Journal of Pharmacological and Toxicological Methods 70 (2014) 276–282

Contents lists available at ScienceDirect

Journal of Pharmacological and Toxicological Methods

journal homepage: www.elsevier.com/locate/jpharmtox

Original article

A comparison of drug-induced cardiotoxicity in rat embryos cultured in human serum or protein free media

Helen E. Ritchie [a,*], Cecilie Haaland Svensson [b], Mats F. Nilsson [c], William S. Webster [b]

[a] Discipline of Biomedical Sciences, Sydney Medical School, University of Sydney, PO Box 170, Lidcombe, NSW 1825, Australia
[b] Discipline of Anatomy and Histology, Sydney Medical School, University of Sydney, Sydney, NSW 2006, Australia
[c] Drug Safety and Toxicology, Department of Pharmaceutical Biosciences, Uppsala University, Sweden

ARTICLE INFO

Article history:
Received 4 April 2014
Accepted 23 July 2014
Available online 1 August 2014

Keywords:
In vitro screens
Teratogenicity
Pharmaceuticals
Methods
Whole rat embryo culture amiodarone
Citalopram
Dofetilide

ABSTRACT

Introduction: Although much reproductive toxicology research is performed in live animals there is increasing use of in vitro techniques primarily to identify potential hazards with human exposure. As many in vitro studies are undertaken using protein free media, the standard protocol is to compare the effect concentration determined in vitro with the predicted therapeutic free plasma concentration in humans. The aim of the present study was to test this rationale by comparing the effect of a small number of therapeutic drugs on heart rate of rodent embryos cultured in human sera or protein free serum. **Methods:** Whole rat embryos were cultured in protein-free media or human serum to which drugs (amiodarone, citalopram, dofetilide, haloperidol, paroxetine, quetiapine, or trazodone) known to induce embryonic bradycardia were added. Embryonic heart rate was observed before and after addition of drugs. **Results:** Most of the tested drugs (5/7) caused a greater decrease in embryonic heart rate in human sera than predicted based on the protein binding of the drug. **Discussion:** The results suggest that there is less unbound drug in the protein free media and/or more unbound drug in the human sera than predicted. Variables such as saturated protein binding and pH cannot fully explain our results. Since the results did not validate the original rationale, reproductive toxicity results obtained using protein free in vitro techniques may not have the large safety factors predicted on the basis of protein binding.

1. Introduction

The advent of Registration, Evaluation, Authorization, and Restriction of Chemicals (REACH) in the European Union (EU) has highlighted the need for the development of suitable in vitro systems particularly with respect to developmental toxicity (Scialli & Guikema, 2012). In vitro studies also play an increasing role in toxicology as they provide a key role in implementation of the 3Rs strategy in research by reducing the number of live animals used. These in vitro studies primarily identify potential hazards when related to human exposure. This in vitro data on drug toxicity can then be used to calculate margins of safety based on human exposure data. When the exposure data is in the form of blood levels, the usual practice is to take protein binding of the drug into account since it is the unbound drug which is the determinant of activity (Kramer et al., 2013; Redfern et al., 2003). However, the validity of this approach has been questioned. For example markers of cardiac toxicity (hERG potency, QT prolongation) of some antipsychotics are better correlated with total plasma concentrations than predicted free concentrations (Kongsamut, Kang, Chen, Roehr, & Rampe, 2002).

In recent studies we have used an in vitro system to identify therapeutic drugs which are potentially hazardous to the function of the embryonic heart. In this assay, whole rat embryos are exposed to therapeutic drugs with ion channel blocking properties and the effect on the function on the embryonic heart is observed and recorded (Ababneh, Ritchie, & Webster, 2011; Abela et al., 2010; Gunnstrom, Ababneh, Webster, Oakes, & Ritchie, 2012). The impetus for these studies is that many therapeutic drugs block ion channels, sometimes as a desired therapeutic effect or as an unwanted side-effect. The potency of the blockade partly determines the risk in human pregnancy (Karlsson et al., 2007; Nilsson et al., 2010).

