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Teratogenic effects of ethanol exposure on zebrafish visual system development

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Abstract

Ethanol intake during pregnancy can produce a wide range of adverse effects on nervous system development including fetal alcohol syndrome (FAS). The most severe congenital malformation observed in newborns with FAS is cyclopia. In this study, we have exposed zebrafish embryos to different ethanol concentrations (2.4%, 1.5% or 1.0%) during eye morphogenesis in four zebrafish strains (AB, EK, GL and TL). In addition, we have studied the survival rate of the cyclopic animals to the end of larval development.

The zebrafish strains GL and AB generated the higher percentage of cyclopic animals after exposure to 2.4% ethanol, while EK showed the higher percent cyclopic animals using 1.5% and 1.0% ethanol. The EK strain showed the higher percent survival during the larval period at all ethanol concentrations (2.4%, 1.5% and 1.0%). Moreover, we have investigated cytoarchitectural alterations in the main components of the visual pathway–retina and optic tectum–and ethanol treatment affects both the retina and the optic tectum. The lamination of neural retina is clearly delayed in treated larvae 3 days postfertilization and the thickness of the pigmented epithelium is considerably reduced. With regard to the optic tectum, treatment with ethanol alters the normal pattern of tectal lamination. The use of zebrafish EK strain is a suitable in vivo vertebrate model system for analyzing the teratogenic effect of ethanol during vertebrate visual system morphogenesis as it relates to both cyclopia and FAS.

Keywords: Alcohol; Malformations; Fetal alcohol syndrome; Cyclopia; Retina; Optic tectum

1. Introduction

Ethanol is a known teratogen and its consumption during pregnancy can produce a wide range of adverse effects on fetal development (alcohol related birth defects), the extreme of which is fetal alcohol syndrome (FAS) [26]. FAS is a constellation of congenital functional anomalies and malformations seen in some newborns (0.5–3/1000 births) exposed to ethanol during development [25]. Regarding functional anomalies, this pathology is characterized by central nervous system (CNS) dysfunctions such as mental retardation and long-term cognitive and behavioral deficits. Cyclopia, due to impaired separation of the eye field into two lateral domains, is the most severe congenital malformation observed in newborns with FAS [8]. There are clinical reports of identical twins being both affected [27] and fraternal twins in which one was affected but not the other despite a similar amount of drinking of the mothers [19,27]. Such data and the information from animal model systems [1,4] suggest genetic components to ethanol sensitivity, although the specific genes remain unknown.

Zebrafish, *Danio rerio*, is an effective model system to investigate the mechanisms of ethanol teratogenicity because the effects of ethanol on zebrafish embryos are similar to those seen in humans, including alterations in neurocranial and craniofacial skeletal development and growth retardation in the appendages [6,16,17,22]. Moreover, zebrafish develop outside the mother eliminating maternal effects on the fetal environment and, thus, offers an excellent model system for studying directly the contribution of fetal genes to ethanol-induced teratogenesis. These maternal effects are a major confounding factor in the discovery of mechanisms regulating fetal sensitivity to ethanol-induced developmental abnormalities. Direct

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treatment of zebrafish with ethanol constitutes a good model for the study of teratogenicity in humans, since it has been demonstrated that ethanol, but not its metabolites, is most likely the primary teratogen in FAS [28].

Several studies have shown that zebrafish constitute an effective physiological [2] and anatomical [3] model system for analyzing the teratogenic effect of ethanol on eye development. Zebrafish embryos exposed to ethanol show alterations in the visual physiology [2] or even develop cyclopia [3]. The results obtained in these reports with zebrafish show effects of ethanol that are similar to other vertebrate species, although there is a large discrepancy between the doses of ethanol that induce similar defects across species [2]. During zebrafish ontogeny, the teratogenic effect of ethanol is determined by chorion permeability [11] and a strain-dependent sensitivity to this teratogen [15]. The purpose of our study was analyzing the generation of the cyclopic phenotype by three ethanol concentrations (2.4%, 1.5% and 1.0%) in four different zebrafish strains (AB, EK, GL and TL). After this analysis, cyclopic animals were employed to determine cytoarchitectural variations in the retinal and tectal patterning as affected by the ethanol treatment.

