Effects of zinc deficiency on morphogenesis of the fetal rat eye

JOHN M. ROGERS* and LUCILLE S. HURLEY

Department of Nutrition, University of California, Davis, California 95616, USA
* Present address: Perinatal Toxicology Branch, Developmental Biology Division, Health Effects Research Laboratory, United States Environmental Protection Agency, Research Triangle Park, North Carolina 27711, USA

Summary

Maternal zinc deficiency during pregnancy results in a high frequency of fetal eye malformations in the Long-Evans rat. In this study we examine the development of the eye from days 12 through 21 of gestation in conceptuses of dams fed deficient or adequate levels of zinc and also examine maternal plasma and conceptus zinc concentrations during this period. Dams were fed diets containing 0.5 (0.5 Zn group), 4.5 (4.5 Zn group), or 100 (100 Zn AL group) μg zinc per gram diet ad libitum, or 100 μg zinc g⁻¹ diet in amounts restricted on a daily basis to the intake of matched animals from the 0.5 Zn group (100 Zn RL group). Conceptuses were removed and maternal plasma was collected on days 12, 14, 16, 19 and 21 of gestation. Maternal plasma and conceptus zinc concentrations reflected maternal dietary zinc level, with dam plasma Zn concentrations in the order of 0.5 Zn group < 4.5 Zn group < 100 Zn group on all days. A similar pattern held for embryo/fetus zinc, except for days 19 and 21, at which times the 0.5 Zn and 4.5 Zn fetuses had similar zinc concentrations. Histological examination of the developing eye of 0.5 Zn fetuses on days 12 and 14 revealed that invagination of the optic cup was often deficient, and that closure of the choroid fissure did not occur, resulting in colobomata and retinal folding visible at term. A very few fetuses were found at term to be anophthalmic or have only remnants of ocular tissue. In the absence of coloboma, retinal histogenesis evaluated on day 19 appeared normal in zinc-deficient fetuses, and in fact, the eye was relatively spared the effects of zinc deficiency in these fetuses. No eye malformations were seen in 4.5 Zn fetuses. Thus, low maternal plasma and embryonic zinc concentrations during organogenesis adversely affect morphogenesis of the optic vesicle and closure of the choroid fissure, leading to anophthalmia or colobomatous microphthalmia with retinal folding at term. A possible mechanism for the latter malformation involving lack of vitreous pressure necessary for normal expansion of the developing eye is discussed. When the choroid fissure does close normally, later prenatal histogenesis of the retina does not seem to be adversely affected by zinc deficiency.

Key words: zinc deficiency, eye development, teratogenesis, rat embryo.

Introduction

The teratogenicity of zinc deficiency was first demonstrated by Turk and coworkers in the chick embryo (Turk, Sunde & Hoekstra, 1959). Hurley & Swenerston (1966) were the first to demonstrate this effect in a mammal, the Sprague-Dawley rat. Fetuses of zinc-deficient rats had multiple skeletal abnormalities as well as soft-tissue malformations affecting the brain, heart, lung and urogenital system. The zinc content of fetuses from zinc-deficient females was significantly lower than that of controls. Mills, Quarterman, Chester, Williams & Dalgarno (1969) and Rogers, Keen & Hurley (1985) have also reported congenital malformations in rats resulting from maternal zinc deficiency. The frequency and severity of congenital malformations resulting from zinc deficiency can be correlated with the concentration of zinc in the maternal diet. Hurley & Cosens (1974) found a decreasing frequency of fetal malformations with increasing maternal dietary zinc during pregnancy. Microphthalmos was seen in chick embryos of zinc-deficient hens by Blanberg and coworkers (Blanberg, Blackwood, Supplee & Combs, 1960). Severe zinc deprivation during embryonic and fetal development in the rat results in profound effects on virtually all derivatives of the neural tube and associated structures including the brain, spinal cord, eyes...
and olfactory tract (Hurley & Shrader, 1972; Warkany & Petering, 1972, 1973; Adeloye & Warkany, 1976). Defects of the eye seen at term included microphthalmia and anophthalmia (Hurley & Shrader, 1972; Warkany & Petering, 1972). In a previous study in the Long-Evans hooded rat, we found anophthalmia or microphthalmia in 38% of live fetuses from dams fed throughout pregnancy a diet containing 0.5 μg zinc g⁻¹ diet (Rogers et al. 1985). Recently, postnatal zinc deficiency induced in rats by low-zinc diet (Leure-du-Pree & McClain, 1982) or by administration of zinc chelators (Leure-du-Pree, 1981) has been shown to result in degenerative changes in the retinal pigment epithelium and rod outer segments.

