MAINTENANCE OF EMBRYONIC CHICK DUODENUM IN LARGE SCALE ORGAN CULTURE

Submitted by

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I. INTRODUCTION

This method was developed specifically for the study of the vitamin D_3-mediated, intestinal calcium absorptive mechanism under precisely controlled *in vitro* conditions completely isolated from systemic influences. The approach has the distinct advantage of preservation of both structural and functional integrity of large amounts of tissue in a defined medium for reasonably long time periods. Most importantly, results obtained with this system are in accord with results obtained *in vivo* (1-5). The techniques described herein could very likely be applied with some degree of success to a variety of other problems involving intestinal function. The procedure described here is limited to the preparation and maintenance of embyronic chick duodenum, although additional information is provided to conduct assays.

II. MATERIALS

A. Embryonated eggs

One-day-old White Leghorn embryonated eggs[1] are incubated at 37.5° C and 60% relative humidity in an incubator (Petersime No. 1[2]) in which they are automatically rotated nearly 180° every 2 hours. Viability is approximately 90% through day 20 (the day before hatching), the day the embryos are used.

B. Culture medium

The most nearly optimal medium tested thus far consists of Waymouth Medium 752/1 (GIBCO[3]), obtained in powder form without calcium and phosphate salts so that these critical ions may be varied at will.

C. Culture medium preparation

The medium is reconstituted as follows:

Rinse powdered medium (10 liter packet) into clean pressure flask No. XX6700053 (Millipore[4]), with glass distilled water AG-11 (Corning[5]). Maintain stirring with magnetic bar on a magnetic stirrer through all additions.

Add 22.4 g $NaHCO_3$ and 2.5 g KCl (Mallinckrodt A. R. grade[6]).

Add 20 g fatty acid free, bovine serum albumin (Miles[7]), and 5 mg oleate (as Na oleate, Sigma[8]). These latter ingredients have been found to produce optimal growth in a cell culture system (6).

Adjust pH to 7.2 with 10 N-NaOH (Mallinckrodt A. R. grade[6]).

Filter through a sterile 142 mm diameter, 0.22μ Millipore filter with asbestos prefilter held in a 142 mm Millipore filter holder (No. YY22 142 00[4]), into sterile bottles (Vitro 400 Wheaton[9]). Sterile medium may be kept at 4°C for up to 6 months.

Adjust calcium and phosphate concentrations (usually 1.25 and 0.625 mM, respectively) at the time of an experiment using sterile solutions of $CaCl_2 \cdot 2H_2O$ and NaH_2PO_4 (Mallinckrodt A. R. grade[6]) of 1000 x greater molarity than desired in the final medium.

Also, at the time of an experiment make the following additions from 1000 x concentrated solutions to give the following medium concentrations: neomycin sulfate, sodium penicillin G and

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[1] Babcock International Inc., Ithaca, NY
[2] Petersime Incubator Co., Gettysburgh, OH
[3] Grand Island Biological Co., Grand Island, NY
[4] Millipore Corporation, Bedford, MA
[5] Corning Glass Works, Corning, NY
[6] Mallinckrodt Chemical Works, St Louis, MO
[7] Miles Laboratories, Elkhart, IN
[8] Sigma Company, St Louis, MO
[9] Wheaton Scientific, Millville, NJ
[10] E. R. Squibb and Sons, New York, NY
[11] Falcon Plastics, Oxnard, CA
[12] Newark Wire Cloth Co., Newark, NJ
[13] Cole-Parmer, Chicago, IL
[14] Linbro Chemical Co., Inc., New Haven, CT
[15] Baker Co. Inc., Sanford, ME
[16] Clay-Adams Inc., New York, NY
[17] National Appliance Co., Portland, OR
[18] TRI-R Instruments, Inc., Rockville Centre, NY
[19] Amersham-Searle, Chicago, IL

streptomycin sulfate (Sigma[8]) at 100, 50, and 50 µg/ml, respectively, and Mycostatin, Nystatin (Squibb[10]) at 100 U/ml.

D. Culture apparatus

The culture apparatus consists of a 20 x 100 mm plastic Petri dish (Falcon[11]), in which is placed a specially designed, rectangular stainless steel grid (51 x 63.5 mm surface with 63.5 mm legs formed by making 90° angle bends in the long dimension; the grid material is 60 mesh stainless steel, Newark[12]). Wash the grids thoroughly in A.R. grade solvents (EtOH, Acetone, Hexane, Mallinckrodt[6]) and then sonicate with an ultrasonic cleaner[13] in detergent solution (7X O-Matic[14]) for two hours, rinse for 2 hours in tap water, and follow by repeated rinses in glass-distilled water. Repeat detergent wash cycle after use; the grids are reusable indefinitely.

E. Other materials

Zephirin Chloride (0.135%)
Fine scissors, No. C-1223 Clay-Adams[16]
Incubator, CO_2 [17]

III. PROCEDURE

A. Culture technique

The entire procedure, from preparation of experimental media through the setting up of the cultures, is done in a laminar flow hood (Edgeguard[15]), prepared in advance to contain all needed materials.

