FULL-LENGTH ORIGINAL RESEARCH

Fetal hypoxia and hyperglycemia in the formation of phenytoin-induced cleft lip and maxillary hypoplasia

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Abstract

Objective: Phenytoin exposure during the first trimester of pregnancy increases the risk of maxillary hypoplasia and cleft lip. The etiology of phenytoin embryopathy is unknown. Interestingly, phenytoin is also known to induce hyperglycemia in humans as well as rats. This study uses a rat model of fetal phenytoin syndrome to examine the role of hyperoxia, hyperglycemia, and arachidonic acid deficiency in the development of cleft lip and maxillary hypoplasia.

Methods: Pregnant rats were dosed with phenytoin during the critical period of lip development (day 11 of pregnancy) with or without supplemental oxygen, insulin, or arachidonic acid. The fetuses from all studies were examined at term.

Results: The frequency of cleft lip and maxillary hypoplasia was reduced by treating dams at the time of phenytoin exposure with either increased oxygen or insulin. However, in fetuses from phenytoin-treated dams dosed with arachidonic acid, the incidence of severe lip deformities remained the same although there was an increase in normal and mildly affected fetuses. Interestingly, this occurred in embryos from hyperglycemic dams.

Significance: Together, the results from these experiments suggest phenytoin-induced malformations may be a multifactorial process as malformations were not solely linked to a hyperglycemic state of the dam.

KEYWORDS
arachidonic acid, cleft lip, hyperglycemia, hypoxia, phenytoin

1 | INTRODUCTION

Between 1993 and 2007, approximately 1% of people were recorded as having epilepsy and prescribed antiepileptic drugs.[1] Phenytoin (Dilantin, Phenytek) accounts for ~20% of prescriptions, but among women of child-bearing age, the use of phenytoin approaches ~5%.[2] This is related to the fact that phenytoin is a well-established human teratogen. The overall risk of having a child with congenital malformation among epileptic women taking phenytoin either alone or in combination is increased 2-3 times.[3] The fetal hydantoin (phenytoin) syndrome comprises growth retardation, characteristic facies including midfacial hypoplasia and increased risk of cleft lip (CL), limb anomalies specifically hypoplasia of the distal phalanges, small nails, and an increased risk of heart defects.

Phenytoin exposure during the first trimester of pregnancy increases the risk of maxillary hypoplasia with prevalence reported as 16.7% in one study.[4] Maxillary hypoplasia (MH) and CL can be considered as part of a continuum resulting from underdevelopment of the maxillary processes (MP). In the rat model of fetal hydantoin syndrome, litters with CL fetuses were always observed within litters that contain other fetuses with MH.[5] It is postulated that severe underdevelopment of the maxillary process leads to CL while a more moderate reduction results in MH. Deficiencies of MP growth are a common feature in CL.[6,7]

There have been numerous suggestions as to the possible cause of the embryotoxicity of phenytoin. Possible mechanisms include (i) disturbances in folate metabolism,[8] (ii) embryonic hypoxia due to phenytoin-induced bradycardia,[9] or (iii) free radical formation via prostaglandin pathways.[10] Similarities between the malformations induced by phenytoin and those produced by maternal hypoxia or drug-induced embryonic bradycardia led to the proposal of an over-arching fetal hypoxia syndrome.[11] Cleft lip and palate also occur with relatively high frequency in the offspring of type I diabetic mothers.[12] Phenytoin has been shown to reduce the release of insulin from the pancreas and induce hyperglycemia in humans as well as rats.[13]

There have been many intervention strategies in experimental models to reduce the incidence of clefting in phenytoin-exposed rats and mice. These studies provide some mechanistic insights as well as potential strategies for limiting the risk of anticonvulsant use in pregnancy. Approaches have included increasing dietary fatty acids,[14] pretreatment with antioxidant enzymes or stiripentol,[15,16] or hyperoxygenation.[17] The results have been variable, and interpretation is difficult as many were performed in mice which spontaneously develop cleft lip and and/or palate. Compared to the rat, mice (especially A/J mice) are more sensitive to the effects of phenytoin.[11] This complicates the interpretation of pathogenesis in the mouse as the genetic mechanism and the drug mechanism may be different.