2. Methods

2.1. Zebrafish strains

The following zebrafish strains were obtained from fish facilities of the University of Wisconsin-Milwaukee Marine and Freshwater Biomedical Sciences Center in the Great Lakes WATER Institute and used for this study: AB (wild-type, originally acquired from the Zebrafish International Resource Center, Eugene OR), EK (Ekkwill wild-type, Massachusetts General Hospital, Charlestown, MA), GL (golden longfin, Ekkwill Waterlife Resources, Gibsonton, FL) and TL (Tuebingen long fin, Massachusetts General Hospital). All procedures and experimental protocols were approved by the Animal Care and Use Committee of the University of Wisconsin-Milwaukee (USA), as well as in accordance with the guidelines of the European Communities Directive (86/609/EEC), the current Spanish legislation for the use and care of animals (BOE 67/8509-12, 1998), and conformed to NIH guidelines.

2.2. Ethanol treatment, embryonic counting and identification of cyclopic embryos

Zebrafish embryos of the different strains were obtained by natural mating and maintained according to standard procedures [29]. Fertilized embryos were collected 2 h after fertilization and maintained at 28.5 °C in petri dishes in E3 medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM CaCl2, 0.33 mM MgSO4 in distilled water). Methylene blue (50 ppb) was added to E3 medium to inhibit fungal growth. The embryos were counted and grown to the desired stages before treatment.

Embryos (n=1600; 400 per each zebrafish strain) were exposed to different ethanol concentrations (0%, 2.4%, 1.5% or 1.0%) in E3 during the period from the dome/30% epiboly stage to 24 h postfertilization (hpf). After ethanol treatment, embryos were placed in E3 medium without ethanol and dead embryos were removed. During the second day of development, embryos were assessed for cyclopia, as indicated by a single medial eye or partially fused eye vesicles. Cyclopic embryos were isolated, counted and grown to 5 days postfertilization (dpf) in E3 medium.

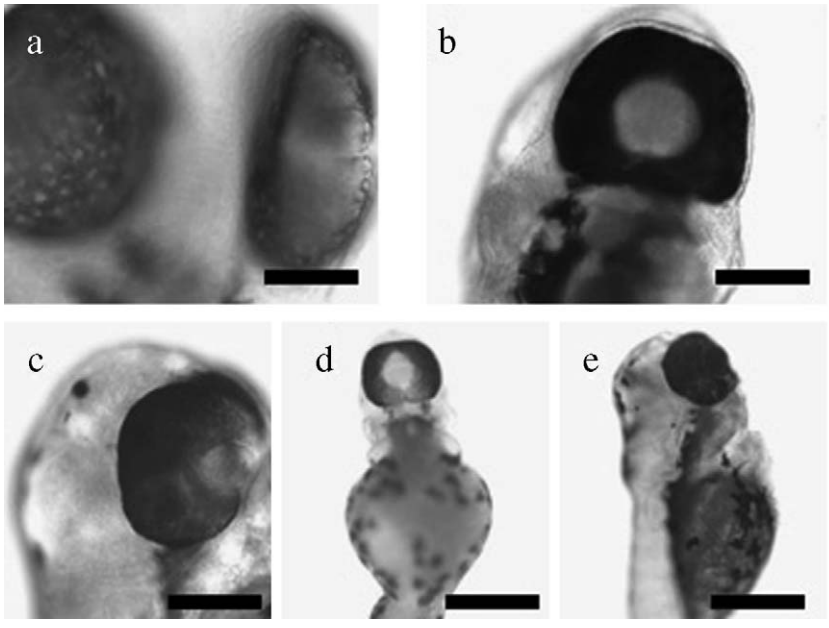

Fig. 1. Whole-mount embryos and larvae of the EK strain in ventral (a, b, d) and lateral (c, e) views. In c and e, dorsal is to the left. (a) Untreated (control) embryo at 48 hpf. (b, c) Ethanol-induced (1.5%) cyclopic zebrafish embryos at 48 hpf. (d, e) Ethanol-induced cyclopic zebrafish larvae at 5 dpf (the end of the larval period). Scale bar (a, b, c)=50 μm, (d, e)=100 μm.