In the present study we examine the development of the eye from day 12 to day 21 of gestation in control and zinc-deficient fetuses. The pathogenesis of anophthalmia or microphthalmia is followed over this period of development and possible mechanisms for these effects are discussed.

**Materials and methods**

Virgin female Long-Evans hooded rats weighing 180–200 g were purchased from a commercial source (Simonsen Laboratories, Gilroy, CA). They were individually housed in stainless steel cages in a temperature (22–23°C) and photoperiod (12 h day⁻¹) controlled room. All animals were allowed to acclimatize for at least 1 week and were fed a complete purified diet containing 100 μg zinc g⁻¹ diet (Table 1) during this time. Females were caged overnight with stock-fed (Purina Rat Chow) males of the same strain. Sperm plugs or sperm in vaginal smears the following morning were indications of a successful mating and dams were considered to be at day 0 of gestation at this time. Sperm-positive dams were assigned to four experimental diet groups: a zinc-deficient group fed a diet containing 4-5 μg zinc g⁻¹ (4-5Zn group); an AL group and a restricted intake control group fed the stock diet (100Zn group) and a low-zinc group fed a diet containing 0.5 μg zinc g⁻¹ (0.5Zn group).

Litters were collected on days 12, 14, 16, 19 and 21 of gestation. Dams were anaesthetized with ether and laparotomy was performed. Blood was collected by cardiac puncture into syringes containing zinc-free heparin (Sigma Chemical Co., St Louis, MO). Blood was centrifuged for 15 min at 3000 g. Plasma was removed with plastic transfer pipettes (W. Sarstedt, Inc., Princeton, NJ) and stored frozen in acid-washed plastic vials until analysed. Three fetuses from each litter were collected for determination of fetal zinc concentrations. On day 12 the embryos were removed with membranes (yolk sac and amnion) intact; on later days the fetuses were removed from the membranes. All embryos were dried at 80°C to constant weight prior to measurement of zinc concentrations, thus these values are given on a dry weight basis. Similarly, eyes removed from day 21 fetuses for zinc determination were dried prior to analysis. Additional whole embryos and fetuses removed for histological examination were preserved in Bouin’s solution. Eyes removed for retinal morphometry were incised at the ora serrata and fixed in 3% glutaraldehyde at 4°C overnight. Whole embryos and fetuses were dehydrated in ethanol, cleared with xylene and embedded in paraffin. Sections were cut at 7 μm and stained with haematoxylin and eosin. Eyes fixed in glutaraldehyde were dehydrated in ethanol and embedded in Spurr’s plastic embedding medium. Sections of the retina (1 μm thick) were cut with an ultramicrotome through the optic nerve and stained with toluidine blue. Retinal measurements were made using an ocular micrometer.

All zinc concentrations were determined by atomic absorption spectrophotometry (Instrumentation Laboratories model 551) following wet ashing of tissues in concentrated (16 M) nitric acid. Using this method recovery of added metal is 98–102% (Clegg, Keen, Lonnerdal & Hurley, 1981).

One-way analysis of variance and Duncan’s multiple-range test (SPSS, Nie, Hull, Jenkins, Steinbrenner & Bent, 1975) were used to evaluate statistical significance. The litter was used as a statistical unit for calculation of fetal values; thus, these values represent means of litter means within each group. A probability of 0.05 or less was considered significant.

**Table 1. Diet composition**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Percent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein (spray dried egg white)</td>
<td>25.0</td>
</tr>
<tr>
<td>Salt mix*</td>
<td>6-0</td>
</tr>
<tr>
<td>Vitamin mix†</td>
<td>1-5</td>
</tr>
<tr>
<td>Sucrose</td>
<td>59.5</td>
</tr>
<tr>
<td>Corn oil</td>
<td>8-0</td>
</tr>
</tbody>
</table>

* Salt mix in g/kg diet: CaCO₃, 18-00; K₂HPO₄, 19-26; NaCl, 10-8; MgSO₄, 3-6; CaHPO₄, 3-6; FeSO₄ 7H₂O, 1-5; KI, 0-048; ZnCO₃, 0-192; CuSO₄ 5H₂O, 0-018; CrK(SO₄)₂ 12H₂O, 0-021; and Mn(SO₄)₂ H₂O, 0-138.
† Vitamin mix in g/kg diet: inositol, 0-375; ascorbic acid, 0-075; calcium pantothenate, 0-0375; thiamin HCl, 0-0225; pyridoxine HCl, 0-0225; nicotinic acid, 0-0225; menadione, 0-01875; riboflavin, 0-0075; p-aminobenzoic acid, 0-0075; folic acid, 0-00045; biotin, 0-0039; vitamin E (Rovimix E-50), 0-1785; vitamin A (Rovimix A-250), 0-04905; vitamin D (Rovimix AD₃₂₅/3₂₅), 0-00345; vitamin B₁₂ (Merck 12-mannitol), 0-0225; choline chloride (70 % solution), 1-0725 (sources: Rovimix, Hoffman-LaRoche, Nutley, NJ; Merck & Co., Rahway, NJ).
Results

