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PHENYTOIN COVALENT BINDING AND EMBRYOPATHY IN MOUSE EMBRYOS CO-CULTURED WITH MATERNAL HEPATOCYTES FROM MOUSE, RAT, AND RABBIT*

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Abstract—The anticonvulsant drug phenytoin is teratogenic in a variety of species including humans. Traditional embryo culture studies have employed the addition of 9000 *g* supernatant (S-9) or microsomal fractions from induced rat or mouse liver as an exogenous bioactivating system to approximate a maternal contribution. However, cellular fractions, unlike cultured intact hepatocytes, may themselves be embryotoxic, and do not reflect the *in vivo* balance of bioactivation and detoxification. To evaluate *in vitro* the known *in vivo* differential species susceptibility to phenytoin teratogenesis, day 9.5 (day of plug = day 1) mouse embryos either were cultured alone for 24 hr or were co-cultured with hepatocytes from maternal mice, rats or male rabbits, thereby exposing the embryos to the effects of potential species-specific phenytoin metabolism. In the absence of hepatocytes, phenytoin embryotoxicity was concentration dependent (0, 10, 20 and 60 μg/mL), with decreases in embryonic growth, reflected by reduced yolk sac diameter and crown rump length, apparent within the maternal therapeutic range (20 μg/mL). Covalent binding of the radiolabeled drug to live embryonic tissue was significantly higher than in control embryos previously killed by fixation, suggesting that the embryo can bioactivate phenytoin to a toxic reactive intermediate. Mouse embryos grew equally well with hepatocytes from all three species, indicating interspecies tissue compatibility. The addition of rat and rabbit hepatocytes, but not mouse hepatocytes, significantly enhanced the phenytoin-induced impairment of mouse embryonic development, as demonstrated by reductions in somite number. The phenytoin-induced impairment of mouse embryonic growth was not enhanced by the addition of rat or rabbit hepatocytes, while mouse hepatocytes conferred protection. The covalent binding of phenytoin to extracellular proteins in the culture medium was not enhanced by the addition of mouse hepatocytes. These results suggest that mouse embryos intrinsically can bioactivate phenytoin to a toxic reactive intermediate, with embryopathic consequences. The protection conferred by maternal mouse hepatocytes suggests a species-specific maternal biochemical balance favouring detoxification that is not shared by rat and rabbit hepatocytes, which enhanced phenytoin embryopathy. Thus, while phenytoin teratogenicity likely involves embryonic bioactivation, maternal determinants may contribute variably to teratologic susceptibility in a species-specific manner.

Key words: phenytoin teratogenicity; phenytoin embryotoxicity; co-culture of hepatocytes with embryos; interspecies comparisons

Phenytoin (diphenylhydantoin), a drug of choice for the treatment of epilepsy, is teratogenic to different degrees in mice [1], rats [2], rabbits [3], chickens [4], and cats [5], as well as in humans, in whom a constellation of anomalies has been termed the FHS‖ [6–10]. In addition to species variability, there are remarkable strain differences in susceptibility to phenytoin teratogenesis [11–14], but the mechanism(s) underlying these differences, and the relative maternal and embryonic contributions, remain to be elucidated. An understanding of these teratologic determinants could help to explain the apparent predisposition of particular humans to the FHS [8, 9].

Several mechanisms have been proposed to explain the teratogenicity of phenytoin, including the reversible interaction of phenytoin itself with several possible receptors, and the enzymatic bioactivation of phenytoin via several pathways, producing a toxic reactive intermediary metabolite that reacts irreversibly with essential embryonic cellular macromolecules [for reviews, see Refs. 15–17].

According to the first hypothesis involving a reactive intermediate, phenytoin is bioactivated by cytochromes P450 (likely CYP2C9) to an electrophilic arene oxide intermediate that, if not detoxified by epoxide hydrolases, and possibly by glutathione *S*-transferases, can covalently bind to embryonic protein, thereby initiating teratogenesis. In studies of children exposed to phenytoin *in utero*, lymphocytes from children with major anomalies were more susceptible *in vitro* to phenytoin-induced cytotoxicity than those from children with minor or no anomalies, but only when cells were incubated with an exogenous source of P450 and essential cofactors, implicating P450-catalysed bioactivation as the mechanism of teratogenicity [8]. While these lymphocyte studies did not demonstrate a deficiency in epoxide hydrolase as the determinant of individual predisposition, a more recent human study of amniocytes from pregnancies involving phenytoin therapy found low

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‖ Abbreviations: FHS, Fetal Hydantoin Syndrome; PHS, prostaglandin H synthase; LPO, lipoxygenase; FBS, fetal bovine serum; and ANP, anterior neuropore.

(<30% of controls) epoxide hydrolase activity in all cases where children subsequently showed features of the FHS, whereas no evidence of the FHS was observed in children from pregnancies with higher epoxide hydrolase activity [9].

In the second hypothesis involving a reactive intermediate, phenytoin may be bioactivated by embryonic peroxidases, such as PHS and LPOs, producing a free radical intermediate that results in the formation of reactive oxygen species, which oxidize embryonic DNA, protein and lipid, thereby initiating teratogenesis [reviewed in Refs. 18–21]. Alternatively, an electrophilic reactive intermediate also may be produced via peroxidase-catalysed bioactivation, initiating teratogenesis via the covalent binding of phenytoin to DNA and protein [22]. Whether via covalent binding or oxidation, DNA is a particularly interesting potential molecular target, since transgenic "knock-out" mice deficient in the p53 tumor suppressor gene, which is required for DNA repair, are more susceptible to the embryotoxicity of phenytoin [23] and another DNA-damaging teratogen, benzo[*a*]pyrene [24].

