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BRIEF COMMUNICATION

Preliminary Findings of a Reduction of Otoconia in the Inner Ear of Adult Rats Prenatally Exposed to Phenytoin

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MINCK, D. R., L. C. ERWAY AND C. V. VORHEES. *Preliminary findings of a reduction of otoconia in the inner ear of adult rats prenatally exposed to phenytoin.* NEUROTOXICOL TERATOL **11**(3) 307–311, 1989.—Pregnant Sprague-Dawley CD® rats were administered phenytoin by gavage on days 7–18 of gestation in doses of 0 or 200 mg/kg. Following completion of a series of behavioral tests (20), progeny (18 months of age) were examined for otoconia in the vestibular labyrinth of the inner ear. None of the controls (N=22) had reduced otoconia, while 23.3% of phenytoin-treated offspring (N=43) had reductions. None of the controls, but 44.2% of phenytoin-treated offspring exhibited abnormal circling behavior during systematic examinations conducted in dry and swimming environments. Of the phenytoin-treated offspring exhibiting circling, 21.0% had reduced otoconia in either the utricle or saccule of one ear, while 25.0% of phenytoin-treated offspring not circling exhibited similar reductions. Conversely, 79% of phenytoin-treated offspring exhibiting circling did not exhibit any otoconial reductions. Thus, otoconial reduction cannot account for the majority of the cases of circling. The 21% vs. 25% otoconial reduction difference was not significant, however, when ratings of the magnitude of reduction were analyzed, circling offspring had significantly lower scores in their utricles than those not circling. More specifically, otoconial reduction in the right utricle and circling behavior were significantly related, although the number of concordant cases was small. Otoconial ratings did not differ for saccules. No differences in regional brain weights were found at the time of otoconial examination (560 days). The evidence provide preliminary support for the idea that prenatal exposure to phenytoin induces a reduction in otoconial crystals of the vestibular labyrinth in some of the exposed offspring, but it cannot account for most of the behavioral effects that have been observed in these offspring.

Phenytoin-induced otoconial reduction Phenytoin-induced teratogenesis of the inner ear
Phenytoin and congenital damage to utricle and saccule Prenatal phenytoin and postnatal vestibular dysfunction
Phenytoin-induced in utero injury of the vestibular labyrinth in rats Circling behavior

PHENYTOIN is a behavioral teratogen in rats (2, 14, 16, 18, 19). One of the behaviors previously observed (16, 19, 20) was abnormal circling locomotion in a minority of phenytoin exposed offspring. The possibility of middle ear infection as a cause of this behavior was ruled out previously (17).

The preceding paper (20) reports the results of an experiment on the relationship between phenytoin-induced circling and some of the behavioral abnormalities seen in these offspring. Circling was found to account for some but not all of the behavioral differences. In the present study the hypothesis was tested that phenytoin-induced circling was the result of inner ear damage to vestibular structures.

This hypothesis was based on the following evidence. Although milder in degree, phenytoin-induced behavioral aberrations resemble the abnormal circling, disrupted swimming, and impaired air-righting described in the pallid mutant line of inbred mice (12,13). The behavioral abnormalities in pallid mice are associated with a congenital absence of otoconia within the inner ear (1, 3, 5, 6, 10). Otoconia are composed of calcite crystals of calcium carbonate (9) attached to the otoconial membrane of the utriculus and sacculus of the vestibule. The otoconia provide mass to the otolithic maculae for detection of gravity for orientation and inertia for detection of linear acceleration (11).