1. Rinse the incubated eggs on an egg tray with a zephirin chloride (0.135%) solution in 50% EtOH before placing them in the hood.

2. Crack open the eggs with large forceps and remove the embryo headfirst. Remove the yolk sac with a second pair of forceps and drop the embryo on its back into a sterile, dry, plastic Petri dish.

3. Excise the duodenum, transfer to a small amount of basal medium in a Petri dish and remove the pancreas using scissors and fine curved forceps, grasping only the cut ends of the duodenum with the forceps.

4. Transfer the duodenum to the surface of a grid inside a Petri dish into which has been poured approximately 40 ml of appropriate medium (just sufficient to wet the bottom of the grid). Slit the duodenum lengthwise with fine scissors and arrange mucosal-side-up. A total of four duodena (approx. 400-500 mg tissue) may be placed on each grid. Arrange the tissue such that the medium makes only bare contact with its serosal surface.

5. Cover the dishes and place in a humidified incubator at about 90% relative humidity, 37.5°C, continuously gassed with a CO_2, O_2, air mixture containing 5% CO_2 and 50% O_2.

6. The pH of the medium at the start varies from 7.4-7.8 and should never drop below pH 7.1 during incubation. The effect of lower pH with this technique is unknown, but lower pH's were detrimental (reduced vitamin D-responsiveness) in early organ culture techniques using sealed flasks containing only air.

7. The cultures should not be disturbed for up to 48 hours during which there is a linear production of the vitamin D_3-induced calcium-binding protein (CaBP) (3). Changes of medium have been found deleterious in terms of CaBP induction.

B. Assays

Duodena cultured as described have been used to study the vitamin D_3-mediated calcium absorptive mechanism (1-5). A specific calcium-binding protein (CaBP), originally discovered in rachitic chick duodenum after an acute dose of vitamin D_3 (7), is induced *de novo* (2) in the cultured duodenum when incubated in the presence of vitamin D_3 or related sterols (3). In the absence of vitamin D_3-sterol (EtOH diluent at 0.1% of the medium; Control cultures), CaBP is not induced; there is no CaBP in the embryonic chick duodenum during development until after hatching (8).

A number of assays have been performed on the cultured duodena. First, rinse some of the duodena from each treatment with an ice-cold buffer solution, and lightly blot on tissue and weigh. Homogenize the duodena individually in several volumes of ice-cold buffer in all ground glass, homogenizer tubes with motor-driven, ground glass pestles (TRI-R[18]). During the 90 second homogenization, keep the material in an ice bath.

1. CaBP

CaBP is assayed by a radial immunodiffusion technique as described in detail elsewhere (3).

2. cyclic AMP

Recently, the action of vitamin D has been linked to the adenyl cyclase system

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and ambient calcium concentration (4, 5). Specific details for cAMP assay of freeze-dried, cultured duodenum have been published (4, 5).

3. Alkaline phosphatase
   Both *in vivo* and in organ culture, vitamin D has been shown to stimulate alkaline phosphatase activity. Details of alkaline phosphatase assay on homogenates of cultured duodenum have already appeared (3).

4. Total protein, total DNA and radiolabeled DNA and protein synthesis have been monitored in the cultured duodenum, as a function of treatment, by previously published techniques (3, 4).

5. Other assays on the duodenal homogenates that have been performed:
   a. Maltase (9)
   b. Acetyl cholinesterase (10)
   c. Thymidine kinase (11)

   Of these latter assays, only thymidine kinase activity has been found to increase in response to vitamin D_3 or 1,25-$(OH)_2$-vitamin D_3 (R. A. Corradino, 1975; unpublished).

   In all assays, levels of activities found in cultured duodena compare favorably with those found in uncultured duodena of the same embryonic age.

6. Radiocalcium uptake
   After a period of culture, transfer the duodenum to a 50 ml Erlenmeyer flask with 3 ml of a buffer solution containing a trace amount of ^{45}Ca (Amersham/Searle[19]). After 30 minutes, remove the tissue, rinse with ice-cold buffer (no ^{45}Ca), blot and weigh. Count the tissue by techniques already detailed (3). Duodena maintained for as little as 6 to 12 hours of culture in the presence of vitamin D-like sterols exhibit increased uptake of radiocalcium over controls (1-5).

   Uptake of other radiolabeled nutrients, including ^{32}P and ^{59}Fe have also been measured and been shown to be stimulated by vitamin D_3 (3).

IV. DISCUSSION

The described culture technique has proven uniquely suited to the study of the vitamin D_3-mediated calcium-absorptive mechanism (1-5). There is every reason to expect that the procedure would be adaptable to the study of factors affecting intestinal cell structure and/or a variety of intestinal functions. The system is one for maintenance of duodenal structure and function, however, since cell proliferation is reduced (3).

The clear advantages of the system are that the tissue survives intact for a sufficiently long period of time under rigidly defined conditions, isolated from systemic influences at least from the initiation of culture, such that specific intestinal functions can be assessed and that direct vs. indirect actions of treatment on the intestine can be easily distinguished.

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