Ocular Malformations of *Xenopus laevis* Exposed to Nickel During Embryogenesis*†

ODED HAUPTMAN, M.B.,‡§ DANIEL M. ALBERT, M.D.,‡¶ MARILYN C. PLOWMAN, B.S.,¶ SIDNEY M. HOPFER, Ph.D.,¶ and F. WILLIAM SUNDERMAN, JR., M.D.¶

‡Howe Laboratory of Ophthalmology, Massachusetts Eye and Ear Infirmary, Boston, MA 02114
and
¶Departments of Laboratory Medicine and Pharmacology, University of Connecticut School of Medicine, Farmington, CT 06030

**ABSTRACT**

The pathogenesis of eye anomalies induced by exposure to Ni$^{2+}$ (as nickel chloride) during embryogenesis was studied in the frog, *Xenopus laevis*. Eyes of control and Ni$^{2+}$-exposed tadpoles were examined without staining using a dissecting microscope, by light microscopy of histological sections, and by electron microscopy. The ocular abnormalities of Ni$^{2+}$-exposed tadpoles included (a) microphthalmia, (b) hypopigmentation, (c) hernias and cysts of the choroid and retina, and (d) iris coloboma; cataracts were uncommon. The pathogenesis of the ocular lesions appears to involve diffuse or focal dysplasia and loss of the retinal pigment epithelium, with dystrophy of photoreceptor outer segments and protrusion of neuroretina through gaps in the pigment epithelium. This study confirms that Ni$^{2+}$ is a potent ocular teratogen for *Xenopus* embryos and points to the retinal pigment epithelium as a primary cellular target for Ni$^{2+}$-induced embryotoxicity.

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† Send reprint requests to F. William Sunderman, Jr., M.D., MC-2225, University of Connecticut Health Center, 263 Farmington Avenue, Farmington, CT 06030.
‡ Present address: Berwick Eye Centre, 5 Lansdown Street, North Balwyn 3104, Australia.
¶ Present address: Department of Ophthalmology, University of Wisconsin School of Medicine, Madison, WI 53792.
Introduction

Increased incidence of malformations occurs in rats, mice, hamsters, chickens, and frogs exposed to nickel compounds during embryogenesis. Nickel teratogenesis affects the eyes, craniofacial structures, neural tube, notochord, skeleton, lungs, heart, and gut. The eyes are a paramount target for nickel teratogenesis in rats and frogs. After pregnant rats were exposed to inhalation of nickel carbonyl on the seventh day of gestation, ocular anomalies (primarily microphthalmia and anophthalmia) were found in 28 percent of the progeny. After tadpoles of the South African frog, *Xenopus laevis*, were exposed to nickel chloride on the second day post-fertilization, more than 80 percent developed ocular malformations, including open choroid fissure, trans-scleral hernias and cysts, and microphthalmia.

*Xenopus laevis* is well suited for experiments on ocular teratogenesis because of the frog's fecundity, its ability to provide embryos year-around, the extensive data base on *Xenopus* in reproductive biology and molecular genetics, and the resemblance of ocular development in *Xenopus* to higher vertebrates, including humans. The present paper describes the ocular pathology of *Xenopus* tadpoles exposed to Ni<sup>2+</sup> in the FETAX test (Frog Embryo Teratogenesis Assay: *Xenopus*), a standardized assay for teratogenic effects of chemicals. This study is a step in our research program to probe the cellular and molecular mechanisms of teratogenesis and embryotoxicity induced by exposures to toxic metals.