In addition to reactive intermediate-mediated irreversible molecular damage, there is evidence that unmetabolized phenytoin may interact reversibly with receptors to produce, for example, some minor anomalies in the FHS [8], and the incidence of human congenital anomalies has been correlated with maternal plasma concentrations of phenytoin [25].

In summary of the major postulated mechanisms of phenytoin teratogenicity, there is evidence for the involvement of both a receptor-mediated contribution from unmetabolized phenytoin, and several embryopathic reactive intermediary metabolites of phenytoin. It is possible that the teratogenicity of phenytoin could involve multiple mechanisms, the relative contributions of which could vary with gestational age, target tissue, level of drug exposure, coexisting exposure to drugs and other xenobiotics, environmental conditions, species, strain and other genetic factors, particularly with regard to biochemical pathways of maternal elimination, maternal/embryonic bioactivation, and embryonic detoxification, cytoprotection, and repair. The use of an *in vitro* embryo culture system can be useful in attempting to discriminate the toxicological relevance of such variables to chemical teratogenesis.

Using the mouse embryo as a "target," the objectives of this study were to: (1) confirm the intrinsic capacity of the embryo to bioactivate phenytoin; (2) estimate the potential maternal contribution to phenytoin bioactivation and embryopathy by the addition of maternal mouse hepatocytes possessing both phase I and phase II drug-metabolizing enzymes; and (3) evaluate, by the addition of hepatocytes from male rabbits and maternal rats, potential species differences in the maternal contribution to phenytoin embryopathy. Previous embryo culture models have employed hepatocellular fractions, such as the 9000 *g* supernatant or microsomal pellet, from adult animals to approximate a maternal source of cytochromes P450 [26, 27]. However, homogenates may not reflect the true metabolic profile of the liver cell with respect to the balance of phase I and phase II enzymes [28], and may themselves be embryotoxic [27]. In contrast, hepatocytes are not embryotoxic [29, 30], and because the three-dimensional structure of the cell and cofactor availability are maintained, they may represent more closely the *in vivo* balance among xenobiotic elimination, bioactivation, and detoxification. Therefore, we co-cultured mouse embryos with hepatocytes from maternal mice and rats and male rabbits. The embryo-hepatocyte co-culture technique initially was developed using the rat [29]; it was modified subsequently in our laboratory using the mouse, and this mouse model was then validated for another putative proteratogen, cyclophosphamide [30], which is known to require bioactivation for teratologic expression. Concentrations of phenytoin employed in the co-culture medium were within the human therapeutic range in maternal plasma (10–20 μg/mL) [31]. These studies provided evidence for: (1) the involvement of enzymatic bioactivation in the mechanism of phenytoin teratogenicity; and (2) differential maternal contributions to phenytoin embryotoxicity that were species dependent, as well as corroborating previous reports [27, 32] that embryonic phenytoin bioactivation may have teratological relevance.

MATERIALS AND METHODS

Animals and housing

Male and female Sprague-Dawley rats and CD-1 mice were obtained from Charles River Canada Ltd. (St-Constant, Québec), and male New Zealand White rabbits from Maple Lane Farms Canada Ltd. (Clifford, Ontario). Animals were exposed to a 12-hr light–dark cycle (7:00 a.m.–7:00 p.m.) with food (Rodent Chow No. 5001 or Lab Rabbit Chow No. 5321, Purina Mills Inc., St. Louis, MO) and tap water available *ad lib.* Rats were housed individually, females in suspended wire mesh cages, breeders in plastic cages with softwood sawdust bedding (Ontario Sawdust Supply Ltd., Holland Landing, Ontario). Mice were placed in plastic cages with hardwood bedding (Beta-Chip®, Northeastern Products Corp., Warrenburg, NY), females in groups of four, breeders alone. Rabbits were caged individually in hanging wire mesh cages.

Nulliparous females were bred in the animal facility of the Faculty of Pharmacy commencing at 8:00 p.m., and insemination verified the following morning at 8:00 a.m. One female rat was placed with one male, and the presence of a sperm-positive vaginal smear designated as day 1 of gestation. Three female mice were housed with one breeder, a vaginal plug confirming insemination and designated as day 1 of gestation.

Co-culture of hepatocytes and mouse embryos

Hepatocyte isolation. Rat embryos implant approximately one to one-and-a-half days later than mouse embryos [33], and accordingly are delayed in their development relative to the mouse. Because pregnancy alters phenytoin metabolism in the rat [34] and the mouse [13], hepatocytes were obtained from dams whose embryos were at the same stage of development as those in culture. Therefore, gestational day 10 rats and gestational day 9 mice were the source of rodent hepatocytes. While rodents produce sex-specific phenytoin metabolites [35, 36], sex-and pregnancy-specific rabbit metabolites of phenytoin to our knowledge have not been described. Therefore, rabbit hepatocytes were obtained from untreated males killed for other studies.

