—Technology Report—

A Self-Contained Culture Platform Using Carbon Dioxide Produced from a Chemical Reaction Supports Mouse Blastocyst Development *In Vitro*

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Abstract. Elevated CO₂ is required for *in vitro* embryo culture to maintain proper media pH and to supply embryo metabolic pathways. As an alternative to current approaches using gas cylinders, we examined use of a chemical reaction to supply CO₂. A closed culture system was constructed and chemicals added to generate CO₂, which was then supplied to developing embryos. This system was shown to provide a stable pH (7.2–7.4) over 4 days of use. One-cell mouse embryos were cultured in the device and no difference in blastocyst formation or cell number was apparent between embryos grown in a closed system with CO₂ supplied by a chemical reaction or positive controls grown in an open system in a CO₂ incubator. This approach provides a highly purified, inexpensive, and easily obtainable gas source and offers potential for development of new, self-contained culture platforms.

Key words: Blastocyst, Carbon dioxide, pH

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In attempts to improve efficiency, approaches to *in vitro* embryo culture have evolved over time. The majority of change has involved manipulations of culture media substrate composition, however, other environmental factors dramatically impact embryo quality, such as culture atmosphere, and also require examination.

While research concerning atmospheric impact on embryo development is not completely lacking, most of the available literature focuses on impact of oxygen concentration [1–7]. Perhaps more importantly, CO₂ gas is critical to support embryo development *in vitro*. Carbon dioxide gas dissolves in the aqueous culture medium to produce carbonic acid, which reaches equilibrium with sodium bicarbonate in the medium to set the external pH (pHe), which can alter internal pH (pHi) and subsequent embryo development and function [8–12]. Also, independent of pHe, CO₂ is required for embryo development *in vitro*, as carbon from external CO₂ is fixed by the embryo and used for various metabolic processes [12–14]. Thus, while there are various culture systems which permit embryo development in both open and closed vessels by utilizing zwitterionic buffers to maintain pHe and do not utilize external CO₂ supplied by an incubator (see review [15]), when proper comparative studies are performed, embryo development is often compromised in these systems, in part, due to lack of external carbon/CO₂ [16].

Though levels of CO₂ utilized for embryo culture may vary from laboratory to laboratory, depending upon target pHe and relevant factors, one consistent variable is that CO₂ is supplied via cylinders of compressed gas. This is often true even when utilizing various portable systems using small incubators or closed culture vessels

[17, 18]. This current approach of gas supply can be inefficient and expensive. Liters of gas are pumped into often large incubators to maintain the pHe of microliter volumes of media. Furthermore, maintaining this gas atmosphere in traditional incubators is hindered by repeated openings/closings, which utilizes/wastes more gas. These perturbations in gas environment impact media equilibration and subsequent embryo quality. Additionally, the expense involved in the initial purchase of an incubator and the associated space/supplies to maintain cylinders of gas is problematic, especially if using a portable field system. Furthermore, and perhaps of more concern, purity of gas in cylinders can be questionable and detrimental to embryo development [19].

In an attempt to explore a more efficient alternative to current methods of supplying CO₂ for embryo culture, use of a chemical reaction, supplied as Alka Seltzer® tablets, in a closed embryo culture system was examined.

Initial experiments focused on ability to obtain and maintain an appropriate pHe in a closed system with chemically generated CO₂. Varying portions of Alka Seltzer® were removed and weighed to allow addition to the closed culture system. Traditional bicarbonate media (25 mM NaHCO₃) was tested and titrations indicated between 0.025 and 0.05 g of the tablet are needed to achieve appropriate pHe for embryo culture (7.20–7.40) (Fig. 1A). Additionally, the closed-system apparatus was able to maintain a stable pHe over 96 h, with no significant changes in pHe compared to 24 h (Fig. 1B). To determine if pH stability could be improved, similar experiments were performed with dual-buffered HEPES:MOPS media with 25 mM NaHCO₃ and similar pHe values were obtained (data not reported). Therefore, 0.03 g of the tablet was used in subsequent embryo development experiments.