METHOD

Subjects were offspring of Sprague-Dawley CD® rats admin-

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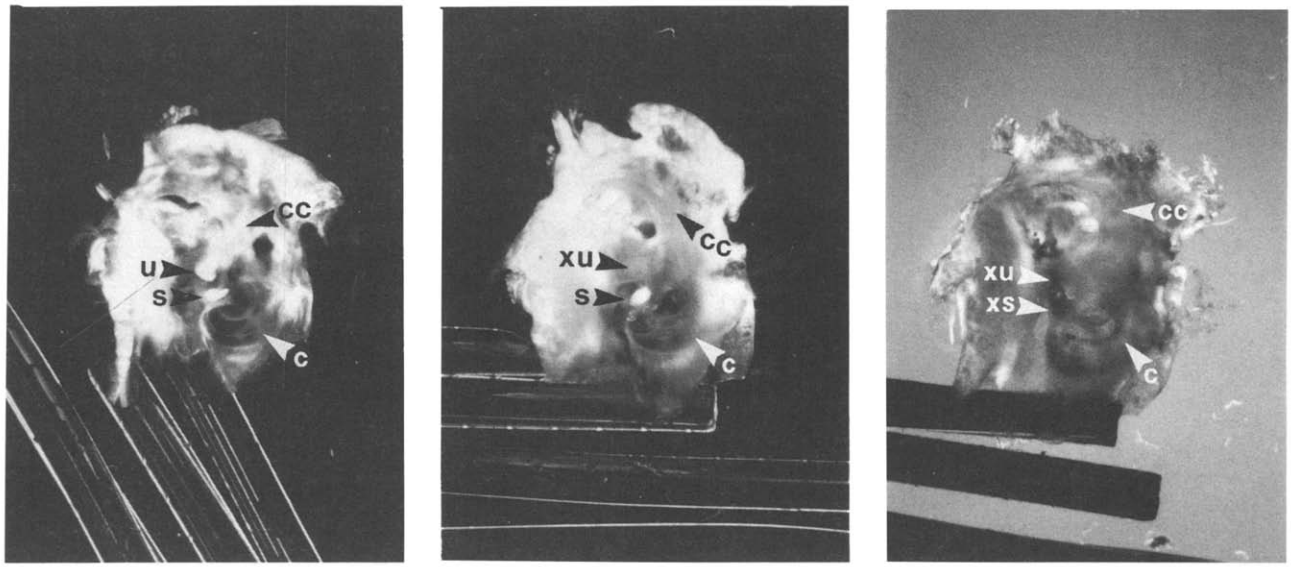

FIG. 1. Right otic capsules from three adult Sprague-Dawley CD rats. Left, otic capsule from vehicle control rat. Middle and right, otic capsules from rats exposed to 200 mg/kg of phenytoin during gestation. u = normal utricle, s = normal saccule, xu = position of missing utricle, xs = position of missing saccule, cc = common crus of the semicircular canals, c = cochlea. Magnification ×75.

istered phenytoin at a dose of 0 or 200 mg/kg (>99% purity, Aldrich Chemical, Milwaukee) by gavage on days 7–18 of gestation (vaginal plug=day 0) suspended in propylene glycol (2 ml/kg). At 18 months progeny were euthanized by methoxyflurane overdose. Rats available for inner ear examination were from the behavioral experiment reported in the preceding paper (20). At weaning, there were 8 litters of phenytoin-treated offspring and 12 litters of vehicle-treated control offspring. The 8 phenytoin litters contained 51 offspring after weaning. Between weaning and euthanasia, 4 were killed at day 85 (the original age designated to end the experiment prior to the decision to extend it) and 4 died, leaving 43 available for inner ear examination. All 43 of the surviving phenytoin offspring had their inner ears examined. The 12 control litters contained 90 offspring after weaning. Between weaning and euthanasia, 6 were killed at day 85 for the reason described above, 10 died, and 2 were killed because of illness, leaving 72 available for inner ear examination. Of these, only representative animals were examined. The method of selection was that the male and female pair in each litter that had been randomly assigned the letter code A on day 7 were chosen for inner ear examination. Had all litters had 100% survival, 24 rats would have been used for inner ear dissection. Since this was not the case, the procedure followed was that if either the male or female A animal had died or been euthanized, the B rat was taken instead. If the B rat was not available, the C rat was taken, and if the C rat was unavailable, the D rat was taken. Three control litters had no surviving males, but only one extra male from one of the complete litters was taken as partial compensation. The final number of control rats was 22, rather than the 24 planned. Brains were removed and otic capsules (semicircular canals, vestibule, and cochlea) were separated and placed in 70% buffered formalin for 5 days. Capsules were transferred for fixation to a solution of 70% followed by 85% ethanol for 2 days at each concentration, then placed in 100% ethanol for 5–10 days. Finally, tissues were cleared in methyl salicylate for 7–14 days. To facilitate examination, surrounding tissues were trimmed from otic capsules. Removed brains of the females were dissected by the method of Glowinski and Iversen (7) and each region weighed.