Materials and Methods

The experimental animals were *Xenopus laevis* tadpoles that were grown in Petri dishes at 23° to 24°C under the prescribed conditions of the FETAX test. The protocol was approved by the Animal Experimentation Committee of the University of Connecticut Health Center. Most tadpoles whose eyes were examined in this study (*N* = 152) were drawn from previously reported FETAX assays of NiCl<sub>2</sub> including a control group (*N* = 18, Group A) grown from 5 to 101 h post-fertilization in FETAX medium (NaCl, 10.7 mmol/L; NaHCO<sub>3</sub>, 1.14 mmol/L; KCl, 0.40 mmol/L; CaCl<sub>2</sub>, 0.14 mmol/L; CaSO<sub>4</sub>, 0.35 mmol/L; MgSO<sub>4</sub>, 0.62 mmol/L; pH 6.8), and eight test groups of 13 to 19 tadpoles (Groups B to I) exposed from 5 to 101 h post-fertilization to various concentrations of NiCl<sub>2</sub> added to FETAX medium (table I). The tadpoles were killed by tricaine anesthesia at 101 h post-fertilization (stage 42, Nieuwkoop/Faber classification) and fixed in buffered formalin (3% v/v). The eyes were inspected with a dissecting microscope under bright- and dark-field illumination, and the ocular diameters were measured from the choroid fissure to the opposite pole, using a calibrated micrometer eyepiece. Eleven controls from Group A and 18 NiCl<sub>2</sub>-exposed tadpoles with ocular anomalies (two to four tadpoles from Groups E to I) were embedded in paraffin. Histological sections (5 μm), cut transversely through the eyes, were stained with hematoxylin-eosin and examined by light microscopy.

To study the time-course for development of ocular deformities, six groups of 18 embryos were grown in FETAX medium, with or without added NiCl<sub>2</sub> (180 μmol/L), during the following intervals after fertilization: 5 to 53 h (controls, Group J; NiCl<sub>2</sub>-treated, Group K), 5 to 77 h (controls, Group L; NiCl<sub>2</sub>-treated, Group M), or 5 to 101 h (controls, Group N; NiCl<sub>2</sub>-treated, Group O). At the end of the exposures (i.e., at Nieuwkoop/Faber stages 26, 36, and 42), six embryos from each group were fixed for 30 min at 0°C in Karnovsky's cacodylate-buffered paraformaldehyde-glutaraldehyde, post-
### TABLE I
Ocular Findings in Control and Ni²⁺-Exposed Tadpoles at 101 Hours Post-fertilization

<table>
<thead>
<tr>
<th>Group</th>
<th>Ni²⁺ Concentration in the Medium (µmol/L)</th>
<th>Number of Tadpoles Examined</th>
<th>Tadpoles with Ocular Malformations N (%)</th>
<th>Ocular Diameter (Mean ± SD) (mm)</th>
<th>Incidence of Microphthalmia N (%)</th>
<th>Severity of Depigmentation (Mean ± SD)</th>
<th>Incidence of Retinal Hernia and/or Cyst N (%)</th>
<th>Incidence of Iris Coloboma N (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>0.0</td>
<td>18</td>
<td>1 (6)</td>
<td>0.94 ± 0.06</td>
<td>0 (0)</td>
<td>1.1 ± 0.5</td>
<td>1 (6)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>B</td>
<td>1.8</td>
<td>21</td>
<td>0 (0)</td>
<td>1.02 ± 0.04</td>
<td>0 (0)</td>
<td>1.3 ± 0.7</td>
<td>0 (0)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>C</td>
<td>3.6</td>
<td>18</td>
<td>3 (17)</td>
<td>0.94 ± 0.14</td>
<td>2 (11)</td>
<td>1.4 ± 0.6</td>
<td>0 (0)</td>
<td>1 (6)</td>
</tr>
<tr>
<td>D</td>
<td>4.5</td>
<td>16</td>
<td>2 (13)</td>
<td>0.90 ± 0.12</td>
<td>2 (13)</td>
<td>3.7 ± 1.6</td>
<td>1 (6)</td>
<td>1 (6)</td>
</tr>
<tr>
<td>E</td>
<td>10</td>
<td>19</td>
<td>19 (100)</td>
<td>0.86 ± 0.10</td>
<td>3 (16)</td>
<td>3.6 ± 1.0</td>
<td>6 (32)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>F</td>
<td>30</td>
<td>18</td>
<td>18 (100)</td>
<td>0.82 ± 0.12</td>
<td>8 (44)</td>
<td>5.5 ± 1.3</td>
<td>4 (22)</td>
<td>2 (11)</td>
</tr>
<tr>
<td>G</td>
<td>100</td>
<td>19</td>
<td>19 (100)</td>
<td>0.79 ± 0.10</td>
<td>15 (79)</td>
<td>5.9 ± 1.3</td>
<td>2 (11)</td>
<td>3 (16)</td>
</tr>
<tr>
<td>H</td>
<td>180</td>
<td>13</td>
<td>13 (100)</td>
<td>0.77 ± 0.06</td>
<td>10 (77)</td>
<td>5.4 ± 0.9</td>
<td>3 (23)</td>
<td>3 (23)</td>
</tr>
<tr>
<td>I</td>
<td>270</td>
<td>13</td>
<td>13 (100)</td>
<td>0.78 ± 0.08</td>
<td>10 (77)</td>
<td>5.6 ± 0.9</td>
<td>2 (15)</td>
<td>8 (62)</td>
</tr>
</tbody>
</table>