Due to the possibility of limited access to Alka Seltzer® and to further define the system, use of the raw chemical active ingredients were also used to generate CO₂. The approximate amounts of citric acid (20 mg) and sodium bicarbonate (20 mg) found in 0.05 g

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Fig. 1. A: Graphical display of dose titration to determine amount of chemical tablet needed to obtain appropriate media pH (7.2–7.4) following 24 h incubation. B: Verification that the closed culture system maintained a stable pH over 96 h of incubation using 0.03 g of tablet. Data are presented as the mean ± SEM.

of the Alka Seltzer tablet were added to the closed system and resulting pH measured of bicarbonate buffered media. The pH of 3 replicates indicate the raw chemicals yielded a pH of 7.35 ± 0.02 (mean ± SEM).

Examination of embryo development using 0.03 g of tablet demonstrated no differences in blastocyst formation between embryos grown in positive controls in an open system in a CO₂ incubator or treatments, which consisted of KSOMAA or KSOMAA + HEPES:MOPS in a closed system with CO₂ supplied by a chemical reaction (92.6, 85.2 vs. 85.2%, respectively). Negative control embryos grown in a closed system with no CO₂ supply yielded significantly lower rates of blastocyst formation compared to treatment or positive control (0%) (Fig. 2A). Rates of blastocyst hatching were not significantly different between positive controls or treatments (KSOMAA, KSOMAA+HEPES:MOPS), though rates were slightly less in closed test tubes with chemically generated CO₂ (29.6 ± 9.3 , 14.8 ± 3.9 , 18.5 ± 5.3 , respectively) (Fig. 2A). Examination of blastocyst cell numbers between positive controls and treatments (KSOMAA or KSOMAA + HEPES:MOPS) revealed no difference (47.44 ± 5.37 n=18, 52.81 ± 7.70 n=16, 44.7 ± 5.2 n=14, respectively) (Fig. 2B).

It is shown here that 1-cell mouse embryos can be cultured to the blastocyst stage using CO₂ supplied via a simple chemical reaction. Chemically produced CO₂ used in a closed system offers a pure and easily accessible gas source and offers an alternative to traditional incubators and gas cylinders. It is known that purity of ambient atmosphere can impact embryo development and pregnancy [19–

Fig. 2. A: Graphical display of resulting blastocyst development and hatching following culture in CO₂ produced via a chemical reaction, cylinders of gas connected to an incubator, or no CO₂. B: Total cell number of blastocysts developed from two sources of CO₂. Data are presented as the mean ± SEM. * represents a significant decrease in blastocyst development and hatching compared to other treatments, $P < 0.01$.

21]. The same holds true for supplied gases. Levels of benzene, freons, aldehydes, toluene, acetone and isopropanol have been identified in compressed gas tanks [19]. Though charcoal activated filters in the incubator [22] or in the gas-line can remove some potential contaminants, impurities in atmosphere/gas composition directly from the tank or picked up during transit to the incubator through tubing, can still compromise embryo development. Chemically provided CO₂ offers a potential solution to these impurities, as well as an alternate backup supply source of gas if needed due to inability to obtain/store cylinders of gas or equipment failure.

Interestingly, an early study examining ability to culture mouse embryo *in vitro* utilized exhaled alveolar air as an alternate to supplying external CO₂ gas from medical cylinder [23], though this approach limits ability to adjust CO₂ levels and pH. use of CO₂ generated from a more complex chemical reaction has been previously used to culture bovine embryos. Prior studies have utilized effervescent granules of tartaric acid, carbonated hydrogen sodium and silicone fiber, granules reacted with water to produce CO₂ for use during *in vitro* oocyte maturation and culture of bovine IVF embryos and nuclear transfer blastocysts [24–27]. In a similar

Fig. 3. A: Schematic of the closed culture device and supply of CO₂ to media and embryos. B: Image of the culture device.