Hepatocytes from rats and rabbits were isolated by modification [29] of the slicing technique [37], while maternal mouse hepatocytes were obtained by a modi-

fication [30, 38] of a retrograde perfusion technique [39].

Cell viability and culture. Cell viability was determined by 0.4% trypan blue exclusion. Rat and rabbit preparations were of greater than 95% viability, while mouse preparations were greater than 85% viable. Hepatocytes were seeded into 25 cm^2 tissue culture flasks (Corning Glassware Inc., Corning, NY) that had been precoated with 2 μg human fibronectin/cm^2 (Sigma Chemical Co., St. Louis, MO, or Collaborative Research Inc., Bedford, MA). Seeding was at a density of either 3 × 10^6 (rat and mouse) or 2 × 10^6 (rabbit) viable cells per flask in "attachment" medium (100 mL Waymouth's MB 752/1 medium supplemented with 17% FBS, 9 mM sodium bicarbonate, 5 mM HEPES buffer, 2 mM L-glutamine). The 2-hr attachment period allowed 1.6 to 2.0 × 10^6 (rats and mice) and 1.1 to 1.6 × 10^6 (rabbit) hepatocytes to adhere per 25 cm^2 flask.

Embryo-hepatocyte co-culture. Embryos were selected, co-cultured with hepatocytes, and evaluated as previously described [30]. In brief, gestational day 9.5 mouse embryos within 4–6 somites were dissected in "embryo" medium (100 mL Waymouth's MB 752/1 supplemented with 14 mM $NaHCO^3$, 2.5 mM HEPES, 1.0 mM L-glutamine; Gibco Laboratories, Burlington, Ontario) and subsequently maintained in heat-inactivated male rat serum while hepatocytes were undergoing the 2-hr stationary period to allow attachment. Co-culture medium [50 mL "embryo" medium, 35 mL male rat serum, 15 mL embryo-compatible FBS, penicillin (50 U/mL) and streptomycin (50 mg/mL); Gibco Laboratories] was gassed for 15 min (40% O_2, 55% N_2, 5% CO_2) and 10 mL was added to each flask, along with 2 embryos and the test chemical. Embryos and hepatocytes were incubated at 37° for 24 hr on a rocker platform at 32 oscillations/min. The following morning at 8:00 a.m., the flasks were oxygenated for 1 min.

At 22–24 hr, the cultures were terminated and the embryonic morphological and developmental parameters were assessed using a dissecting microscope with 10× magnification and an eyepiece reticle micrometer. Morphological assessment included yolk sac diameter, crown-rump length, and the presence or absence of a yolk sac circulation. Developmental parameters included the change in somite number, ANP closure, and dorsal-ventral flexure (turning).

Preparation of test chemicals

A 400 μg/mL stock solution of phenytoin (Sigma Chemical Co.) was prepared in 0.02 N NaOH immediately prior to the appropriate experiment, and was added to the co-culture medium to yield a final phenytoin concentration of 10, 20 or 60 μg/mL. *In vitro* radiolabeled studies employed 3.5 × 10^6 dpm of 5,5-[4-^{14}C]diphenylhydantoin (sp. act. 1.7 GBq/mmol, Dupont) per culture flask.

Covalent binding

Covalent binding of phenytoin to tissue protein is a direct measure of its bioactivation to an embryotoxic reactive intermediate, and hence is thought to reflect potential toxicological initiation. Phenytoin covalent binding was determined by modification of an exhaustive washing procedure [40] that permitted accurate determinations in microgram quantities of tissue samples, and facilitated rapid processing of a large number of samples. Irreversible protein binding of sub-pharmacological concentrations (0.93 μg/mL) of radiolabeled phenytoin was determined in the fibronectin coating the culture flasks, embryos, and hepatocytes, and from 1 mL of the culture medium. Dilution with unlabeled drug to bring the total phenytoin concentration to 20 μg/mL may have rendered the assessment of covalent binding less accurate as a result of competition between labeled and unlabeled drug for the relatively small quantities of embryonic tissue and bioactivating enzyme. To control for nonspecific binding, metabolically inactive tissue was required. Boiled extracts are employed traditionally as inactive metabolic controls in *in vitro* studies, but the use of boiled tissues in these studies was precluded due to the degradation of embryos during the 24-hr culture period, which prevented their subsequent recovery from the medium [27]. Instead, tissues from liver and embryo were fixed in Carnoy's solution. Whole embryos (N = 8/sample), hepatocytes (1 culture flask/sample), and fibronectin (1 culture flask/sample) were homogenized as described above, and the total volume was adjusted to 1000 μL with Hanks' Balanced Salt Solution. From these samples a 50-μL aliquot was removed for Coomassie Brilliant Blue G-250-based protein determination (Bio-Rad Protein Assay). Protein samples were placed on separate, 25 mm diameter, 0.45 μm Nylon 66 filter membranes (Ultipor® N66, Pall Trinity Micro Corp., Cortland, NY; Scientific Products & Equipment Ltd., Rexdale, Ontario), which were housed in separate wells on a vacuum suction apparatus (Millipore Ltd., Mississauga, Ontario). Each cell was washed continuously with hot (50°) methanol (HPLC Grade, Caledon Laboratories, Georgetown, Ontario) until no radioactivity could be detected in the filtrate. The filter membrane and precipitate were placed into a loosely covered 20 mL glass scintillation vial and dried overnight in a glassware drying oven. To each vial, 0.750 mL of BTS-450 tissue solubilizer (Beckman Instruments, Fullerton, CA) was added, and the vials were returned to the oven for several hours to allow digestion and dissolution of the precipitate. To each sample, 15 mL of scintillation fluid (Ready Organic, Beckman Instruments) was added, and the vials were vortexed. The samples were quantified by using a liquid scintillation spectrometer with automatic quench correction (Beckman model LS 5000 TD).