2.3. Statistical analyses

For the different ethanol treatments (2.4%, 1.5% and 1.0%), intergroup (among zebrafish strains) differences regarding frequency of cyclopia and survival of cyclopic embryos at the end of larval development were analyzed by one-way analysis of variance (ANOVA) followed by multiple comparison test of Bonferroni. Critical values of $*P<0.05$ and $**P<0.01$ were used for all analyses.

2.4. Histological processing

Larvae of 3 dpf were anesthetized with 0.03% tricaine methanesulfonate (MS-222, Sigma, St. Louis, MO). Five specimens of untreated EK zebrafish and five cyclopic specimens of EK treated with 1.5% ethanol were fixed by immersion with 1% paraformaldehyde and 1.5% glutaraldehyde in 0.05 M cacodylate buffer, pH 7.4, for 2 h at 4 °C. All specimens were postfixed in 1% osmium tetroxide for 1 h at 4 °C. The larvae were oriented and embedded with EMbed-812 resin (Electron Microscopy Sciences, Fort Washington, PA). Parasagittal serial sections of 1 μm thickness were cut in an ultramicrotome Reichert-Jung Ultracut E (Nussloch, Germany) and stained with an aqueous solution containing 1% toluidine blue and 1% borax.

2.5. Image analyses

Sectional material and whole embryos were analyzed using a Leica microscope equipped with brightfield condensers. Brightfield digital images were obtained with an Apogee KX digital camera (Apogee Instruments, Inc., Tucson, AZ) coupled to an Olympus Provis AX70 photomicroscope. The capture software was connected to a trichromatic sequential filter (Cambridge Research and Instrumentation Inc., Boston, MA). The original images were processed digitally with Adobe® Photoshop® 7.0 (Adobe Systems, San Jose, CA) software. After conversion into black and white, the sharpness, contrast and brightness were adjusted to reflect the appearance seen through the microscope.

3. Results

3.1. Strain and dosage-dependent teratogenic effects of ethanol exposure

In this study, we analyzed the frequency of cyclopia after exposure to different ethanol concentrations (2.4%, 1.5% or 1%) in four zebrafish strains (AB, EK, GL and TL). Embryos were assessed for cyclopia during the second day of development, with embryos having either a single medial eye or partially fused eye vesicles being scored as cyclopic. Embryos with two distinct and separate eyes were scored as normal despite any possible narrowing of the region between the eyes (Fig. 1).

The zebrafish strains GL and AB generated the higher percentages of cyclopic animals after 2.4% ethanol exposure (70.4% and 46.5%, respectively), followed by EK (36.1%) and TL (31.7%). However, no specific significant intergroup (zebrafish strains) differences were observed after statistical analyses. Using a 1.5% ethanol treatment, only EK and AB strains produced cyclopic embryos (16.1% and 1.1%,