Otoconia were examined microscopically under bright and dark field, with and without polarizing lenses, to optimize visualization of crystals. The utricle and saccule of each ear were rated for otoconia using the following scale: dense otoconia=3 (normal), slight reduction in density=2, severe reduction=1, and absence=0. Four ratings were thus obtained for each animal, which were then summed; thus, a completely normal animal would obtain the maximum possible rating of 12. Otoconia were rated independently by two observers who did not know the rats treatment condition. Minor scoring discrepancies were resolved by using the more conservative (least abnormal) rating.

Because the rats were older when examined, their bone density was high, making dissection difficult. Specifically, one of the two otic capsules fractured during dissection in 26% of the phenytoin-treated and 32% of control offspring. Moreover, in the phenytoin group, 42% of fractured capsules were among those identified as circlers. This resulted in incomplete data for analysis of otoconial ratings.

Overall otoconial scores were analyzed by analysis of variance (general linear model), combined right and left utricle, right and left saccule scores, and weight data by *t*-test for independent samples (two-tailed), and frequency data were analyzed by Chi-square contingency analyses.

RESULTS

Changes in the phenytoin-treated (N=43) offspring's inner ears ranged from no apparent change to complete absence of otoconia in one ear. Examples are shown in Fig. 1. Absence of otoconia sometimes involved only one otolithic structure, and in the most severe cases involvement was complete, with absence of otoconia in both the utricle and saccule of one ear with lesser reductions in the other ear. No reductions or absences of otoconia were found among any of the controls (N=22).

The proportion of phenytoin-treated rats exhibiting circling is shown in Table 1 (left column). There was a significant increase in the proportion of rats exhibiting circling (44.2%) in the phenytoin-treated group as compared to controls, $\chi^2(1)=13.7$, $p<0.001$; this

TABLE 1

NUMBER AND PERCENTAGE OF AFFECTED AND UNAFFECTED PROGENY HAVING OTOCONIAL REDUCTION OR EXHIBITING ABNORMAL CIRCLING BEHAVIOR

Group	Number (%) Exhibiting Abnormal Circling		Number (%) With Reduced Otoconia*		Number (%) With Severely Reduced Otoconia†	
Control	0/22	(0.0)	0/22	(0.0)	0/22	(0.0)
Phenytoin	19/43	(44.2)‡	12/43	(27.9)§	6/43	(14.0)¶

*Reduction was defined as having at least 1 of the 4 inner ear structures with a rating of 0, 1 or 2.

†Severe reduction was defined as having at least 1 ear structure with a rating of 0 or 1.

‡$p<0.001$; §$p<0.02$; ¶$p<0.07$.

is comparable to that seen in the experiment as a whole where 42.3% of phenytoin-treated offspring exhibited circling (20).

Fractured capsules were arbitrarily assumed to be normal (assigned ratings of 3). When this was done an analysis of variance of ratings summed across all four structures showed no significant group effect. It is unlikely, however, that all of the phenytoin-treated offspring with fractured otolithic labyrinths were normal.