Statistical analysis

Significance for all statistical tests was determined *a priori* to be $P < 0.05$. Both Epistat software (Tracy Gustafson, Round Rock, TX) and SAS/STAT™ (SAS Institute Inc., Cary, NC) provided the statistical analysis and were resident on an IBM-compatible personal computer. Flexure and ANP closure were analyzed on Epistat using Fisher's exact test for non-parametric binomial data. Non-random selection of samples necessitated all other data to be analyzed using Kruskal–Wallis one-way analysis of ranks of non-parametric data. This latter test and the Tukey's post hoc analysis for non-parametric multiple comparison testing were calculated using SAS/STAT™.

RESULTS

Hepatocyte isolation

Rabbit and rat hepatocytes were much more resistant than mouse hepatocytes to the deleterious effects of the

isolation process. Using the modified slicing technique, digestion with type II collagenase (Worthington Biochemical Corp., Freehold, NJ) liberated 40–50 × 10^6 cells from 4–5 g of rat or rabbit liver tissue with viabilities of greater than 95%. Typically 40–50 × 10^6 cells of 85–90% viability were harvested from a single maternal mouse liver with the perfusion method. Although some lots of collagenase did consistently provide better preparations than others, viabilities and yields of rabbit and rat hepatocytes were consistently superior to those of the mouse (data not shown).

Phenytoin concentration–response

Embryos were incubated in the co-culture medium with various concentrations of phenytoin to determine a threshold embryotoxic concentration. Using phenytoin concentrations (0, 10, 20 and 60 μg/mL) that included the therapeutic plasma range (10–20 μg/mL) [31], and similar to those employed by other investigators (10–20 μg/mL [27]; 60 μg/mL [41]), embryos were examined following 24 hr of culture. Significant reductions of yolk sac diameter and crown rump length occurred at phenytoin concentrations of 20 μg/mL and greater, while significant decreases in the change in somite number became apparent only at 60 μg/mL (Fig. 1). No toxicity in any of the parameters was noted at concentrations of phenytoin below 20 μg/mL. This concentration was employed in subsequent studies.

Interspecies co-culture experiments

Growth and development of mouse embryos co-cultured with 20 μg/mL phenytoin and maternal mouse hepatocytes (gestational day 9.5), maternal rat hepatocytes (gestational day 10.5) and male rabbit hepatocytes are depicted in Tables 1, 2, and 3, respectively. In the absence of phenytoin, the inclusion of hepatocytes from mouse (maternal), rat (maternal), and rabbit (male) had no ill effect on embryonic growth and development. Em-