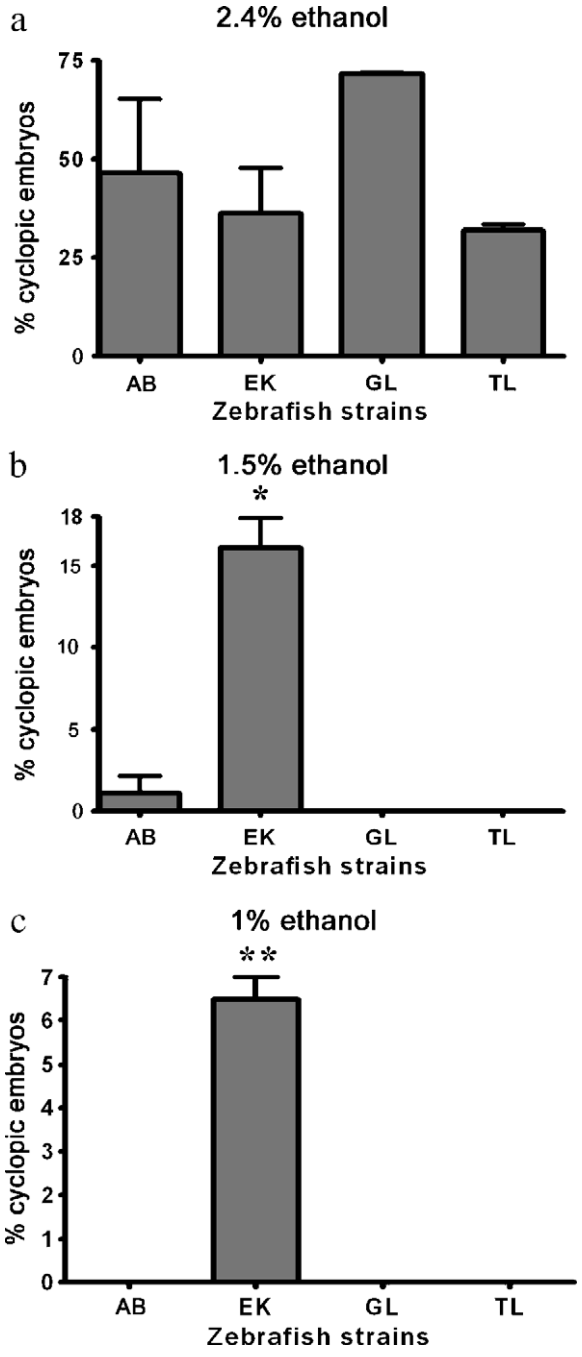

Fig. 2. Development of cyclopia after ethanol treatment in the different zebrafish strains. For all ethanol solutions, the intergroup differences were analyzed by one-way analysis of variance (ANOVA) followed by multiple comparison test of Bonferroni. Critical values of $*P<0.05$ and $**P<0.01$ were used for all analyses. (a) Following 2.4% ethanol exposure, the higher percentages of cyclopic animals were observed in the GL and AB zebrafish strains. (b) Following 1.5% ethanol exposure, cyclopic animals were obtained only in the EK and AB strains. A significant intergroup difference was observed in the EK strain. (c) Following 1.0% ethanol exposure, the cyclopic phenotype was only observed in the EK strain, showing a marked significant intergroup difference.

respectively). A statistically significant intergroup difference was observed in the EK strain following 1.5% ethanol exposure. Finally, following exposure to 1.0% ethanol, EK was the only strain that produced the cyclopic phenotype (6.5%) showing a marked specific significant intergroup difference (Fig. 2, Table 1).

We then analyzed the survival rate of the cyclopic animals at the end of larval development, 5 dpf. The survival of cyclopic zebrafish larvae was determinated by observing the free-swimming larvae or the beating heart of those larvae that were not swimming. The GL strain, which produced the highest percentage of cyclopic embryos following 2.4% ethanol treatment, was the only strain with no surviving cyclopic larvae at this ethanol concentration. The AB and TL strains showed a low rate of cyclopic larvae survival (26.3% and 19.6%, respectively), while the EK strain had the highest survival rate during the larval period (41.6%). However, no specific significant intergroup (zebrafish strains) differences were observed following treatment with 2.4% ethanol. With regard to the 1.5% and 1% ethanol solutions, only the EK strain showed surviving cyclopic larvae (78.6% and 85.7%, respectively). After statistical analysis, the EK strain showed specific significant intergroup differences using both 1.5% and 1% of ethanol solutions (Fig. 3, Table 1).

3.2. Visual system morphogenesis

The neural retina is constituted by six layers: the optic nerve fiber layer, the ganglion cell layer, the inner plexiform layer, the inner nuclear layer, the outer plexiform layer and the outer nuclear layer. Placed at the interface between the outer nuclear layer and the choroid layer, the pigmented epithelium is a multifunctional and indispensable component of the vertebrate eye. During normal zebrafish visual system ontogeny, the neural retina acquires the lamination into six layers by 3 dpf (Fig. 4a). The optic axons project topographically onto the

Table 1
Strain and dosage-dependent teratogenic effects of ethanol exposure on cyclopia induction and survival rate

Ethanol solutions	% cyclopic embryos	% survival of cyclopic larvae
AB		
2.4%	46.5	26.3
1.5%	1.1	0
1%	0	0
EK		
2.4%	36.1	41.6
1.5%	16.1 (*)	78.6 (*)
1%	6.5 (**)	85.7 (*)
GL		
2.4%	70.4	0
1.5%	0	0
1%	0	0
TL		
2.4%	31.7	19.6
1.5%	0	0
1%	0	0

Induction of cyclopia (%) and survival of cyclopic larvae (%) after exposure to three different alcoholic solutions (2.4%, 1.5%, 1%) illustrated in Figs. 2 and 3 in all zebrafish strains. For all ethanol solutions, intergroup (among zebrafish strains) differences regarding frequency of cyclopia and survival of cyclopic embryos at the end of larval life were analyzed by one-way analysis of variance (ANOVA) followed by multiple comparison test of Bonferroni using $*P<0.05$ and $**P<0.01$ as critical values.