This study uses a rat model which does not normally display CL, to examine the role of hyperoxia, hyperglycemia, and arachidonic acid deficiency in the development of CL and MH. Our results could be of clinical significance to those managing pregnant women using phenytoin and provides some insights into the causes of the induced malformations.

2 | MATERIALS AND METHODS

2.1 | Animals

Sprague-Dawley (SD) rats (Animal Resource Centre, Perth, WA) were kept under constant day/night cycle in a climate-controlled room. All the animal studies were approved by the

Key Points
- Phenytoin increases risk of cleft lip
- Phenytoin induces hyperglycemia
- Maternal normoglycemia reduces frequency of phenytoin malformations
- Maternal hyperoxia reduces frequency of phenytoin malformations
- Arachidonic acid supplementation reduces severity of phenytoin malformations

University of Sydney Animal Ethics Committee. In line with the Basel declaration, care has been taken in the planning of these experiments to reduce animal numbers where possible. Sprague-Dawley rats were mated overnight and examined the next morning for positive vaginal smear. Rats with a sperm-positive smear were separated, and this day was considered gestational day (GD) 0.

2.2 | Test chemicals

Sodium Phenytoin was obtained as an intravenous formulation (Phenytoin Injection BP; Mayne Pharma Pty Ltd, Mulgrave, Australia) containing phenytoin sodium (50 mg/mL, pH 10.0-12.3). Insulin was obtained as insulin glargine as an intravenous formulation (Sanofi 100 IU/mL). Arachidonic acid (>95%) was obtained as a solid diluted in sunflower oil to give a final concentration of 100 mg/mL (Sigma-Aldrich Pty Ltd, Castle Hill, Australia).

2.3 | Gestational interventions and study design rationale

Phenytoin intraperitoneal (ip) doses between 150 and 200 mg/kg have previously been shown to induce a high incidence of craniofacial malformations when exposed during the period of upper lip development occurs (GD11 to GD12 in the rat).[5,18,19] Timing and dose is critical with maximum effects at midnight on GD11 following a 180 mg/kg ip dose.[5,11] The pharmacokinetic profile is unknown at this dose. However, phenytoin is known to induce significant bradycardia in rat embryos in vitro at unbound concentrations >100 µmol/L.[20] To achieve this concentration in vivo in the rat requires high parenteral doses. Plasma concentration of phenytoin after 150 mg/kg has been shown to reach 240 µmol/L (33 µmol/L unbound) and 100 mg/kg ip reached 150 µmol/L.[11] These are similar to the maximum human therapeutic plasma levels of ~133 µmol/L (unbound) although the average is closer to 63 µmol/L.[21] However, the lower dose was not teratogenic, highlighting the roll duration of exposure as well as concentration in inducting malformations.

For the hyperoxia study, the highly teratogenic 180 mg/kg dose was chosen in order to maximize the frequency of craniofacial malformations. Subsequent studies used the lower teratogenic dose of 150 mg/kg due to concerns relating to potential maternal toxicity. Care was taken to ensure that the rats in the intervention groups were weight matched as there is a strong positive correlation between weight of the pregnant rat and the severity of effect (ie, prevalence of CL and MH) even when administered as mg/kg body weight.[18]

2.4 | Hyperoxia exposure

On GD 11, each dam was weighed and matched with a dam within 10% body weight. These two dams were randomly allocated into either normoxia or hyperoxia group until there were 5 weight-matched dams. On GD 11 at 12:00 midnight, rats were injected with phenytoin (180 mg/kg ip). The volumes used did not exceed 1.5 mL.