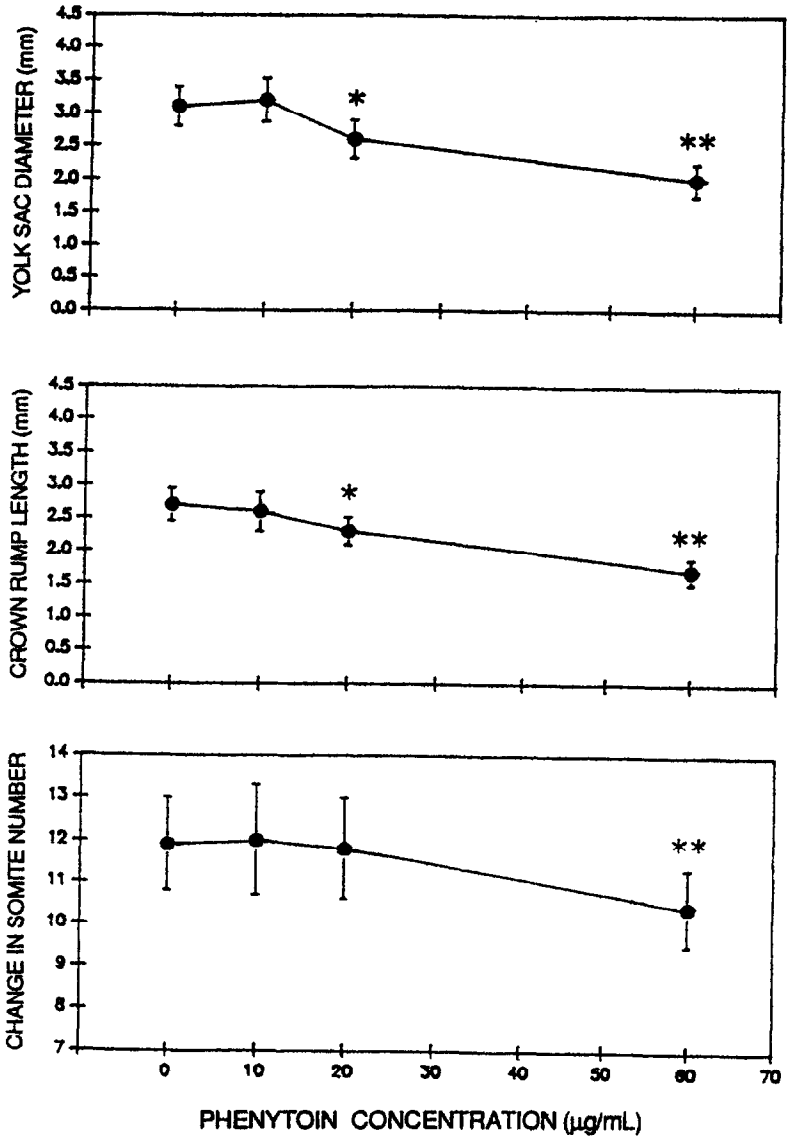

Fig. 1. Effects of phenytoin concentration on mouse embryos co-cultured for 24 hr with maternal mouse hepatocytes. Each point is the mean ± SD of 10 embryos. Key: (*) $P < 0.05$ from controls (0 μg/mL phenytoin), and (**) $P < 0.01$ from controls.

Table 1. Effect of murine hepatocytes on murine phenytoin embryotoxicity

Group	Culture conditions: Hep	Culture conditions: P	Embryos (N)	Yolk sac diameter (mm)	Crown-rump length (mm)	Change in somite number	Flexure (%)	ANP closure (%)
1	–	–	16	3.2 ± 0.21*	2.8 ± 0.22	11.8 ± 1.3	100	100
2	+	–	16	3.4 ± 0.20	2.9 ± 0.21	12.3 ± 1.2	100	100
3	–	+	16	2.4 ± 0.16†‡	2.0 ± 0.16†‡	12.2 ± 1.1	94	94
4	+	+	16	2.8 ± 0.28§	2.3 ± 0.17§	11.8 ± 1.2	88	88

Embryotoxicity from co-culturing gestational day 9.5 murine embryos (N) with maternal day 9.5 murine hepatocytes (Hep) and 20 μg/mL phenytoin (P). The change in somite number was determined by subtracting the number of somites at the initiation of the culture period from the number of somites following the 24-hr culture period for each embryo. In embryos achieving at least 13 somites, flexure denotes those that had rotated completely and assumed a convex curvature ("fetal position"). Of embryos achieving 16 somites, those with closed anterior neuropores (ANP) are depicted by ANP closure.

* Values are means ± SD.

† $P < 0.001$ vs Groups 1 and 2.

‡ $P < 0.05$ vs Groups 1, 2 and 4.

§ $P < 0.05$ vs Groups 1 and 2.

bryos grow adequately in co-culture medium, with or without hepatocytes, indicating that there is no requirement for growth factors produced by maternal tissues. Phenytoin without hepatocytes caused significant reductions in yolk sac diameter and crown rump length when compared with embryos not exposed to the drug. This suggests that phenytoin may have acted directly via a receptor-mediated event, and/or that it was bioactivated by the embryo to a toxic reactive intermediate.

The addition of rat and rabbit hepatocytes did not potentiate phenytoin-mediated reduction of crown-rump length and yolk sac diameter. However, unlike with phenytoin alone, the addition of hepatocytes from these two species significantly enhanced the phenytoin-induced reduction in somite number. Addition of rat and rabbit hepatocytes also tended to enhance the phenytoin-induced impairment of turning and ANP closure, but this was not statistically significant.

The phenytoin-induced deficits in yolk sac diameter and crown-rump length persisted when embryos were incubated with mouse hepatocytes, but the severity was reduced significantly when compared with embryos exposed to phenytoin without hepatocytes. Mouse hepatocytes, in contrast to rat and rabbit hepatocytes, did not enhance phenytoin-induced reductions in somite growth, suggesting that, at least *in vitro*, mouse hepatocytes may be embryoprotective, while those of rats and rabbits are not. Alternatively, the embryoprotective effect could be indicative of poor hepatocyte preparation and culture, in which case the hepatocyte tissue may act as a tissue "sink" effectively reducing embryonic exposure to the drug. This appeared unlikely, since we had employed the same mouse hepatocyte-embryo co-culture system [29, 30] to demonstrate the requirement for maternal bioactivation in the teratologic expression of cyclophosphamide, a proteratogen known to require exogenous P450 bioactivation to exert toxic effects in whole rat embryo culture [26] and limb bud organ culture [42]. Covalent binding studies with radiolabeled phenytoin described below were designed to address this issue.