Tadpoles with an ocular diameter ≤ 0.8 mm.

Severity of depigmentation, based on a relative scale from 1 to 10.

p < 0.05 versus the corresponding value for controls (Group A) by t-test or Chi-square test.
fixed for 1 h at 0°C in Palade’s veronal-buffered osmium tetroxide, stained en bloc for 2 h at 25°C with Kellenberger’s buffered uranyl acetate, and embedded in epon resin. Transverse semi-thin sections through the eyes were stained with methylene blue and examined by light microscopy. Thin sections were cut with a microtome using a glass knife, mounted on slot grids coated with plastic resin, post-stained with Kellenberger’s uranyl acetate and Reynold’s lead citrate, and examined with an electron microscope* at 80 kV.


**Results**

Specific ocular malformations of Xenopus tadpoles in Groups A to I at 101 h post-fertilization are summarized in table I and illustrated in figures 1 and 2. Only one ocular anomaly, a small retinal cyst, was found in the controls (Group A). Ocular abnormalities were observed in all tadpoles that were exposed to Ni²⁺ at concentrations ≥4.5 μmol/L (Groups D to I). The common abnormalities were microphthalmia, focal hypopigmentation, hernias or cysts of the choroid and retina that protruded through the sclera, and iris colobomas (incomplete closure of the choroid fissure). Cataracts were seen in a
few severely malformed eyes, but the lenses were generally normal. In one Ni$^{2+}$-exposed tadpole, the optic nerve was markedly thickened. The types, severity, and incidences of the ocular abnormalities depended upon the nickel concentration in the FETAX medium. Hernias or cysts of the choroid and retina were most frequent at Ni$^{2+}$ concentrations of 4.5 or 10 μmol/L (Groups D and E), microphthalmia and hypopigmentation were most severe at Ni$^{2+}$ concentrations ≥30 μmol/L (Groups F to I), and the highest incidence of iris colobomas was observed at 270 μmol Ni$^{2+}$/L (Group I). Many Ni$^{2+}$-exposed tadpoles also had craniofacial anomalies, with rostral, lateral, or dorsal displacement of the eyes (figures 1 and 2).

The ocular histology of control and Ni$^{2+}$-exposed tadpoles at 101 h post-fertilization is illustrated in figure 3. In a typical control tadpole (Panel A), the lens is circumferentially enveloped by retinal layers, comprising the ganglion cell layer, the inner nuclear layer, the photoreceptor outer segments, and the pigment epithelium. A corresponding histological section of an eye from a Ni$^{2+}$-exposed tadpole (Panel B) shows moderate disarray of the retinal layers, with focal absence of photoreceptor outer
segments and pigment epithelium. The eyes of Ni$^{2+}$-exposed tadpoles consistently revealed such lesions of the photoreceptor outer segments and pigment epithelium. Development and maturation of photoreceptor outer segments occurred only at sites where the subadjacent pigment epithelium was normal and intact. Another histological section of an eye from a Ni$^{2+}$-exposed tadpole (Panel C) shows extensive loss of the pigment epithelium and diffuse malformation of the retinal layers. Upon histological examination, the crystalline lens and optic nerve were usually unremarkable. The primary ocular lesion in the Ni$^{2+}$-exposed tadpoles appeared to be diffuse or focal dysplasia of retinal pigment epithelial cells, associated with secondary dystrophy of photoreceptor outer segments, and localized protrusion of neuroretina through gaps in the pigment epithelium.