Generation of the cardiac action potential in both adult and embryonic hearts is dependent primarily on sodium, potassium and calcium ion channels but the adult and embryo do not necessarily use the same channels or have the same sub-types of the channels. For instance, during the organogenic period in rats the embryonic heart is highly dependent on the potassium channel hERG[1] for normal repolarization (Abrahamsson et al., 1994; Danielsson et al., 2013) while the adult rodent heart relies mainly on different potassium ion channels (Nerbonne & Kass, 2005). The situation in humans is somewhat different with an apparent reliance on the hERG potassium channel in both adults and fetuses (Danielsson et al., 2013). If an ion channel blocking drug affects

* Corresponding author. Tel.: +61 2 9351 9476; fax: +61 2 9351 9520.
E-mail address: Helen.ritchie@sydney.edu.au (H.E. Ritchie).

[1] Abbreviations: hERG: human ether a-go-go-related gene; DMEM, Dulbecco's Modified Eagle's Medium, EHR, embryonic heart rate; DMSO, dimethyl sulfoxide; GD, gestational day.

http://dx.doi.org/10.1016/j.vascn.2014.07.008
1056-8719/

the functioning of the embryonic heart at the same or lower exposure levels than the adult heart then the drug represents a risk in pregnancy assuming equivalent exposure. An example is the antiarrhythmic drug dofetilide which blocks the hERG potassium ion channel. If the drug is administered to a pregnant rat during the organogenic period it causes bradycardia and arrhythmia of the embryonic heart without any adverse effects to the dam (Ritchie, Ababneh, Oakes, Power, & Webster, 2013). The resultant circulation disturbances and hypoxia in the embryo are responsible for the congenital malformations observed at term.

We have examined over 40 drugs, mostly antidepressants and antipsychotics using this in vitro rat embryo culture system. All the tested drugs blocked hERG potassium and/or sodium ion channels (not necessarily as their therapeutic effect) and caused a concentration-dependent bradycardia in the rat embryonic heart (Ababneh et al., 2011; Abela et al., 2005; Gunnstrom et al., 2012). For each drug we determined the concentration that caused severe bradycardia down to the no-effect concentration. This methodology clearly identified these drugs as potential hazards for human pregnancy since unwanted embryonic bradycardia can potentially lead to growth retardation, malformation and/or embryonic death (Ritchie et al., 2013; Webster & Abela, 2007).

We then calculated the margin of safety by comparing the concentration that caused bradycardia in vitro with human therapeutic plasma concentrations. Since the embryos were cultured in a protein free solution it was assumed that the tested drugs were in the "free (unbound) state" and hence the correct comparison was with "free drug" concentration in the human. When this comparison was made most of the drugs showed a very large margin of safety between concentrations affecting the embryonic heart and therapeutic concentrations.

In this report we examine a small number of therapeutic drugs that caused bradycardia in the embryo culture system and compare the effect on embryonic heart rate (EHR) between embryos cultured in human sera or in a protein free solution (Dulbecco's Modified Eagle's Medium, DMEM). The margin of safety is then calculated based on in vivo reported maximum free drug plasma concentrations for each compound. For the embryos in DMEM all the added drug is assumed to be free. If the estimation method is accurate then the calculated margins of safety should be similar for embryos cultured in human serum and protein free DMEM.

2. Materials and methods

2.1. Animals

The University of Sydney Animal Ethics Committee approved all animal work in this study. Sprague-Dawley rats were mated overnight and examined the next morning for a sperm-positive vaginal smear. The rats that had mated were separated and this day was considered gestational day (GD) 0.

2.2. Test chemicals

The drugs chosen for study covered a range of protein binding (64–99.9%). Paroxetine, haloperidol, trazodone and citalopram were purchased from Sigma Aldrich Australia Pty (Castle Hill, Australia). Dofetilide was obtained from Pfizer Australia (West Ryde, Australia). Quetiapine fumarate was obtained from Hangzhou Dayangchem Co. Ltd. (China). Quetiapine fumarate contains two molecules of quetiapine so 2.5 μM of quetiapine fumarate was considered to be equivalent to 5 μM of quetiapine. Amiodarone (Cordarone Sanofi-Aventis, Australia Pty Ltd., Macquarie Park, Australia) was obtained as an IV formulation. One milliliter contained 50 mg of amiodarone hydrochloride, 20.2 mg of benzyl alcohol, 100 mg of polysorbate 80, and water. Dofetilide, paroxetine, and citalopram were dissolved in distilled water. Amiodarone was diluted with distilled water. Haloperidol, quetiapine and trazodone were dissolved in dimethyl sulfoxide (DMSO).