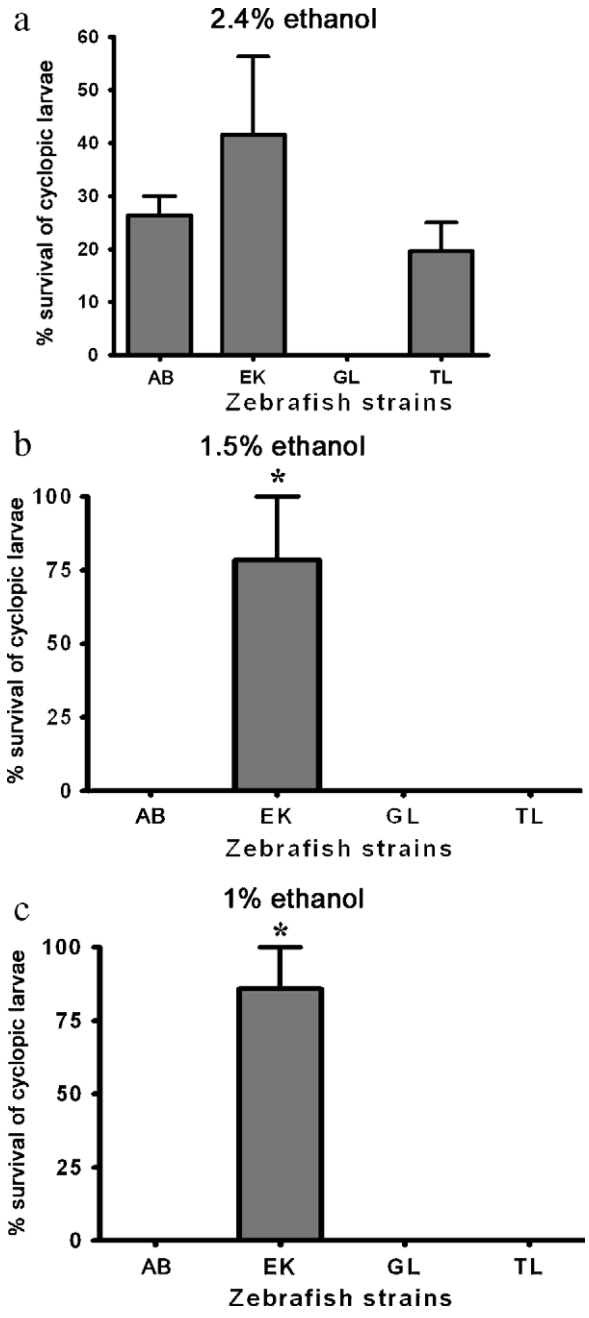

Fig. 3. Survival of the cyclopic animals at the end of larval development. For all ethanol treatments, the intergroup differences were analyzed by one-way analysis of variance (ANOVA) followed by multiple comparison test of Bonferroni. Critical values of $*P<0.05$ and $**P<0.01$ were used for all analyses. (a, b, c) Following treatment with varying concentrations of ethanol, cyclopic larvae of the EK strain showed a higher percent survival. Statistically significant intergroup differences were observed in the case of 1.5% and 1% of ethanol treatments.