In order to obtain another estimate of a possible drug-related effect, the data were dichotomized such that rats with any reduction in otoconia (rating of 0, 1 or 2) in any of the 4 structures examined were considered affected, and all rats with normal otoconia (rating of 3) were considered unaffected. Table 1 (column 2) shows the number and percentage (27.9%) of affected rats irrespective of whether they exhibited circling or not. There was a significant difference in the proportion of rats with reductions of otoconia in the phenytoin group (27.9%) compared to controls (0%), $\chi^2(1)=6.0$, $p<0.02$. Also shown in Table 1 are the results of an analysis for severe otoconial reduction (ratings of 0 or 1) compared to those with ratings of 2 or 3. Severe reductions only occurred among phenytoin-treated offspring, but the effect in the 6 rats showing this severe reduction (14.0%) fell slightly short of significance, $\chi^2(1)=3.4$, $p<0.07$.

In order to determine the concordance between circling behavior and otoconial reduction, additional analyses were conducted on the contingency of circling and otoconial deficits among phenytoin-treated offspring. This is shown in Table 2. An analysis of variance on affected rats with all utricular and saccular findings pooled showed no group effect, but there was a significant disproportionality in contingency analyses of some ear structures when examined separately. The structure most frequently reduced was the right utricle and it was affected significantly more often in circlers than in noncirclers, $\chi^2(1)=4.85$, $p<0.05$. No significant effect was seen for the left utricle or the left or right saccule (data not shown). The fact that there was a difference in frequency of right utricular reductions, whereas there were no differences when all structures were analyzed together, led us to further examine the ratings. Table 2 (right two columns) shows the results of examining the ratings for the right and left utricles and right and left saccules combined for phenytoin circlers and noncirclers. This analysis revealed that there was a difference between the ratings of circlers and noncircles. The difference was only significant for utricular ratings, $t(8)=2.94$, $p<0.02$, but not for saccular ratings, $t(8)=1.71$, $p=0.13$, although the latter difference was in the same direction as that for the utricles.

Finally, body and regional brain weights for the female controls and at least one female phenytoin rat from each litter are shown in Table 3. Weights were compared by *t*-tests and no significant differences were found.

DISCUSSION

The present data demonstrate that prenatal phenytoin exposure at 200 mg/kg on days 7–18 of gestation induces a reduction in otoconia in the utricle and saccule of the inner ear in some rats. Because of a problem with otic capsule fracture encountered in these older rats, the data were incomplete, and this in turn prevented more detailed analyses. The data were sufficient to demonstrate that otoconial reduction was significantly related to abnormal circling behavior, but insufficient to determine the strength of the relationship. The data suggest that the utricles (particularly the right utricle) are more often affected than any of the other otoconial structures, but the relationship was not strong. Because of this, the question of whether rats with predominantly right-sided otoconial reductions reliably circle ipsilaterally or contralaterally to the lesion side cannot currently be addressed.

If phenytoin-induced otoconial reduction ultimately proves to be analogous to that seen in the pallid mutant, then one would predict that no clear relationship would exist between lesion side and direction of circling. The data for pallid mice would also lead to a prediction that otoconia in the utricles would be affected more severely than in the saccules (5). Further experiments will be designed to address such questions in greater detail. The present

TABLE 2

NUMBER AND PERCENTAGE OF PHENYTOIN OFFSPRING EXHIBITING ABNORMAL CIRCLING BEHAVIOR, OTOCONIA REDUCTION AND RATINGS OF OTOCONIAL REDUCTIONS

Categorization of Phenytoin Offspring Circling Behavior	Number (%) of Phenytoin Rats with Normal or Reduced Otoconia*				Number (%) of Phenytoin Rats With Severely Reduced Otoconia in the Right Utricle†				Right and Left Utricular and Saccular Rating Score¶	
	Normal		Reduced		Normal		Reduced		Utricles	Saccules
Noncirclers	18/24	(75.0)	6/24	(25.0)	22/22	(100.0)	0/22	(0.0)	4.8 ± 0.3	5.3 ± 0.5
Circlers	15/19	(79.0)	4/19	(21.0)	13/16	(81.2)	3/16	(18.8)‡	3.2 ± 0.5§	4.8 ± 0.9

*Reduction was defined as having at least 1 of the 4 structures with a rating of ≤2.