[^{14}C]Phenytoin covalent binding

To further investigate the nature of the apparent protective effect of maternal mouse hepatocytes, the covalent binding of a radiolabeled reactive intermediate of phenytoin to mouse hepatocytes and embryos, as well as to other culture components such as serum proteins and fibronectin, was measured (Fig. 2). The nonspecific tissue binding of phenytoin to embryos and hepatocytes previously fixed in Carnoy's solution served as a control with no metabolic activity, particularly with respect to bioactivation. Prior to exhaustive washing with hot methanol, which removes all non-covalently bound phenytoin, tissue homogenates from fixed and live embryos

Table 2. Effect of rat hepatocytes on murine phenytoin embryotoxicity

Group	Culture conditions: Hep	Culture conditions: P	Embryos (N)	Yolk sac diameter (mm)	Crown-rump length (mm)	Change in somite number	Flexure (%)	ANP closure (%)
1	–	–	16	3.4 ± 0.31*	2.9 ± 0.27	12.1 ± 1.5	100	100
2	+	–	16	3.5 ± 0.23	2.9 ± 0.24	11.8 ± 1.5	94	94
3	–	+	16	2.6 ± 0.36†	2.0 ± 0.37†	11.9 ± 1.9	94	94
4	+	+	16	2.7 ± 0.25†	2.3 ± 0.25†	10.1 ± 1.2‡	88	88

Embryotoxicity from co-culturing gestational day 9.5 murine embryos (N) with day 10.5 rat hepatocytes (Hep) and 20 μg/mL phenytoin (P). Parameters are described in Table 1.

* Values are means ± SD.

† $P < 0.01$ vs Groups 1 and 2.

‡ $P < 0.01$ vs Groups 1, 2 and 3.

Table 3. Effect of rabbit hepatocytes on murine phenytoin embryotoxicity

Group	Culture conditions		Embryos (N)	Yolk sac diameter (mm)	Crown-rump length (mm)	Change in somite number	Flexure (%)	ANP closure (%)
	Hep	P						
1	–	–	16	3.4 ± 0.18*	2.9 ± 0.16	12.0 ± 0.17	94	94
2	+	–	16	3.3 ± 0.19	2.8 ± 0.21	11.4 ± 1.7	100	94
3	–	+	16	2.6 ± 0.18†	2.0 ± 0.17†	11.9 ± 1.4	94	88
4	+	+	16	2.5 ± 0.21†	1.9 ± 0.25†	9.8 ± 1.2‡	88	75

Embryotoxicity from co-culturing gestational day 9.5 murine embryos (N) with male (3.1 kg) rabbit hepatocytes (Hep) and 20 μg/mL phenytoin (P). Parameters are described in Table 1.

* Values are means ± SD.

† $P < 0.001$ vs Groups 1 and 2.

‡ $P < 0.0001$ vs Groups 1, 2 and 3.

had similar concentrations of phenytoin, indicating that fixation was a valid control for non-covalent tissue disposition of phenytoin.

In the absence of hepatocytes, covalent binding of phenytoin in live embryos (0.713 pmol/mg protein) was 40% higher than that in the fixed (dead) control embryos (0.510 pmol/mg protein) ($P < 0.05$), providing evidence that embryos intrinsically are capable of bioactivating phenytoin to a reactive intermediate.

Live hepatocytes did not increase significantly the covalent binding of phenytoin in fixed embryos (0.638 pmol/mg protein); therefore, live embryos were not similarly employed. Live hepatocytes also failed to cause increases in phenytoin covalent binding to either culture medium proteins or fibronectin, which could be indicative of either the generation of a highly reactive metabolite that is unable to leave the cell, or a cellular balance of phase I and phase II drug-metabolizing enzymes favouring detoxification over bioactivation.

DISCUSSION

In our mouse embryo culture model, exposure to 20 μg/mL phenytoin was the threshold of embryotoxicity, and therefore this drug concentration in the co-culture medium was selected for all subsequent experiments. The therapeutic plasma concentration of phenytoin in humans has been described to be in the range of 10–20 μg/mL [31]. In the quaking C57BL/6J (qk/qk) mouse, spontaneous seizures are controlled by oral administration of 60 mg/kg/day phenytoin, resulting in a mean steady-state phenytoin plasma concentration of approximately 14 μg/mL [43]. While the level of mouse embryonic exposure remains unknown, the rapid equilibrium achieved between maternal and fetal tissues as demonstrated by whole body autoradiography [44] suggests that maternal plasma drug concentrations may reflect the concentration of phenytoin in the amniotic fluid to which the embryo would be exposed *in vivo*. Therefore, the exposure of embryos to 20 μg/mL phenytoin in our *in vitro* studies was not only appropriate for the co-culture model, but also probably represented *in vivo* therapeutic conditions.

Results from Tables 1–3 demonstrate that phenytoin-induced embryopathy occurred in the presence or absence of hepatocytes from any of the three species employed. At concentrations of 20 μg/mL, phenytoin caused reductions in yolk sac diameter and crown rump length, which corroborated previous results in mouse embryo culture [27]. However, unlike in previous studies [27, 32], the apparent trends for phenytoin-initiated deficits in turning, ANP closure, or somite growth in the absence of hepatocytes were not statistically significant. The reason for these apparent differences in the effects of phenytoin on developmental parameters remains obscure. One possible explanation is that, while the previous studies [27, 32] employed pure male rat serum, the co-culture medium in this study contained 35% male rat serum and 17% FBS to accommodate the differing needs of embryos and hepatocytes *in vitro*.