After phenytoin treatment, the rat was placed either in an open top plastic cage with solid sides (normoxia) or in a similar cage placed within a solid box into which medical oxygen (99.5% O_2) was piped at 0.5 L/min for 36 hours (hyperoxia). This duration encompasses the period of lip fusion in the rat embryo. Following phenytoin dosing, the rats experienced a period of sedation lasting 6-12 hours. Body temperature was maintained using an infrared light over the period of sedation. After 36 hours, the rats were transferred to their normal cages and maintained under normal oxygen conditions until GD 20.

2.5 | Insulin study

Weight-matched rats were randomly assigned to phenytoin (Phe) or phenytoin and insulin (PheIns) until there were 10 weight-matched dams. At midnight on GD 11, all rats were injected ip with phenytoin (150 mg/kg). After dosing, the phenytoin and insulin (PheIns) rats were given a subcutaneous injection of 8 IU insulin glargine (Sanofi 100 IU/mL) into the thigh.

Blood samples were collected from the tail before dosing with phenytoin and/or insulin as well as 2 and 9 hours after dosing, and glucose concentration was measured using blood glucose test strips and a FreeStyle Optium Neo meter (Abbott) with a detection range of 1.1-27.8mmol/L.

2.6 | Arachidonic acid supplementation

On GD 11, the pregnant dams were randomly assigned to arachidonic acid (ARA) and phenytoin and arachidonic acid (PheARA) groups. The PheARA group (n = 11) were given an intraperitoneal dose of phenytoin (150 mg/kg) followed immediately by a single subcutaneous injection of 200 mg/kg of ARA. The ARA group (n = 8) were given a subcutaneous injection of 200 mg/kg of arachidonic acid. Blood samples were collected from the tail before dosing with phenytoin as well as two further collections after dosing, and glucose concentration was measured as described previously.

2.7 | Fetal Collection and measurements

On GD20, the pregnant dams were killed by CO_2 inhalation and cervical dislocation and the fetuses were removed, weighed, and placed in Bouin's fixative. The fetuses were subsequently examined using a dissecting microscope for external malformations. Calipers were used to measure crown-rump length. The length of fusion (lip height) in the midline of the two maxillary processes was measured using a graticule eyepiece (Figure 1A). Using the same methodology as described previously,[18] "mild maxillary hypoplasia" was defined as any fetus with a length of fusion less than the minimum length in GD20 Sprague-Dawley fetuses (1.1 mm).

2.8 | Statistics

Statistical analysis was carried out using SPSS version 24 software.

To compare blood glucose levels, fetal crown-rump length, fetal and maternal weight, lip height, a one-way ANOVA (ARA study) was performed or independent samples t test (Hyperoxia, Insulin studies). Homogeneity of variance was tested using Levene's test. For post hoc analyses, a Dunnett 2-sided t test was used. If the variance was not homogenous, a Mann-Whitney U-test was used.

Pearson's chi-squared test was utilized to compare the number of implantations, live born, or frequency of resorptions and abnormal fetuses occurring between control and treated groups. If sample size was less than 5, then Fisher's exact test was used.

In all comparisons, differences were considered statistically significant at $P < 0.05$.

3 | RESULTS

3.1 | Hyperoxia study

The results of this study are shown in Table 1. Mean body weight of the dams was not significantly different between treatment groups. There was no significant difference in number of implantations or frequency of resorptions.

