gas equilibration of culture media drops overnight at 4°C is superior to equilibration at 37.5°C.

Materials and methods

Collection of embryos

Cycling female hamsters were superstimulated with 25 IU of pregnant mare's serum gonadotrophin (PMSG) on the day of the post-oestrus discharge (day 1) then mated 3 days later (day 4), as previously described (McKiernan *et al*., 1995). Approximately 27 h post-egg activation, 2-cell embryos were collected by flushing excised oviducts.

Experimental design

Experiment 1

This experiment was performed to ascertain whether incubating embryos within sealed bags filled with gas mixture would produce similar results to culturing embryos in the conventional way using an O_2/CO_2 controlled incubator. There were two treatments. In both treatments, 60 mm Petri dishes (Falcon Plastics no. 1007; Becton Dickinson, Franklin Lakes, NJ, USA) were prepared each containing several 50 µl drops of HECM-9 (McKiernan and Bavister, 2000) under a mineral oil overlay (Sigma Chemical Co., St. Louis, MO, USA, cat no. M-8410). The HECM-9 culture medium was prepared by adding 11 amino acids (0.01 mM each of asparagine, aspartate, cysteine, glutamate, glycine, histidine, lysine, proline, serine; 0.20 mM glutamine; 0.5 mM taurine) and pantothenic acid (3 µM pantothenate) to basic salt solution containing lactate (BM-3) that had been prepared up to 3 days earlier. In treatment A, culture dishes were placed in the lower chamber of a standard tissue-culture incubator (Thermo Forma/Forma Scientific, Marietta, OH, USA, model no. 3130) that was set to provide 5% O_2 and 10% CO_2 using N_2 and CO_2 injection. Using 10% CO_2 produces a culture medium pH of 7.2 and supports optimal blastocyst development of hamster embryos (McKiernan and Bavister, 1990). For treatment B, dishes were inserted into a 6.5 inch × 8.0 inch plastic bag (SealPak Pouch, no. 402; Kapak Corp., Minneapolis, MN, USA) that was then filled with premixed gas (5% O_2/10% CO_2/85% N_2) from a tank and heat-sealed (**Figures 1–3**). To facilitate insertion and removal from the bags, each dish was placed on the inverted lid of a multi-well dish (Falcon Plastics, nos 3043 or 3047) that was used as a tray. A 30 mm Petri dish (Falcon Plastics no. 1008) containing approximately 2 ml of the same culture medium with 1 mg/ml phenol red was included in each bag to visually indicate integrity of the seal during culture. This dish also served to maintain high humidity inside the bags.

At first, an automated gas injection/bag sealer device (K-Systems; KIVEX Biotech Ltd, Birkerød, Denmark) was used, but later a long needle was employed to inject gas into the bags (**Figure 2**), then they were sealed using a standard laboratory heat sealer (Pouch Sealer; Kapak Corp.). The bags were placed in the upper chamber of the incubator at 37.5°C without gas injection, i.e. with ambient air filling the chamber. All dishes (A and B) were allowed to equilibrate for 2 h before adding the embryos. This was ample time for equilibration with CO_2 because when freshly prepared culture drops containing phenol red under an oil overlay were incubated at 37.5°C in 5% CO_2, the colour equivalent to pH 7.2 (i.e. complete equilibration with CO_2) was reached in 1 h. Two-cell embryos collected from one female were distributed into the culture drops in approximately equal numbers between the two treatments. For treatment A, embryos were simply inserted into the culture drops. For treatment B, the bags were cut open to allow embryos to be added, then the dishes were placed into fresh bags that were gassed with 5% O_2/10% CO_2/85% N_2 and heat-sealed. Both sets of dishes were returned to their respective incubator chambers, which were either filled with gas (5% O_2/10% CO_2/85% N_2, treatment A) or contained ambient air (treatment B), as before, and incubated for 48 h at 37.5°C after which the embryos were examined for blastocyst development. There were four replicates across days of this experiment, each using one or two females/day, for a total of six females.

Experiment 2

In this experiment, only the sealed bags were used. The design is shown in **Figure 4**. The afternoon before an experiment, four to six 60 mm Petri dishes (Falcon no. 1007) were prepared, each containing several 50 µl drops of HECM-9 under a mineral oil overlay. The HECM-9 was prepared that day from BM-3 as described for experiment 1. Each dish was placed on a plastic tray and inserted into a plastic bag (Kapak Corp.) that was filled with gas (5% O_2/10% CO_2/85% N_2) and heat sealed as in experiment 1, using a needle for gas injection and a laboratory bag sealer. Half of the bags were placed in a refrigerator at 4°C (treatment A), the other half at 37.5°C (treatment B) in a standard tissue-culture incubator (Forma Scientific model 3130). Only ambient air was present in the incubator chamber. The prepared dishes were incubated under these two conditions overnight, for approximately 16–18 h. The remaining BM-3 solution was kept at 4°C in a plastic tube overnight; next morning, 11 amino acids and pantothenic acid were added to make HECM-9. Using this freshly prepared culture medium, several 50 µl drops were made in two or three 60 mm Petri dishes, overlaid with oil then sealed in bags filled with the same gas mixture (treatment C). All the dishes in treatments A and C were placed in the 37.5°C incubator chamber (which already contained treatment B dishes) for 2 h