In figure 4 are shown photomicrographs of thin sections of tadpoles killed at 53 h post-fertilization (Groups J and K). In the eye from a control tadpole (Panel A), the cytoplasm of pigment epithelial cells contains many melanin granules, and the subadjacent photoreceptor outer segments are developing in a regular array. In contrast, the eye from a Ni$^{2+}$-exposed tadpole (Panel B) shows a few melanin granules in the cytoplasm of pigment epithelial cells and marked dystrophy of the nascent photoreceptor outer segments.

In figure 5 are shown electron micrographs of the junction between the pigment epithelial cells and photoreceptor outer segments in eyes of control and Ni$^{2+}$-exposed tadpoles. In control tadpoles at 77 and 101 h post-fertilization (Groups L and N; Panels A and C), the photoreceptor outer segments have formed and become attached to the pigment epithelium, obliterating the subretinal space. The pigment epithelial cells contain abundant melanin granules in juxtaposition to invaginating photore-
Figure 4. Photomicrographs of coronal sections through the eyes of control and Ni\textsuperscript{2+}-exposed tadpoles at 53 h post-fertilization (thin section, methylene blue stain, 200× magnification). Panel A (control): normally developing retinal pigment epithelium (′P′) and lens (′L′). Cytoplasmic melanin granules are present in the pigment epithelium. The photoreceptor outer segments (′O′) are beginning to develop. Panel B (Ni\textsuperscript{2+}-exposed) shows severe dystrophy of the pigment epithelium (′P′) and photoreceptor outer segments (′O′).

Receptor outer segments. In contrast, in Ni\textsuperscript{2+}-exposed tadpoles at 77 and 101 h post-fertilization (Groups M and O; Panels B and D), the subretinal space is open, the pigment epithelium is attenuated, and the photoreceptor outer segments are stunted and dystrophic. These findings suggest that the pathogenesis of the ocular lesions in Ni\textsuperscript{2+}-exposed tadpoles involves dysplasia of the pigment epithelium and dystrophy of the photoreceptor outer segments.

Discussion

This study confirms that Ni\textsuperscript{2+} is a potent ocular teratogen for Xenopus embryos in the FETAX assay. Eye anomalies are often observed in FETAX assays of teratogenic chemicals, but the ocular malformations induced by Ni\textsuperscript{2+} are exceptionally frequent and severe. Five divalent metal ions have given positive results for teratogenesis in FETAX assays.\textsuperscript{8,15–17} At the respective EC\textsubscript{50} levels (i.e., the concentrations that caused malformations of all sorts in 50% of tadpoles), the relative frequency of eye anomalies was Ni\textsuperscript{2+} \textgreater Co\textsuperscript{2+} \textgreater Cu\textsuperscript{2+} \textgreater Zn\textsuperscript{2+} = Cd\textsuperscript{2+}. The ocular malformations of Co\textsuperscript{2+}-exposed tadpoles resemble those induced by Ni\textsuperscript{2+}, including microphthalmia, hypopigmentation, hernias and cysts of the choroid and retina, and iris colobomas, however the teratogenic potency of Co\textsuperscript{2+} is only one-tenth that of Ni\textsuperscript{2+}.\textsuperscript{15} Both Ni\textsuperscript{2+} and Co\textsuperscript{2+} cause tissue injury by redox reactions that generate oxygen free radicals, but the biochemistry of the peroxidative damage is better understood for Ni\textsuperscript{2+} than for Co\textsuperscript{2+}.\textsuperscript{18–22} For these reasons, Ni\textsuperscript{2+} is the divalent metal ion that appears to be best suited for research on ocular teratogenesis in Xenopus.
Although some ocular anomalies of Ni<sup>2+</sup>-exposed tadpoles may be secondary to extrinsic craniofacial malformations, this study suggests that most of the ocular anomalies involve primary damage to the pigment epithelium of the retina and secondary dysplasia of photoreceptor outer segments. The intrinsic ocular malformations appear to be sequellae of focal dysplasia or destruction of melanocytes in the pigment epithelium.