The pups exposed to phenytoin and hyperoxia were significantly larger (crown-rump length and weight) than normoxia treatment group ($P < 0.005$). The spectrum of facial defects induced by phenytoin was mainly associated with the extent to which craniofacial processes had fused.
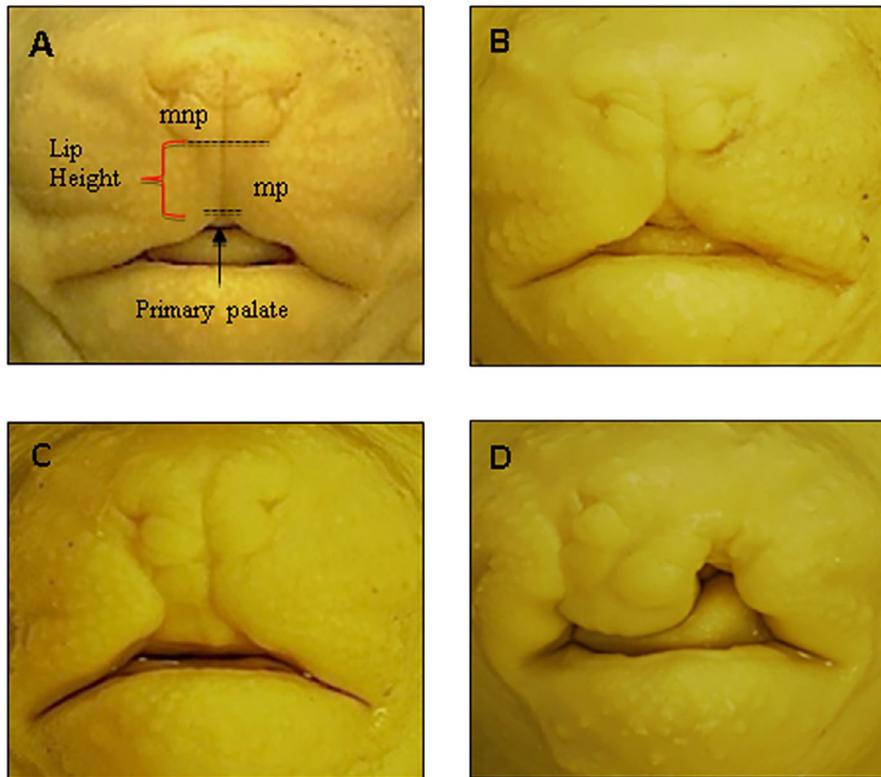
FIGURE 1 GD 20 rat fetuses after exposure to a teratogenic dose of phenytoin (180 mg/kg) on GD 11. A, Normal fetus; B, Fetus showing mild MH; C, Fetus showing right severe MH; D, Fetus showing left CL. mnp: medial nasal process, mp: maxillary process. Lines demarcate distance used to measure lip height

At the most severe, there was complete failure of fusion between the maxillary and medial nasal processes (cleft lip; Figure 1D). Embryos were classified as having "severe MH" (Figure 1C) when the maxillary process failed to reach the midline of the upper lip while "mild MH" (Figure 1B) was defined as reduced contact of the maxillary processes in the midline (<1.1 mm). The frequency of facial malformations was significantly lower in the hyperoxia group compared to the normoxia group (60% normal foetuses in hyperoxia group compared to 13% normal foetuses in normoxia group, $P = 0.001$). Lip height was also greater in the phenytoin and hyperoxia group.

3.2 | Insulin study

The results of this study are shown in Table 2. Mean body weight of the dams was not significantly different between treatment groups. There was no significant difference in number of implantations or frequency of resorptions.

Blood glucose was measured before phenytoin dosing as well as 2 and 9 hours after dosing (Table 2). There was no significant difference in blood glucose levels before dosing. Two hours after dosing, Phe rats had significantly higher blood glucose levels than dams dosed with PheIns ($P < 0.001$). By 9 hours, blood glucose levels were similar between the two groups.

There was no significant difference in crown-rump length or fetal weight between Phe and PheIns groups.

As expected, due to the lower phenytoin dose used in this study, the frequency of cleft lip and severe maxillary hypoplasia was lower than in the previous study. However, the spectrum of defects was the same. When treatment groups were compared, there was a significant difference in the distribution of deformations of the maxillary processes with fewer abnormal embryos (severe or mild MH) in the PheIns group and more normal fetuses ($P = 0.001$). PheIns pups also had significantly greater lip height ($P = 0.004$).

3.3 | Arachidonic acid study

The results of this study are shown in Table 3. There was no statistically significant difference in maternal weights, number of implantations, or percent resorptions between all three groups. There were no statistically significant differences in maternal glucose levels before any intervention. After 2 hours, blood glucose was significantly higher in the PheARA and Phe groups compared to the ARA control group ($P < 0.005$).