Effect of dietary zinc on maternal plasma and embryonic zinc concentrations
Pregnant rats fed from mating 0.5 µg zinc g⁻¹ diet had significantly lower plasma zinc concentrations than controls on days 12–21 of gestation. Dams fed 4.5 µg zinc g⁻¹ diet had intermediate plasma zinc concentrations, which were significantly lower than controls on all days and significantly higher than the 0.5 Zn group through day 16, but not on days 19 or 21 (Fig. 1). Similarly, embryo/fetus zinc concentrations in the 0.5 Zn and 4.5 Zn diet groups were lower than controls on all days tested, but the 0.5 Zn group was lower than the 4.5 Zn group only until day 16 (Fig. 2).

Effects of zinc deficiency on eye development
In control embryos on day 12 invagination of the optic cup and formation of the lens vesicle was complete, although the primary lens fibres were not elongated (Fig. 3A). By day 14 the eyes in 100 Zn AL and 100 Zn RI control embryos were well formed, with elongation of the primary lens fibres almost complete and establishment of a well-defined vitreous space (Fig. 3C). In contrast, in many 0.5 Zn embryos invagination of the optic vesicle was incomplete on day 12 and the lens remained at the placode stage. Furthermore, large numbers of necrotic cells were seen in the diencephalic neuroepithelium near the optic cup (Fig. 3B). At day 14 the optic vesicle was invaginated in most 0.5 Zn embryos but the choroid fissure often remained open, resulting in an unclosed and poorly defined vitreous space. The lens vesicle was formed by this time, but fibre elongation was retarded, resulting in a flattened lens (Fig. 3D). The nonclosure of the choroid fissure seen in zinc-deficient embryos on day 14 persisted, resulting in colobomata in many of the zinc-deficient fetuses at term. The colobomata were clearly visible in whole eyes at term (Fig. 4). Sectioning of colobomatus eyes of 0.5 Zn fetuses revealed a large amount of retinal folding and lack of a vitreous space, while eye development in 4.5 Zn embryos was comparable to controls (Fig. 5).

The degree to which invagination of the optic vesicle and closure of the choroid fissure occurred in zinc-deficient fetuses was variable, and resulted in fetuses with varying degrees of microphthalmia or apparent anophthalmia at term (Rogers et al. 1985). Histological examination of fetuses in the present study revealed that some were completely anophthalmic, while others contained small remnants of ocular tissues, including lens, retina and pigment epithelium (Fig. 6).

Eye growth was not adversely affected in zinc-deficient fetuses without colobomata. Indeed, when expressed as a percentage of body weight, it is apparent that noncolobomatous eyes were somewhat spared the growth-inhibiting effects of zinc deficiency compared to overall growth effects. Furthermore, unlike effects on the whole fetus (Rogers et al. 1985), maternal zinc deficiency did not result in a lower...
concentration of zinc in whole eyes than that observed in restricted intake controls. (Table 2).

**Effects of zinc deficiency on the differentiation of the fetal retina at term**

Zinc deficiency did not appear to affect the prenatal histogenesis of the retina in zinc-deficient fetuses without colobomata. Even among zinc-deficient fetuses with colobomata the folded retina often showed normal histogenesis (Fig. 5B).

Measurements of the thickness of the whole retina and retinal layers at term showed no differences among the various diet groups, even without correcting for effects on fetal size (Table 3). Thus, the major effect of zinc deficiency on eye morphogenesis was the inhibition of optic vesicle invagination and choroid fissure closure that occurred during organogenesis. When this did not occur, subsequent prenatal histogenesis of the retina appeared to be largely spared.

![Image of developing eye in control and zinc-deficient embryos](image)

Fig. 3. The developing eye in control and zinc-deficient embryos. (A) Control day 12. (B) Zinc-deficient day 12. (C) Control day 14. (D) Zinc-deficient day 14. Note lack of invagination of optic cup on day 12 and coloboma on day 14 in zinc-deficient embryos. pr, pigmented retina; nr, neural retina; lv, lens vesicle; nc, necrotic cells; co, coloboma. A-C ×165; B,D ×180.
Zinc deficiency and eye morphogenesis

Fig. 4. Coloboma in an eye from a 0-5 Zn fetus on day 21. *on*, Optic nerve. ×35.