The citalopram concentrations used in this study were determined from preliminary studies which established a no-effect concentration and a concentration that caused approximately 50% reduction in heart rate. The concentrations to be tested for the other drugs were selected from previous publications as concentrations that caused a significant reduction in heart rate when the drug was administered in DMEM (Ababneh et al., 2011; Abela et al., 2010; Gunnstrom et al., 2012).

Each drug stock solution was made up at the start of each experiment and then serially diluted with DMSO or water so that a standardized volume of 50 μl was added for each tested concentration. The following stock solutions were made: amiodarone (8 mM), citalopram (2.46 mM), dofetilide (200 nM), haloperidol (1 mM), paroxetine (1 mM), quetiapine (4 mM), and trazodone (2.5 mM).

DMEM (D1145, Sigma–Aldrich, St Louis, USA) Dulbecco's Modified Eagle's Medium (DMEM) is a cell culture medium containing amino acids, vitamins, inorganic salts supplemented with 4500 mg/L, glucose. The pooled, normal human serum (Innovative Research, Novi, MI 48377) was heat inactivated (56 °C for 30 min) and supplemented with glucose (3 mg/ml).

The pH of the DMEM and human serum was measured after 1 h of gassing with 95% $O_2$and 5% CO_2 for each of the drugs at the highest concentration used. For DMEM the pH values were all ~7.8 and for human serum 7.6–7.7.

2.3. Whole embryo culture

On GD 13, the pregnant dams were anesthetized by CO_2 inhalation and killed by cervical dislocation. The decidua and Reichart's membrane were removed from each embryo, leaving the embryo with an intact yolk sac and ectoplacental cone. A small incision was made in the yolk sac avoiding damage to major blood vessels. The embryo was then eased through this incision and the amnion was opened. Each embryo was cultured singly in a glass culture bottle containing either 2.5 ml DMEM (Sigma–Aldrich, St Louis, USA) or pooled, normal human serum (Innovative Research, Novi, MI 48377). The embryos in their culture bottles were placed in a rotating culture system (BTC Engineering, Cambridge, UK). The culture bottles were rotated at 11 rotations per minute and were continuously gassed with 95% O_2 and 5% CO_2 at 38 °C (Abela et al., 2010).

After 1 h in the incubator each embryo, still in its culture bottle, was examined under a dissecting microscope (Leica M 420, Leica Microsystems Ltd., Heerbrugg, Switzerland), equipped with a heating stage to maintain the temperature of the culture fluid at 38 °C. The dissecting microscope was also equipped with a video recorder (Olympus DP70, Olympus Australia Pty Ltd., Melbourne, Australia). The heart of each embryo was recorded for 20 s. Each culture bottle with its single embryo was administered one of the test chemicals or control vehicle (water, DMSO or IV solvent) at a standardized volume of 50 μl and then replaced in the incubator for a further 1 h before re-examination. Ten embryos at each concentration in each medium were used and the treatment order was randomized prior to administration of chemical.

2.4. Image analysis of cardiac effects

Embryos that had a heart rate of ≥180 beats/minute (bpm) after the first hour in culture (pre-treatment) were considered undamaged and were used in the experiment. The video recordings of the embryonic hearts were made at 30 frames/second and analyzed using software developed at the University of Uppsala (Khan, Nilsson, Danielsson, & Bengtsson, 2008). The atria and ventricles of the heart were identified on the video image and were manually marked. Movement of that region was then detected by the program and recorded as a plot during a 15-s period. The program measures light–dark intensity changes on a relative scale (0–1) in the selected area of the heart against time (s). Traces of atrial and ventricular activity are ultimately created by the image analysis program with one (1) representing a filled compartment (end-diastole) and zero (0) representing a fully emptied compartment (end-systole).