Fetal weight was statistically significantly lower in the PheARA group compared to either the PHE or ARA groups ($P < 0.005$). This difference was maintained after adjustment for maternal weight. CRL was also reduced in the PheARA group compared to ARA control ($P = 0.028$). Lip height was significantly greater in the PheARA group compared to the Phe group ($P < 0.005$). Both the PheARA and PHE only group were significantly shorter than ARA control group ($P < 0.005$).

TABLE 1 The effect of supplemental oxygen on the teratogenicity of phenytoin (180 mg/kg) administered to GD 11 pregnant rats

	Phenytoin	Phenytoin + Hyperoxia
Number of treated rats	5	5
Mean maternal weight (g) (GD 11) ± SD	367.6 ± 19.78	370.0 ± 22.53
Mean no. of implantations ± SD	13.0 ± 5.57	10.6 ± 4.45
Resorptions (%) ± SD	4.6 ± 7.28	5.0 ± 11.18
Number of fetuses	63	52
No. of fetuses with CL (%)	19 (30.2%)	0.0*
No. of fetuses with severe MH (%)	21 (33.3%)	5 (9.6%)
No. of fetuses with mild MH (%)	15 (23.8%)	16 (30.8%)
No. of normal fetuses (%)	8 (12.7%)	31 (59.6%)
Mean fetuses weight (g) ± SD	3.1 ± 0.36	3.6 ± 0.36*
CRL (mm) ± SD	31.5 ± 1.97	34.1 ± 2.33*
Lip height (mm) ± SD	0.87 ± 0.30	1.13 ± 0.29*

*Significant difference $P < 0.05$, compared to Phe.

TABLE 2 The effect of insulin on the teratogenicity of phenytoin (150 mg/kg) administered to GD 11 pregnant rats

	Phenytoin	Phenytoin and Insulin
Number of treated rats	10	10
Mean maternal weight (g) (GD 11) ± SD	335.0 ± 41.43	325.0 ± 36.29
Blood glucose levels (mmol/L) ± SD		
0 h	7.7 ± 1.43	8.0 ± 1.48
2 h	19.7 ± 4.14*	7.8 ± 2.52
9 h	10.8 ± 4.88	9.4 ± 5.44
Mean no. of implantations ± SD	13.0 ± 3.74	14.2 ± 3.52
Resorptions (%) ± SD	16.1 ± 23.08	4.0 ± 5.87
Number of fetuses	124	136
No. of fetuses with CL (%)	2 (1.6%)*	0.0
No. of fetuses with severe MH (%)	37 (29.8%)	13 (9.6%)
No. of fetuses with mild MH (%)	74 (69.1%)	94 (69.1%)
No. of normal fetuses (%)	11 (8.9%)	29 (21.3%)
Mean fetuses weight (g) ± SD	3.54 ± 0.69	3.66 ± 0.45
CRL (mm) ± SD	32.1 ± 3.42	32.9 ± 1.93
Lip height (mm) ± SD	0.73 ± 0.25*	0.87 ± 0.25

*$P < 0.05$, compared to PheIns.

There was a strong association between treatment and lip score ($P < 0.005$). All ARA litters produced normal fetuses. Litters treated with Phe and ARA had twice as many normal fetuses (25% compared to 10%), fewer fetuses with mild MH compared to the Phe only group while the frequency of severe MH were similar.

4 | DISCUSSION

Exposure to phenytoin during pregnancy is associated with a distinctive pattern of dysmorphic features common to humans,[22,23] rabbits,[24] rats, and mice[18,25,26], suggesting a common, as yet unknown, teratogenic mechanism. One possible mechanism involves the induction of embryonic bradycardia and consequent hypoxia following dosing. The subsequent malformations are hypothesized to be a result of the subsequent reoxygenation episode. Evidence for this comes from work with the sensitive A/J mouse strain, showing generation of damaging free reactive oxygen species when heart rate had recovered after dosing.[26] Others have suggested that malformations result from the generation of reactive toxic intermediates via P450 enzymes although the embryo has negligible P450.[27]