Discussion

Fetuses of dams fed 0.5 µg zinc g⁻¹ diet throughout gestation had microphthalmia of varying degree at term, ranging from microscopic remnants of ocular tissue to eyes with colobomata and extensive retinal folds. In rare instances no sign of ocular tissue could be found by histological examination. Thus it appears that invagination of the optic cup and closure of the choroid fissure are the processes that are adversely affected by developmental zinc deficiency. In anophthalmic fetuses it is likely that there was an even earlier error in eye development, such as lack of optic vesicle formation; however, no such observation was made in this study.

Retardation of optic cup invagination and choroid fissure closure may be related to the large number of necrotic cells seen in the adjacent neuroepithelium in zinc-deficient embryos on day 12. During the time that these processes are occurring, maternal plasma and embryonic zinc concentrations are lower in embryos of the 0-5 Zn group than in those in the 4-5 Zn group. This difference in embryonic zinc concentration probably explains why the 4-5 Zn fetuses do not have eye malformations at term, despite zinc concentrations at term are similar to those in 0-5 Zn fetuses. Likewise, the similar zinc levels in whole fetuses of these two groups during the latter part of gestation provides some explanation for the relative lack of effect on ocular histogenesis in severely deficient fetuses at term, as histogenesis is occurring during this period. Dams fed zinc-deficient diet throughout pregnancy are virtually anorexic during the last 3-4 days of pregnancy (Rogers et al. 1985). This inanition induces a catabolic state in the dam, freeing maternal tissue zinc which is then available to the fetus (Masters, Keen, Lonnerdal & Hurley, 1983). Indeed, fetuses in the 4-5 Zn group near term appear to be able to accumulate zinc in the eyes to a level similar to that of restricted intake controls. The effects of severe zinc deficiency on fetal eye size were much less severe than effects on body weight. This relative sparing of eye size in relation to effects on body weight was also seen in the restricted intake control group and is consistent with the well-established sparing of brain growth in fetuses of rats subjected to nutritional deficiencies (Winick, 1976).

The microphthalmia and retinal folding seen in zinc-deficient fetuses at term probably results from...

Fig. 5. (A) Section through a normal eye of a 100 Zn fetus on day 21. (B) Section through a colobomatous eye from a 0-5 Zn fetus on day 21. Note folding and eversion of retina, and lack of vitreous space. *rf*, retinal fold; *re*, retinal eversion. ×33.
Table 2. Effects of maternal dietary zinc on fetal weight, eye size and eye zinc concentration on day 21 of gestation

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>No. litters</th>
<th>Fetus weight (g)</th>
<th>Fetal eye weight (mg)</th>
<th>Eye/body weight (%)</th>
<th>Fetal eye zinc (µg g⁻¹ dry weight of eye)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 Zn</td>
<td>10</td>
<td>3.13 ± 0.13*</td>
<td>13.5 ± 0.7*</td>
<td>-435 ± 0.15*</td>
<td>66.2 ± 0.9b</td>
</tr>
<tr>
<td>4.5 Zn</td>
<td>9</td>
<td>5.29 ± 0.09*</td>
<td>20.9 ± 0.3*</td>
<td>-391 ± 0.09*</td>
<td>60.4 ± 1.7*</td>
</tr>
<tr>
<td>100 Zn AL</td>
<td>7</td>
<td>5.56 ± 0.16*</td>
<td>21.7 ± 0.8*</td>
<td>-391 ± 0.09*</td>
<td>71.9 ± 2.3*</td>
</tr>
<tr>
<td>100 Zn RI</td>
<td>10</td>
<td>4.52 ± 0.18*</td>
<td>21.0 ± 0.6*</td>
<td>-423 ± 0.10*</td>
<td>62.6 ± 0.9a</td>
</tr>
</tbody>
</table>

Values shown are means ± S.E.M.
Values in the same column not sharing a common superscript are significantly different by Duncan's multiple-range test (P ≤ 0.05).
Fetus weights indicate means of litter means; eyes (one from each of five fetuses per litter) were pooled for determination of weights and eye zinc concentrations.

