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A novel whole-embryo culture model for pharmaceutical and developmental studies

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ABSTRACT

Introduction: This report introduces a new vertebrate whole-embryo culture model for the direct application of pharmaceuticals and/or toxins into developing embryos. This method uses a terrestrial amphibian system and therefore has eliminated the problem of mammalian placental and uterine concerns. To test the utility and effectiveness of this method, we investigated the effect of fluoxetine on craniofacial development. Fluoxetine is one of the most commonly prescribed selective serotonin reuptake inhibitor (SSRI) on the market and treatment of depression during pregnancy is commonly deemed necessary. Previous studies have shown that SSRIs may promote developmental defects and congenital malformations of the heart. **Methods:** This model utilized the egg/embryos of the directly developing Puerto Rican coquí frog, *Eleutherodactylus coqui*. The *E. coqui* embryo clutches were placed on filter paper in a Petri dish and were directly exposed (chronically) to fluoxetine concentrations ranging from 0.10 mM to 1.0 mM. Traditional whole-mount bone (Alizarin red) and cartilage (Alcian blue) staining was utilized to show the effect of fluoxetine on craniofacial development. **Results:** Whole-mount staining revealed profound defects in cartilage development, particularly in the nasal capsule, mandible, and the brain case. Further, fluoxetine-treated embryos developed significantly slower compared to control animals. **Discussion:** We found that the *E. coqui* culture model was an effective and sensitive technique for pharmaceutical studies, particularly since it allows the direct application of drugs and toxins into the developing embryo without the hindrance of the uterus and placenta. Chromatographic analysis revealed that fluoxetine infiltrated and penetrated embryonic tissue. It was found that altering serotonergic activity during development, via fluoxetine, stunted craniofacial development and organization.

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1. Introduction

Investigations have shown that monoaminergic systems, particularly serotonin, are essential for normal neuronal development (Buznikov, Shmukler, & Lauder, 1996; Kruzelock & Barbato, 1991; Lauder, 1993; Lauder & Bloom, 1974; Nebigil et al., 2001; Revilla, Díez-Alarcia, Mostany, Pérez, & Fernández-López, 2001; Siuciak, Gamache, & Dubocovich, 1992; Ten Eyck, Jermakowicz, Chinn, & Summers, 2005). Serotonin (5-HT) has been found in both vertebrate and invertebrate zygotes and has been shown to regulate early embryological events (Buznikov et al., 1996; Lauder, 1993) and bone morphogenesis (Yadav et al., 2008). In particular, 5-HT, 5-HT_{1A} and 5-HT_2 receptors, and 5-HT_T (transporter) are detectable as early as blastocoele and gastrulation events in amphibians, chicks, fish, rodents, and sea urchins (Moiseiwitsch & Lauder, 1995; Nebigil et al., 2001; Ten Eyck et al., 2005).

It has been proposed that the use of antidepressant drugs alters neural development (Choi, Ward, Messaddeq, Launay, & Maroteaux, 1997; da Silva, Gonçalves, Manhaes-de-Castro, & Nogueira, 2010; Hensler, 2002; Moiseiwitsch & Lauder, 1995). Selective serotonin reuptake inhibitors (SSRIs) are the most commonly prescribed antidepressant drugs in the United States (Homberg, Schubert, & Gaspar, 2010; Pastuszak et al., 1993). Fluoxetine (Prozac®) is one of the most widely prescribed SSRIs, and it is frequently prescribed for pregnant women as it has longer half-life than any other SSRIs in the market, which equates as a lower risk of discontinuation syndrome (Lattimore et al., 2005). However, the longer half-life also means long duration of effect, which could increase the risk of endangering the fetus due to chronic drug treatment during pregnancy.

Previous studies have examined the effect of SSRIs in developmental studies in mammalian models. Moiseiwitsch and Lauder (1996) investigated the effect of SSRIs on craniofacial development using mouse embryos and showed that fluoxetine prevented dental development. Another study using mouse whole-embryo cultures demonstrated that exposure to SSRIs, including but not limited to fluoxetine, can cause craniofacial malformations (Shuey, Sadler, & Lauder, 1992). These models have yielded valuable information; however, due to uterine and placental barriers, mammalian culture techniques require

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extraction of embryos and/or portions of the fetus from the uterus. This extraction procedure can prove risky, laborious, and may impose stressful conditions on the embryo or tissues.

We have developed a culture technique that allows the direct application of drugs or other pharmaceuticals into the developing vertebrate embryo. This culture model utilizes eggs of the Puerto Rican coquí frog, *Eleutherodactylus coqui*. These frogs possess many features that make them ideal as animal models. The eggs/embryos from this amphibian are laid by the female, but the free-swimming, aquatic tadpole stage has been eliminated. The egg/embryo is covered by a gelatinous sac containing perivitelline fluid; these embryos directly develop into the adult phenotype, hatching as terrestrial froglets (Jennings & Hanken, 1998). This eliminates the need to extract the embryos in any manner and eliminates additional stressful conditions. Adult frogs are simple to maintain in captivity, require no standing or running water, and readily reproduce in captivity.

The developmental stages of this amphibian have been describe and classified so that all embryos can be staged accurately (Townsend & Stewart, 1985). Many developmental studies have utilized *E. coqui*, and much of their development, particularly neural, bone, and cartilage ontogeny has been described (Elinson, 2013; Hanken, Klymkowsky, Summers, Seufert, & Ingebrigtsen, 1992; Kerney, Gross, & Hanken, 2010; Kerney & Hanken, 2008; Kulkarni, Singamsetty, & Buchholz, 2010; Ten Eyck et al., 2005). Whole-embryo culture and staining could also yield studies that more easily examine developmental stages. The use of living dyes with this model system could yield consecutive sampling possibilities. Thus, this model would also be an excellent system to use retro- and anterograde tracers, etc., to investigate neurological changes that occur in living organisms exposed to toxins and drugs. In addition, in utero models impose the barrier of maternal organ and homeostatic systems. This system offers the advantage of culturing these vertebrate eggs/embryos directly without the hindrance of a mammalian barrier (i.e., uterus and the placenta). These anurans are indigenous to the island of Puerto Rico but were accidently introduced on the island of Hawaii where they are an alien pest, thus making them accessible and readily available for collection.