fashion, bovine embryos were also grown using the Oxoid™ atmosphere generating system [28], a commercially available item normally used for growth of microorganisms. This approach utilizes a paper sachet of ascorbic acid and reacts with air to provide an atmosphere of ~6% CO₂ and 15% oxygen when used in the proper sized Aerojar™. Importantly, chemicals used in previous studies to generate CO₂ are not found in embryo culture media or IVF laboratories, and thus may be difficult to obtain, and are likely embryo toxic. These complex commercial products/reactions may not be widely available or easy to reproduce in a remote laboratory. In contrast, key reagents from our study (citric acid and sodium bicarbonate) are common ingredients in culture media, and thus offer an easily obtainable alternative to prior complex approaches. Products of the chemical reaction (sodium citrate and water), are also common products in culture media and would appear to be a safer alternative than prior chemical approaches. Other acid:base reactions could be utilized in a similar fashion to produce CO₂, though potential for toxic fumes or byproducts should be considered. Furthermore, we compared our approach to positive control embryos grown in an incubator and obtained comparable rates of blastocyst development, which, at >80% from 1-cell, are considered appropriate when using the widely accepted MEA assay standards of various commercial culture media suppliers. Comparison of blastocyst cell number further support efficacy of the system. Future experiments examining development of embryos from other domestic animal species may be insightful in further examining efficacy of this approach. Additionally, no reports exist regarding transfer of embryos from any species after being grown in conditions using chemically generated CO₂, and will likely prove insightful if pursuing further advancement of this approach.

An additional advantage of using the approach of a closed culture platform for 96 h of uninterrupted culture is maintenance of stable pH and CO₂ levels, providing a more stable culture environment. Opening and closing of large incubators results in

evacuation of atmosphere and manipulations of cells in Petri dishes outside the incubator can result in damaging pH rises. It has been reported that use of closed modular chambers improved mouse embryo development compared to culture in large incubators, presumably due to increased stability of environmental condition [29]. The same may hold true for the closed culture system used in this study. However, one limitation with the rudimentary approach used is the reduced ability to adjust/monitor CO₂ levels. We examined use of enhanced buffering media with HEPES:MOPS, but no difference in pH or development were observed from bicarbonate-only buffered media. Furthermore, monitoring of embryo development is restricted. Though optical clarity of the test tubes does allow for visualization of developing embryos, resolution and detail is limited. Additionally, ability to isolate or monitor individual cells is problematic in the current format. However, adjustments could be made to the platform and incorporation of a closed culture system with chemically generated CO₂ in a small sealed Petri dish with individual wells or chambers to house embryos, surrounding a central CO₂ generating well would improve visibility and embryo tracking. Devices could be housed in small, simple, warming boxes to maintain temperature, similar to prior approaches at developing portable field incubators, but without the need for external cylinders of gas. This approach may be amenable for development of a self-contained culture platform, perhaps in conjunction with emerging microfluidic technology.

In conclusion, preliminary findings in this technical report demonstrate that chemically generated CO₂ from a simple reaction using common laboratory ingredient can be used to efficiently culture mouse embryos and offers the opportunity to supply a pure and inexpensive gas source compared to current traditional techniques. This approach may lend itself to establishment of new field laboratories or offer a back-up gas supply mechanism to current labs. Furthermore, this approach may lead to novel environmentally stable embryo culture platforms, without the use of traditional large

gas-filled incubators or external gas cylinders.

Methods

Construction of device

A closed embryo culture system was constructed consisting of two 15 ml screw-cap test tubes, connected by Tygon tubing fitted through holes in the caps (Fig. 3). Holes were sealed with a silicone adhesive and allowed to off-gas.