4.1 | Phenytoin and hypoxia

In this study, oxygen supplementation of the dam reduced the frequency of cleft lip and midfacial hyperplasia. In the original mouse hypoxia studies,[17,28] cleft lip incidence was also reduced by maternal oxygen supplementation. It was postulated that the dam was hypoxic due to a decrease in maternal heart rate. This effect on the dam was also noted in rabbits.[29] Others have reported a drop in oxygen saturation of the blood on GD20 (about 20% after a single intravenous dose of 150 mg/kg phenytoin in the rat) (Juranek, personal communication[30]). Thus, it was hypothesized that oxygen supplementation corrected the maternal-induced hypoxia. Maternal heart rate is unknown in our study, but others[13] have noted no effect on heart rate following similar doses in male SD rats. Thus, it is plausible that embryonic hypoxia is the fundamental cause of the malformations associated with phenytoin teratogenesis in the rat. It is also possible that the cause of the malformations lies not only

	Phenytoin	ARA	Phenytoin and ARA
Number of treated rats	11	8	11
Mean maternal weight (g) (GD 11) ± SD	325.5 ± 37.58	301.9 ± 25.35	319.1 ± 37.27
Blood glucose levels (mmol/L) ± SD			
0 h	7.6 ± 1.66	8.1 ± 1.08	8.0 ± 1.38
2 h	19.3 ± 4.71	8.1 ± 1.08*	19.5 ± 1.96**
Mean no. of implantations ± SD	12.6 ± 3.96	14.0 ± 2.00	14.6 ± 1.96
Resorptions (%) ± SD	10.8 ± 21.87	3.6 ± 3.94	16.5 ± 32.25
Number of fetuses	130	108	133
No. of fetuses with CL (%)	0 (0.0%)	0	3 (2.3%)*
No. of fetuses with severe MH (%)	39 (30.0%)	0	44 (33.1%)
No. of fetuses with mild MH (%)	77 (59.2%)	0	53 (39.8%)
No. of normal fetuses (%)	14 (10.8%)	108 (100%)	33 (24.8%)
Mean fetuses weight (g) ± SD	3.9 ± 0.89	3.9 ± 0.34	3.3 ± 0. 47*,**
CRL (mm) ± SD	33.8 ± 3.46	34.2 ± 2.11	33.2 ± 2.82**
Lip height (mm) ± SD	0.75 ± 0.27**	1.51 ± 0.12*,**	0.96 ± 0.25*,**

TABLE 3 The effect of ARA on the teratogenicity of phenytoin (150 mg/kg) administered to GD 11 pregnant rats

*$P < 0.05$, compared to Phe.

**$P < 0.05$ compared to ARA.

in generalized embryonic hypoxia but also in embryonic hyperglycemia.

4.2 | Phenytoin and hyperglycemia and cleft lip

It is well established clinically that hyperglycemia can be induced in patients treated with phenytoin[31] as well as rats due to the drug's inhibitory effects on glucose-induced insulin release in the pancreas.[13,32] In the current study, glucose levels following phenytoin administration increased while the blood glucose levels in insulin treated dams remained steady. At the same time, control of blood sugar level by insulin in the dam increased the frequency of normal foetuses, eliminated the incidence of cleft lip, and significantly reduced the incidence of severe midfacial hyperplasia.

Strikingly, similar craniofacial malformations are generated in the streptozotocin-induced diabetic rat.[33] Type one diabetes during pregnancy is associated with a two- to ninefold increase in the rate of major congenital defects in the fetus[34] with cleft palate and lip also occurring in relatively high frequency.[12] It is believed that these anomalies are secondary to the teratogenic effect of hyperglycemia in early pregnancy although the diabetic state itself results in a variety of metabolic disturbances including the presence of hyperglycemia and associated hyperketonemia.[35] In our rat model, hyperglycemia was restricted to the period of craniofacial development. This

does not occur in the human or rat diabetic models which experience a chronic state of hyperglycemia.