†Severe reduction was defined as having an otoconia rating of ≤1.

‡$p<0.05$ by chi-square; §$p<0.02$ by *t*-test for independent samples, two-tailed.

¶Scores are for all rats showing a reduction. The maximum possible score for each pair of structures was 6.

TABLE 3

BODY AND REGIONAL BRAIN WEIGHTS OF FEMALE PROGENY PRENATALLY EXPOSED TO PHENYTOIN AND CONTROLS AT THE TIME OF INNER EAR EXAMINATION MEAN ± SE

	Control	Phenytoin*
N	12	11
Body Weight (g)	410.5 ± 11.0	402.9 ± 15.1
Brain Weight (mg)		
Cerebellum	297.2 ± 1.9	292.1 ± 5.3
Medulla-Pons	283.2 ± 5.7	269.9 ± 4.9
Hypothalamus	59.4 ± 2.3	63.0 ± 4.3
Striatum	71.7 ± 4.0	66.3 ± 4.7
Mesencephalon	275.6 ± 9.4	275.4 ± 11.0
Hippocampus	172.9 ± 5.2	173.7 ± 4.1
Cerebral Cortex	761.5 ± 12.7	760.3 ± 10.5
Mean Total Brain Wt. (mg)	1921.5 ± 25.8	1900.7 ± 24.9

*No differences were significant by *t*-test.

data serve as a preliminary finding that a phenytoin-induced otoconial effect exists, but many unresolved questions remain.

From studies in pallid and other mutant mouse strains it is known that otoconial formation is dependent on synthesis of mucopolysaccharides that form the otoconial membrane. Otoconial formation is also dependent, by an as yet unknown mechanism, on the adequate availability of manganese (5,10). Gravid pallid mice administered supplemental manganese beginning shortly before otoconia formation, exhibit normal otoconial ontogeny (5). Nonmutant mice show congenital otoconial deficiencies when administered a manganese-deficient diet during gestation (4). These data suggest that manganese plays a crucial role in formation of otoconia, and it is speculated that phenytoin may also interact with manganese to interfere with its role in otoconial development.

In addition, another mineral, zinc, has been implicated in the maintenance of otoconial crystals (6). Carbonic anhydrase is a zinc-dependent enzyme and it has been shown that the carbonic anhydrase inhibitor, dichlorophenamide, induces otoconial deficiency in C57BL/6J × Swiss/Cox mice progeny in utero (15). This effect is particularly interesting in the present context, because the effect is asymmetrical, with the right utricle being predominantly affected. In the present experiment, phenytoin-treated offspring also showed a predominance of effect in the right utricle. This may suggest a mechanistic link between phenytoin and carbonic anhydrase inhibitors in terms of their effects on otoconial development. Another possible link may develop out of the recent observation that the fungicide dinocap also induces congenital otoconial reduction and abnormal behavior in mice (8). It remains to be seen whether some structure-activity relationship exists among phenytoin, dinocap, and the carbonic anhydrase inhibitors which might link them to interference with otoconial formation.

The present data may be taken as an indication that phenytoin prduces a previously unrecognized teratogenic effect on embryonic inner ear development which may contribute, albeit in a minor way, to some of the functional deficits observed postnatally. The incomplete relationship between the inner ear defect and the functional effects also supports the view that otoconial reduction can at best explain only a small amount of the variance associated with the behavioral abnormalities induced by phenytoin. To the extent that otoconial reduction cannot explain most of the behavioral dysfunctions, the evidence suggests that other mechanisms and sites of action must be involved in phenytoin's embryotoxicity.

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