In vitro embryotoxicity in the absence of an exogenous bioactivating system (hepatocytes) implies that: (1) the embryo may bioactivate phenytoin to an embryotoxic, electrophilic, or free radical intermediate that irreversibly alters critical embryonic macromolecules; and/or (2) phenytoin exerts its deleterious effects via a reversible, receptor-mediated mechanism. In the absence of hepatocytes, phenytoin covalent binding was significantly higher in live embryos than in fixed controls, suggesting that embryos can bioactivate phenytoin to a reactive intermediate. PHS and/or LPOs may play an important role in embryonic bioactivation, as evidenced by the reduction in phenytoin embryopathy by the dual PHS/LPO inhibitor eicosatetraynoic acid in embryo culture [32]. *In vivo* covalent binding of phenytoin to day 16 fetal mouse liver has been shown to correlate positively with the incidence of cleft palates in the same fetuses [45], and the *in vivo* reduction of phenytoin teratogenicity by acetylsalicylic acid pretreatment correlates with a similar reduction in covalent binding of phenytoin to gestational day 13 embryonic tissue [46]. Together, these data support the hypothesis that covalent binding *in vitro* may contribute, at least in part, to the initiation of embryotoxicity. Alternatively, phenytoin bioactivation *in vivo* [19] and in embryo culture [47] causes embryonic DNA oxidation, which may play a role in teratologic initiation, since p53-deficient mice with deficient DNA repair are more susceptible to the teratogenicity of phenytoin [23] and another DNA-damaging teratogen, benzo[*a*]pyrene [24].

While our data support the hypotheses for irreversible binding of phenytoin as a proximate event initiating embryotoxicity, they do not exclude effects of unmetabolized phenytoin via a receptor-mediated mechanism. In our study, the phenytoin concentration-dependent growth deficits were noted at and above drug concen-

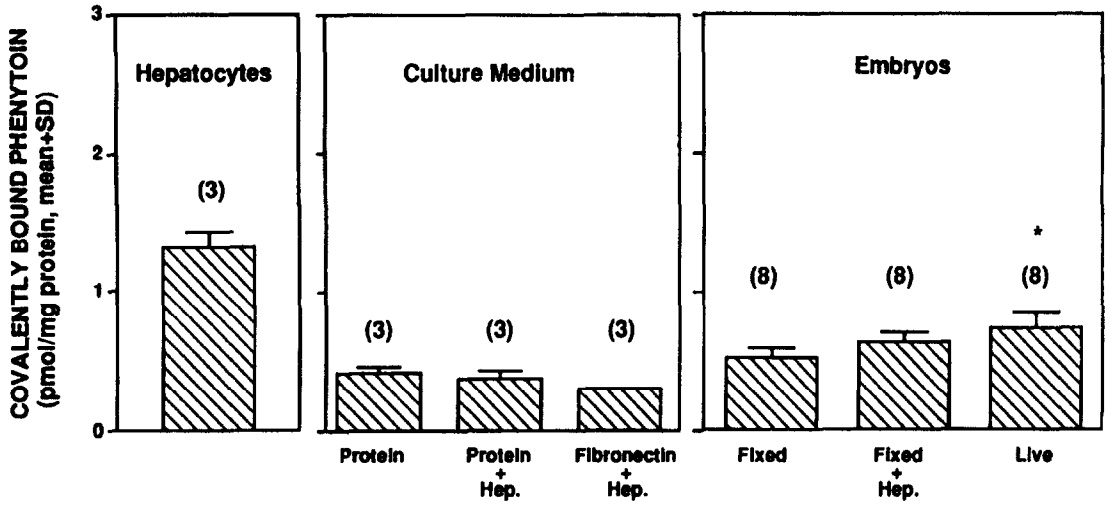

Fig. 2. Covalent binding of radiolabeled phenytoin to co-culture components. Embryos and hepatocytes were co-cultured for 24 hr with radiolabeled [^{14}C]phenytoin (3.5×10^6 dpm; 0.93 μg/mL). Values are means ± SD. The N per sample is as follows: embryonic tissue from 24 embryos was divided into three groups of eight; hepatocytes from three culture flasks were used (1.8×10^6 cells/flask); fibronectin from three flasks was employed; and the culture medium from three culture flasks was employed. The asterisk indicates a significant difference from control embryos that had been killed by fixation prior to culturing ($P < 0.05$).

trations of 20 μg/mL, while developmental deficits as indicated by a decrease in somite number occurred only at 60 μg/mL. This may be indicative of two different concentration-dependent mechanisms of phenytoin toxicity; embryonic bioactivation can occur at or above therapeutic concentrations, initiating some types of embryopathies. However, other, and perhaps some similar, embryopathies resulting from alternative, receptor-mediated mechanisms likely occur only at higher phenytoin concentrations. This would be consistent with clinical observations of concentration-dependent phenytoin teratogenicity [25], and with a correlation of major but not minor phenytoin-induced anomalies with *in vitro* phenytoin-induced cytotoxicity in lymphocytes from children with FHS [8].

While the evidence discussed above supports an embryonic determinant of phenytoin toxicity, a potential maternal contribution to the modulation of *in vitro* phenytoin embryotoxicity also was evident. In the presence of rat and rabbit hepatocytes, phenytoin-mediated growth retardation in the form of reductions in yolk sac diameter and crown-rump length persisted, but was not augmented when compared with embryos cultured with phenytoin alone. However, the addition of rat or rabbit hepatocytes enhanced the phenytoin-induced decrease in somite number, indicative of a developmental deficit. Rabbit and rat hepatocytes in the absence of phenytoin did not impair embryonic growth, precluding the possibility that developmental deficits were the result of species incompatibility. The embryonic and maternal processes modulating phenytoin embryotoxicity may differ mechanistically, and/or diffusional barriers may differ for reactive intermediates generated inside and outside the embryo. *In vitro*, a hepatocyte-generated reactive intermediate need travel only several millimeters to reach the target embryo, distinct from the substantially greater barriers *in vivo*, involving both membranes and distance. It has been suggested that phenytoin may form a membrane-traversable, teratogenic drug-protein complex in the sera of phenytoin-treated maternal monkeys [48]. Whether a reactive intermediate *in vivo* can escape maternal cells of origin, traverse the placenta, and react with embryonic macromolecules remains speculative.