Chemical titration and pH measurement

To initially determine approximate amount of chemical reagent needed to obtain appropriate pH (7.20–7.40), 1 ml of culture medium (KSOMAA; Ho *et al.* 1995) + 10% Serum Substitute Supplement (SSS, Irvine Scientific, Irvine, CA, USA) was placed into one tube, while 3 ml of H₂O was placed into the second tube. Varying amounts of reagent (0.025, 0.05, 0.1 g) were obtained by breaking small portions from the large Alka Seltzer® tablet, then added to the tube containing H₂O to generate CO₂, which was subsequently supplied to the tube containing culture medium by the connecting tubing. The chemical reaction utilizes the acid:base reaction: $\text{HCO}_3(\text{aq}) + \text{H}^+(\text{aq}) \rightarrow \text{H}_2\text{O}(\text{l}) + \text{CO}_2(\text{g})$ and goes to completion in seconds. Therefore, for ease of reagent measurement and ability to add reagents quickly to the tube in a repeatable fashion before closing, the majority of subsequent experiments utilized small portions obtained from the larger tablet of Alka Seltzer®. Each whole tablet contains 1000 mg citric acid, 325 mg acetylsalicylic and 1918 mg of bicarbonate. Because each tablet weighs ~3.24 g, the amounts of tablet used (0.025, 0.05 and 0.1 g) contain ~10.2, 20.4 and 40.9 mg of total acid, respectively, which reacts with equal amounts of sodium bicarbonate (1:1 ratio). Excess bicarbonate in the tablet is not utilized in generating CO₂. Tubes were placed into a 37 C ungasd warming oven and pHe of culture media measured ~24 h and later to allow sufficient equilibration.

Furthermore, to further define the chemical reaction for greater reproducibility, use of 20 mg of citric acid and sodium bicarbonate, were enclosed in a cellulose capsule and added to the closed system to verify whether these raw reagents could reproduce the reaction and provide a reproducible pHe in the range of 7.2–7.4, similar to use of 0.05 g of the tablet. Encapsulation of the reagents allowed for easy addition to the H₂O tube, permitting the tube to be closed before the chemical reaction could proceed/complete, thereby improving repeatability.

Based on initial dose titrations using small pieces of tablet, subsequently 0.03 g of Alka Seltzer was utilized for the remainder of the developmental experiments. pHe was measured following 24 h of culture and to verify pHe stability in the closed-system device, pHe was measured in a second tube 96 h later to verify it remained within the 7.2–7.4 desired range. pHe of all media was measured directly within the test tubes following electrode calibration with pH standards (7 and 10) using a double junction glass KCl filled electrode warmed to 37 C. Experiments were performed 3–5 times and pHe values averaged. Data are presented as the mean ± SEM.

Embryo culture

Subsequently, 0.03 g of the Alka Seltzer reagent was used to equilibrate 1 ml of media for 24 h prior to adding frozen/thawed 1-cell mouse embryos (B6C3F1; Embryotech Havervill, MA, USA).

Five to six embryos were cultured per tube in pre-equilibrated medium, an additional 0.03 g of reagent was then added to the H₂O and tubes re-capped. To reduce variability and ensure appropriate atmosphere, the tube containing the embryos was capped first, while the tube containing the water and chemicals was capped second, immediately following addition of the reagent tablet. Treatments included KSOMAA + 10% SSS and a double-buffered medium consisting of KSOMAA + 10% SSS + 10 mM HEPES + 10 mM MOPS buffers to help further stabilize medium pHe. Both media contained 25 mM bicarbonate and pHe values were similar. Following 96 h of uninterrupted culture, in order to maintain the CO₂ atmosphere and pHe, embryos were removed, graded for development, and assessed for total cell number. Positive controls consisted of an unsealed test tube of KSOMAA + 10% SSS in a CO₂ incubator (pH 7.2–7.4), while negative controls consisted of a sealed test tube and no CO₂ (pH 8.1–8.3). Each treatment utilized a total of 27 embryos, data were collected over 5 replicates and presented as the mean ± SEM.

Blastocyst cell count

For total cells counts, blastocysts were exposed to 0.9% sodium citrate for ~5 min and then placed into methanol:acetic acid:water (3:2:1) for ~10 sec. Blastocysts were placed onto a microscope slide and allowed to air-dry before placement in methanol:acetic acid (3:1) for 24 h. Slides were stained for 10 min with aceto-orcein and cells counted on a brightfield microscope. Data were analyzed using ANOVA followed by Bonferroni multiple comparison test.

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