The mechanism whereby hyperglycemia may induce dysmorphogenesis has not been elucidated. Several theories have been postulated including excess glucose,[36] damage to the developing yolk sac, deficiency states of arachidonic acid or myoinositol, and the generation of free oxygen radicals.[37] This concept of a multifactor origin has been promoted by many investigators[35,38] to acknowledge the fact that factors other than hyperglycemia are important to the pathogenetic mechanism.

4.3 | Hyperglycemia and hypoxia

The incidence of neural tube defects was increased in the GD7.5 diabetic mouse embryo when pregnant mice were kept in a hypoxic environment (12% O_2) or were hyperglycemic (>250 mg/dL).[39] At the same time, O_2 flux, an indicator of O_2 availability, was reduced by 30% in embryos of hyperglycemic mice. This may explain the ameliorative effect of hyperoxia; excess glucose metabolism accelerates the rate of O_2 consumption, thereby exacerbating the hypoxic state that is relieved by increasing oxygen supply. Such a state of pseudohypoxia following hyperglycemia has also been noted in adult tissues such as kidney[40] and retina[41] and is thought to be responsible for the damage observed in diabetic ulcers.

4.4 | Phenytoin, arachidonic acid, and cleft lip

Multiple studies have demonstrated arachidonic acid supplementation decreases the frequency of malformations associated with diabetic embryopathy, both in vivo and in vitro in rodent models.[42–44] The etiology may involve the glucocorticoid pathway. Excess glucocorticoids also induce hyperglycemia[45] and induce similar craniofacial abnormalities such as cleft lip and palate, and midfacial hypoplasia in rodent and primate models.[46,47]

In our study, fetuses exposed to co-administration of ARA with PHE were more likely to have normal craniofacial development and greater lip height compared to PHE only fetuses despite persistent maternal hyperglycemia. The effects in our study were mild compared to the diabetes and glucocorticoid animal models, but this may be because treatment was restricted to the period of upper lip formation (acute exposure) versus the chronic exposures experienced in diabetic models.

Like glucocorticoids, phenytoin competitively binds to a glucocorticoid receptor and induces phospholipase A2-inhibiting proteins (PLIP).[48,49] As PLIPs inhibit arachidonic acid release, this might explain the observation that ARA supplementation reduces the frequency of glucocorticoid-induced cleft palate in mice[42] and rats[50] as well as in our study. Increased lymphocyte glucocorticoid receptor level has been observed in children with fetal hydantoin syndrome when compared to phenytoin-exposed children without abnormal craniofacies.[51] In mice, susceptibility to phenytoin malformations is limited to strains with mutations in a histocompatibility locus resulting in elevated levels of glucocorticoid receptors.[52] Formation of the upper lip requires disintegration of epithelial seams between the MNP and the maxillary processes followed by mesenchymal proliferation and fusion.[53] Cortisone prevented this loss of epithelium in mice resulting in cleft lip formation.[42] Thus, the anti-inflammatory effect of phenytoin (via glucocorticoid pathways) may prevent the epithelial breakdown required for normal lip fusion to occur resulting in the formation of cleft lip.

When pregnant rats are dosed with phenytoin, embryos are exposed to several potentially adverse events including high concentrations of phenytoin, hypoxia, hyperglycemia, and ARA deficiency for several hours. All interventions, normalizing maternal hyperglycemia, co-administration with ARA, or increasing ambient oxygen concentration reduced but did not eliminate malformations. This would suggest that none of these conditions alone are the cause of the malformations implying that malformation-induction is a multifactorial process as malformations were still present in normoglycemic fetuses.

5 | CONCLUSION

Our results could be of clinical significance to pregnant women using phenytoin, suggesting careful monitoring and controlling of blood glucose levels might be a useful strategy in managing fetal outcomes.

DISCLOSURE

None of the authors has any conflict of interest to disclose. We confirm that we have read the Journal's position on issues involved in ethical publication and affirm that this report is consistent with those guidelines.

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