The embryoprotective effect of the mouse maternal hepatocytes in reducing phenytoin embryotoxicity (reduced yolk sac diameter and crown rump length) was somewhat unexpected, suggesting that maternal detoxifying capacity exceeds bioactivating activity in that species. Furthermore, unlike rat or rabbit hepatocytes, maternal mouse hepatocytes did not enhance phenytoin-initiated deficits in somite growth at a phenytoin concentration of 20 μg/mL, but were able to do so at a concentration of 60 μg/mL, as noted in the initial phenytoin concentration–response study. It is not certain whether the phenytoin-initiated decrease in somite growth was dependent upon the presence of hepatocytes, since no embryos were cultured alone in that study. With respect to the cytochromes P450-arene oxide hypothesis, since in vitro hepatocyte P450 content and activities are lower in the mouse, compared with the rat and rabbit [49], then mouse *in vitro* detoxification might exceed bioactivation artifactually compared either with mouse activities *in vivo*, or with *in vitro* co-culture with rabbit or rat hepatocytes. Nevertheless, it appears unlikely that the embryoprotective nature of maternal mouse hepatocytes was the result of commonly encountered decreased bioactivating potential noted particularly with cultured mouse hepatocytes [39, 49]. Evidence for functional hepatocytes included: (a) trypan blue exclusion; (b) generation of para-hydroxylated and glucuronidated phenytoin metabolites [38]; and (c) in other studies using cyclophosphamide concentrations similar to those in published rat studies [29], our mouse hepatocyte-embryo co-culture model demonstrated a similar requirement for maternal P450-catalysed bioactivation of cyclophosphamide for expression of embryotoxicity [30].

While the hepatocyte-embryo co-culture model is useful in exploring mechanisms underlying teratogenesis

and teratologic susceptibility, its direct applicability to *in vivo* teratologic predictions is questionable. *In vivo*, when phenytoin is administered orally, there are substantial species differences in embryotoxicity and teratogenicity, with the following increasing order of susceptibility: humans [7] < rabbits [3] < rats [2] < mice [50] < cats [5]. The toxicological potential of phenytoin may be dependent upon the relative proportions and the stereochemical configurations of the phenytoin metabolites [51, 52], which vary greatly between strains and species [53–60]. Therefore, differences in teratological susceptibility may be due, at least in part, to species and strain differences in phenytoin metabolism [12–14, 60]. The *in vitro* hepatocyte-embryo co-culture system is unlikely to serve as a reliable predictor of *in vivo* susceptibility among animal species, let alone generally in humans. Relative embryotoxic response in culture would be expected to vary with both the hepatocyte and embryo species of origin, as well as with biochemical differences from *in vivo* activities due to culture conditions, although different hepatocyte-embryo species combinations could prove useful in elucidating different mechanisms of individual human teratological susceptibility.

In summary, these studies employed an *in vitro* embryo-hepatocyte co-culture model to approximate at a simplified level the potential relationship between the mouse embryo "target" tissue and the maternal hepatic tissue thought to contribute to the bioactivation of xenobiotics to potential teratogenic reactive intermediates. The results confirmed the intrinsic capacity of the mouse embryo to bioactivate phenytoin to a reactive intermediate, which bound covalently to embryonic proteins. This covalent binding to critical cellular macromolecules is thought to initiate alterations in cellular function or cause cellular death, which ultimately results in *in utero* death or teratogenicity, unless embryonic mechanisms of cytoprotection and repair intervene. It appears that, at least in a hepatocyte-embryo co-culture system, phenytoin metabolites are produced extraembryonically [38], and may traverse hepatocyte membranes, travel distally, cross the embryonic yolk sac membranes, and enter the embryo to exert their deleterious effects, although whether such a process can transpire *in vivo* remains speculative. Under the conditions of our model, marked species differences in the putative maternal contribution to phenytoin embryopathy were evident, in that hepatocytes from maternal rats and male rabbits enhanced mouse phenytoin teratogenicity, while mouse maternal hepatocytes were embryoprotective. It remains to be determined whether such maternal modulation occurs *in vivo*. The predictive application to *in vivo* susceptibility also may differ when rat and rabbit hepatocytes are cocultured with embryos of their respective species, rather than with mouse embryos. *In vivo*, increasing susceptibility to phenytoin teratogenicity is observed from rabbits to rats to mice. In humans, a teratologically susceptible individual mother and her embryo may be more like one species, while the general population more like another. At the present time, there is insufficient evidence to establish the optimal animal hepatocyte-embryo coculture system(s) for human teratological relevance. In conclusion, while phenytoin teratogenicity likely involves embryonic bioactivation, maternal determinants may contribute variably to teratologic susceptibility in a species-specific, and possibly a strain-specific, manner.

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