The objective of this culture technique description is to display the simplicity of the system yet illustrate the versatility and opportunity that the model can bring to developmental and pharmaceutical biology. In this demonstration, we will treat developing embryos with the SSRI fluoxetine using the proposed method to determine the effect of fluoxetine on bone and cartilage development. This paper will also demonstrate the two methods we used to validate the efficacy of our proposed method: (1) gas chromatography/mass spectroscopy (GC/MS) to validate that direct application of the drug penetrated and infiltrated the tissue, and (2) Alizarin red and Alcian blue whole-mount staining to show the effect of fluoxetine on cartilage and bone development.

2. Materials and methods

2.1. Animals

Clutches of *E. coqui* embryos were collected from the University of Hawaii-Hilo Daniel K Inouye College of Pharmacy (UHH DKICP) research field site (250 m); approximately 7 km SW of Hilo, HI. These clutches were the result of spontaneous mating between wild adults in the UHH DKICP experimental *E. coqui* research area. The care of all animals was carried in conformity with the regulations of the National Institutes of Health Guide for the Care and Use of Laboratory Animals and the University of Hawai'i IACUC.

2.2. Chemical and Reagents

Aqueous solutions were prepared using double deionized water (DDW) obtained from a Milli-QTplus Ultra-Pure Reagent Water System (Millipore®, Continental Water Systems, El Paso, TX). Fluoxetine HCl for pharmaceutical treatment was purchased from Sigma (Sigma-Aldridge Co., St Louis, MO). Fluoxetine methanolic standards were purchased from Cerilliant (Cerilliant Corp., Round Rock, TX) at a concentration of 1.00 mg/mL in sealed glass ampules. Fluoxetine-d6 methanolic standard was purchased from Cerilliant at a concentration of 0.100 mg/mL in sealed glass ampules. The derivatization reagent, pentafluoropropionic anhydride (PFPA), was obtained from Sigma (Sigma-Aldridge Co., St Louis, MO). Methanol, acetonitrile, ammonium hydroxide, acetic acid, ethyl acetate, sodium fluoride, and potassium phosphate monobasic were purchased from Fisher Scientific (Pittsburgh, PA). The pH of all solutions was measured using a Denver Instrument UB-5 bench top pH meter (Denver Instrument, Bohemia, NY) connected to a pH electrode.

2.3. Culture technique

Embryos from collected *E. coqui* clutches were staged under a dissecting microscope (Leica S8APO) according to Townsend and Stewart (1985); staging table ranges from TS 1 to TS 15, 1 = newly laid egg, 15 = hatching. These are very specific developmental stages based on both behavioral and morphological criteria. Clutches typically average 28 eggs/embryos (Townsend & Stewart, 1985). The *E. coqui* eggs/embryos were placed on moist filter paper in a polystyrene Petri dish (100 mm × 15 mm). All clutches were split into two groups, control and experiment. Application of control and experimental solutions was applied directly to the eggs/embryos so that the filter paper remained damp and allowed a coat/layer of solution to adhere to the gelatinous covering of the egg/embryo. The top of the Petri dish was secured and cultures were kept in a dark incubator at ambient temperate (Fig. 1). For this study, control embryos from each clutch of eggs were allowed to develop from TS 1 (initiation of experiment) through TS 15 (termination of embryogenesis) and then were fixed in 10% formaldehyde. The respected experimental embryos that corresponded with controls were subsequently staged and fixed in 10% formaldehyde at the same time.

2.4. Pharmaceutical treatment

Fluoxetine HCl was dissolved in embryo culture water (40% DDW and 60% tap water) to prepare the following concentrations: 0.10 mM, 0.25 mM, 0.50 mM, and 1.0 mM. Three sets of 10/embryos (all TS-1) per treatment group were used for both experimental and controls in all treatment concentrations. The embryos were laid directly on filter paper within a Petri dish; embryos and filter paper were kept moist within the culture dish by direct application of fluoxetine solutions. The volume of solution was 1 ml, and treatment was every other day. Cultures were checked daily to ensure that both filter paper, and embryos were coated with solution. Control embryos received culture water only. The treatment ceased once the control embryos reached TS 15; this stage is well defined with specific developmental criteria (Townsend & Stewart, 1985) and is the terminal stage of embryogenesis. Both control and experimental embryos were processed as described below at the termination of the drug treatment.

2.5. Gas chromatographic/mass spectroscopic (GC/MS) conditions

Analyses of fluoxetine standards and embryo samples were performed using a GC/MS, which consisted of a Agilent Technologies 7890A GC, interfaced with an Agilent 7000 Triple Quad MS (Agilent, Palo Alto, CA). The GC/MS was operated with a transfer line temperature of 280 °C and a source temperature of 250 °C. The electron multiplier voltage was set at 1077 eV above the tune value. Chromatographic separation was achieved using an Agilent HP5MS capillary column 30 m × 0.25 mm i.d., 0.25 μm film thickness (Agilent Tech., Santa Clara, CA.). Helium and nitrogen was employed as the carrier gas and used at a flow rate of 2.25 and 1.5 mL/min, respectively. An Agilent

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