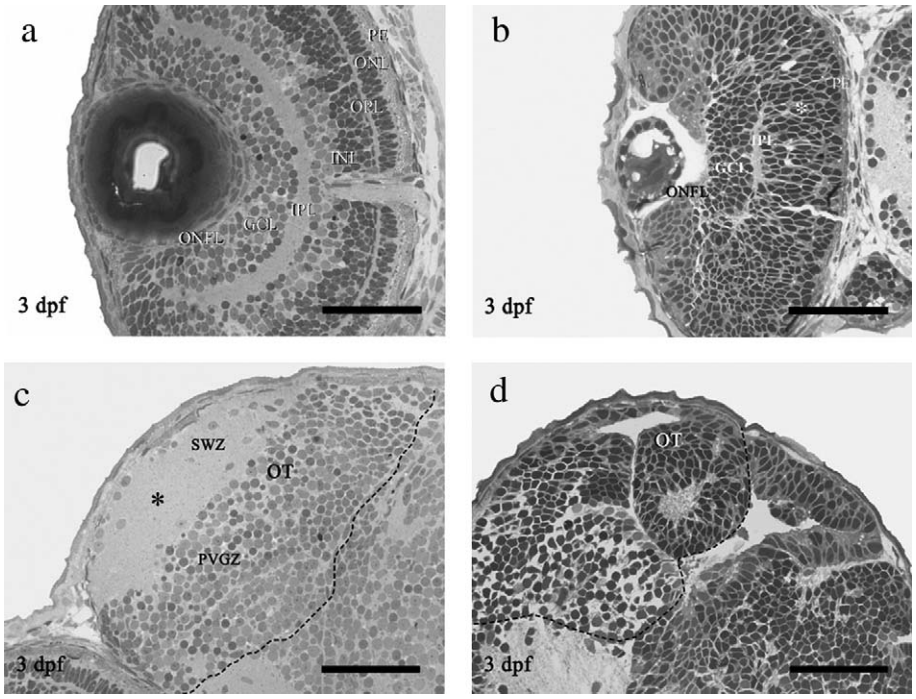

Fig. 4. Parasagittal sections of 1 μm thickness through the retina (a, b) and optic tectum (c, d) of EK strain zebrafish larvae (a, c) and ethanol-induced cyclopic EK larvae (b, d) at 3 dpf stained with an aqueous solution containing 1% toluidine blue and 1% borax. (a) The lamination of the retina into six layers is completed in the untreated larvae at this stage of development. (b) In the retina of the ethanol-induced cyclopic larvae, the laminar differentiation is only partially completed. There are no boundaries among the inner nuclear, outer plexiform and outer nuclear layers (asterisk). The thickness of pigmented epithelium is reduced after ethanol treatment. (c) The optic tectum of untreated zebrafish is characterized by two germinal zones: superficial white zone and periventricular gray zone. The neuropile of the superficial white zone looks like a uniform matrix (asterisk). (d) The optic tectum of ethanol-induced cyclopic zebrafish reveals an impaired lamination, and it is not possible to discern between superficial white and periventricular gray zones. GCL: ganglion cell layer of the retina, INL: inner nuclear layer of the retina, IPL: inner plexiform layer of the retina, ONFL: optic nerve fiber layer of the retina, ONL: outer nuclear layer of the retina, OPL: outer plexiform layer of the retina. OT: optic tectum, PE: pigmented epithelium, PVGZ: periventricular gray zone, SWZ: superficial white zone. Scale bar (a, b, c, d)=50 μm.

optic tectum where they form several bands of terminals. The mesencephalic optic tectum is a multilayered encephalic region constituted by six layers. The different teleostean tectal strata are originated from two mesencephalic portions called the periventricular gray zone and the superficial white zone [23]. The density of cells in the periventricular gray zone is higher than in the superficial white zone, which is mainly constituted by neuropile (Fig. 4c), and neuroblasts from the periventricular gray zone migrate towards the superficial white zone during tectal morphogenesis.

In ethanol-induced cyclopic larvae, lamination of the neural retina is clearly delayed by 3 dpf because only four layers, instead of six, can be discerned. The central region of the inner plexiform layer, the ganglion cell layer and the optic nerve fiber layer are well developed. There are no boundaries among the inner nuclear, outer plexiform and outer nuclear layers, constituting a single retinal layer (Fig. 4b). In addition, the pigmented epithelium is homogeneously distributed, although its thickness is considerably reduced after ethanol exposure (Fig. 4b). Regarding the optic tectum, the germinal zones that give rise to the tectal strata can not be discerned at this stage of development probably due to the lack of neuropile (Fig. 4d). Thus, treatment with ethanol during development alters the lamination of the optic tectum.