Fig. 6. (A) Coronal section through the head in the eye region of a 0.5 Zn fetus on day 19, showing unilateral anophthalmos (empty orbit at arrow) with embedded ocular tissues on contralateral side (double arrow). (B) Higher magnification of embedded tissues, showing lens (l) and pigment epithelium (pe) remnants. A × 16; B × 120.

lack of vitreous pressure due to nonclosure of the choroid fissure. One category of microphthalmos in humans is associated with cystic extension of the vitreous space, often through a colobomatous opening in the eye (Coulombre & Coulombre, 1977). Studies in chick embryos have demonstrated that the buildup of positive vitreous pressure following choroid fissure closure is an essential force for normal eye enlargement and retinal expansion. When vitreous substance was continuously drained from developing eyes, microphthalmic eyes with retinal folds resulted (Coulombre, 1956). A similar role for internal fluid pressure during periods of expansion has been shown to occur in the embryonic brain (Desmond & Jacobson, 1977). These investigators produced folding in the walls of chick embryo brains by placing capillary tubes into the brain ventricles thereby releasing cerebrospinal fluid pressure. Coloboma and retinal folding and eversion have been reported in rat fetuses following maternal folic acid deficiency (Giroude,
Table 3. Measurements of whole retinas and retinal layer thicknesses from day 21 fetuses of dams fed different levels of zinc during gestation*†

<table>
<thead>
<tr>
<th>Experimental group</th>
<th>No. litters</th>
<th>Whole retina</th>
<th>Neuroblast layer</th>
<th>Ganglion Cell+IP*</th>
<th>Nerve fibre layer</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.5 Zn</td>
<td>8</td>
<td>189 ± 9</td>
<td>130 ± 7</td>
<td>34 ± 3</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>4.5 Zn</td>
<td>8</td>
<td>204 ± 10</td>
<td>144 ± 8</td>
<td>32 ± 3</td>
<td>23 ± 3</td>
</tr>
<tr>
<td>100 Zn AL</td>
<td>6</td>
<td>203 ± 8</td>
<td>144 ± 6</td>
<td>36 ± 2</td>
<td>21 ± 1</td>
</tr>
<tr>
<td>100 Zn RI</td>
<td>5</td>
<td>200 ± 3</td>
<td>137 ± 2</td>
<td>32 ± 2</td>
<td>24 ± 4</td>
</tr>
</tbody>
</table>

*Measurements in microns, mean ± S.E.M.
†One retina from each of 2–4 pups per litter was measured and litter means were used as the statistical unit.

Ganglion cell layer+inner plexiform layer.

Delmas, Lefebvres & Prost, 1954; Nelson, 1957; Armstrong & Monie, 1966), and large retinal folds ('double optic cup') were almost always accompanied by colobomata (Armstrong & Monie, 1966). Coloboma, retinal folding and eversion of the retina also occur in rat fetuses subsequent to maternal vitamin A deficiency (Warkany & Schraffenberger, 1946). Coloboma was described in rat fetuses as a result of X-irradiation (Wilson, 1954) and retinal folds were reported following maternal injection of trypan blue (Gillman & Gilbert, 1954). Exposure of pregnant rats to inhalation of nickel carbonyl for only 15 min on day 7 or 8 of gestation frequently resulted in anophthalmia or microphthalmia with thickened, folded or redundant retinas (Sunderman, Alipass, Mitchell, Basel & Albert, 1979).

The mechanism leading to incomplete optic cup invagination and lack of choroid fissure closure in zinc-deficient fetuses is unclear. The necrosis seen in the diencephalic neuroepithelium adjacent to the optic vesicle may inhibit normal optic cup formation, although necrosis was not prevalent in the optic cup per se. Record and coworkers (Record, Tulsi, Dreosti & Fraser, 1985) have recently shown that the embryonic neural epithelium is particularly prone to cell necrosis during periods of maternal zinc deficiency.

Embryonic zinc deficiency results in decreased cellularity of the neural tube and reduced embryonic incorporation of [3H]thymidine (Swenerton, Shrader & Hurley, 1969), and the head region has been shown to be affected to a greater extent than the rest of the embryo (Eckhert & Hurley, 1977). These effects are probably due in part to reduced activity of thymidine kinase and DNA polymerase, zinc metalloenzymes (Dreosti & Hurley, 1975; Duncan & Dreosti, 1975; Duncan & Hurley, 1978; Record & Dreosti, 1979), and effects on RNA or protein synthesis may also be important, as a number of zinc metalloenzymes participate in the processes of transcription and translation (O'Dell, 1974). Thus there are probably multiple biochemical lesions involved in the neuroepithelial necrosis and subsequent ocular malformations seen in this study.

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References


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