Recent studies have shown marked effects of Ni<sup>2+</sup> on melanin formation in mammalian melanocytes. Biosynthesis of melanin, the principal pigment in the eyes and skin of mammals and amphibia, involves, first, conversion of L-tyrosine, via L-DOPA (dihydroxy-L-phenylalanine) and dopaquinone, to dopachrome, and second, intramolecular rearrangement of dopachrome to form dihydroxyindole and dihydroxyindolecarboxylic acid, which undergo polymerization to eumelans. The Ni<sup>2+</sup> affects several steps of this pathway in vitro, activating enzymatic hydroxylation of L-tyrosine, inhibiting decarboxylation of dopachrome, and converting red dopachrome to colorless dihydroxyindolecarboxylic acid. The Ni<sup>2+</sup> also forms stable complexes with L-DOPA and related products, and such metal complexes can generate oxygen free radicals by the Fenton reaction. As one hypothesis for the pathogenesis of the ocular anomalies, the authors propose that Ni<sup>2+</sup> damages retinal pigment epithelial cells by (a) toxic effects on mela-
nin biosynthesis, (b) complexation with DOPA derivatives, and (c) local formation of oxygen free radicals.

As a second hypothesis, the authors suggest that Ni\(^{2+}\) induces ocular teratogenesis by interacting with \(pNiXa\), a Ni\(^{2+}\)-binding protein that is abundant in *Xenopus* embryos until Nieuwkoop/Faber stage 36.\(^{30}\) Presence of \(pNiXa\) in the embryos coincides with their susceptibility to Ni\(^{2+}\)-induced teratogenesis.\(^{8,30}\)

Based on amino acid sequence, \(pNiXa\) belongs to the serpin (serine proteinase inhibitor) superfamily and shows sequence identity to Ep45, an estrogen-regulated serpin that is secreted by *Xenopus* liver.\(^{30,31}\) The \(pNiXa\) is an inducer of oocyte maturation, and thus can influence the events associated with germinal vesicle breakdown and cell division.\(^{32}\)

The \(pNiXa\) has sequence homology to human PEDF (pigment epithelium derived factor), a recently discovered serpin that is secreted by the pigment epithelium of the fetal human retina, that induces a neuronal phenotype in cultured human retinoblastoma cells.\(^{33}\)

Steele et al.\(^{33}\) proposed that the retinal pigment epithelial cells of human embryos secrete PEDF into the interphotoreceptor matrix, and that PEDF modulates retinal neurite outgrowth and maturation by a paracrine effect. This hypothesis is consistent with evidence that extracellular proteins and their inhibitors regulate the histogenesis of the neural retina in chick embryos.\(^{34}\)

The present authors speculate that \(pNiXa\) may have a role analogous to PEDF, and that Ni\(^{2+}\)-binding to \(pNiXa\) may induce ocular malformations by blocking an effect of \(pNiXa\) on retinal maturation.

Combined occurrence of iris colobomas and retinal dysplasia, often noted in the Ni\(^{2+}\)-exposed tadpoles, mimics the clinical syndrome of congenital retinal dystrophy with bilateral macular dysplasia.\(^{35}\) The pathogenesis of colobomas and microphthalmia has been studied in an animal model, the cinnamon mouse, which is homozygous for the gene for microphthalmia.\(^{36,37}\)

The murine ocular malformations may either involve failure of secondary vitreous formation,\(^{36}\) or dystrophy of the retinal pigment epithelium, with overgrowth of the outer layers of the stratum nervosum.\(^{37}\)

References

12. DAWSON, D. A. and BANTLE, J. A.: Development of a reconstituted water medium and pre-