4. Discussion

In this study, we have extended the analysis of ethanol-induced cyclopia in zebrafish embryos [3] to include different ethanol concentrations (2.4%, 1.5% and 1.0%) and several zebrafish strains (AB, EK, GL and TL). We have demonstrated that generation of the cyclopic phenotype depends on both ethanol concentration and strain-dependent sensitivity. In addition, we have demonstrated in cyclopic larvae from the EK strain that ethanol exposure (1.5%) affects the retinal and tectal cytoarchitecture.

We have obtained cyclopic embryos from the EK strain after treatment with ethanol concentrations of 1.5% and 1%. Cyclopia has only been previously reported in zebrafish embryos following treatment with 2.4% ethanol [3]. Experiments for analyzing the permeability of the zebrafish chorion have demonstrated that only about 8% of a glycerol solution and 3% of DMSO solution actually enters the embryonic tissue [11]. Studies investigating the permeability of the zebrafish chorion to ethanol have shown that approximately 30% of the ethanol reaches embryonic tissues [18]. This could explain the higher concentration of ethanol required to produce FAS-like defects in zebrafish as compared to rodent species [2].

Embryos of the wtOX zebrafish strain (derived from the Goldfish Bowl, Oxford, UK) were exposed to 2.4% ethanol

starting at different stages of development to analyze the production of the cyclopic phenotype [3]. In this study, the existence of a narrow time window of susceptibility to ethanol teratogenesis in the late blastula and early gastrula stages was determined. In addition, the expression pattern of several genes at early developmental stages was analyzed and compared to the *cyclops* and the *one-eyed-pinhead* mutants [12,21,24] concluding that cyclopia occurs because ethanol impairs migration of the precordal plate during ontogeny [3]. In the present study, we have shown the existence of both a dosage- and strain-dependent sensitivity to the ethanol-induced cyclopic phenotype.

After 1.5% ethanol treatment, the EK strain showed a high survival rate at the end larval period, when anatomical [5] and physiological [14] maturation of visual system occurs in wild type zebrafish. This experimental group is suitable for analyzing the long-term teratogenic effect of ethanol on this sensory pathway.

We have observed that the retinal lamination of ethanol-induced cyclopic zebrafish larvae at 3 dpf is clearly delayed. Regarding the pigmented epithelium, the embryonic exposure to ethanol reduced its thickness, although it appeared homogeneously distributed. Development of the ventral midline of the neural keel is blocked in *cyclops* mutant zebrafish embryos, causing a deletion in the ventral forebrain, with two partial retinas joined in the ventral midline [12]. In the *cyclops* mutants, the nuclear and plexiform layers, as well as the pigmented epithelium, are constituted at this stage of development, although the pigmented epithelium is only present dorsolaterally [10]. The fact that retinal morphogenesis is more affected in the ethanol-induced cyclopic zebrafish larvae than in the *cyclops* mutants could be a consequence of the loss of expression of certain genes (*shh*, *axl* and *nk2.2*) that specify the ventral morphogenesis of the forebrain and midbrain of ethanol-induced cyclopic zebrafish embryos, as has been previously reported by Blader and Strähle [3].

The optic tectum of ethanol-induced cyclopic zebrafish larvae showed an impaired lamination. However, there is no data about cytoarchitectural organization in the optic tectum of the *cyclops* or the *one-eyed-pinhead* mutants to compare with present results. The neuropathology of the effects of ethanol on the developing CNS is similar to those of patients with mutations in L1 [7], which is a neural cell adhesion molecule involved in neuroblast migration [20], neurite outgrowth, axonal pathfinding and neurite fasciculation [13]. This suggests that dysregulation of L1 could be involved in the impaired lamination of the optic tectum in ethanol-induced cyclopic zebrafish.

We can conclude from our results that induction of the cyclopic phenotype by ethanol exposure depends on both the concentration of ethanol and the genetic background of each zebrafish strain. The EK zebrafish strain is a useful in vivo vertebrate model for analyzing the late stages of visual pathway ontogeny in cyclopic larvae after treatment with a 1.5% solution of ethanol, which is the ethanol concentration reported to induce physiological problems such us alterations in the electroretinogram recordings and lower visual acuity [2]. The present study complements experiments carried out in mammalian species investigating the basis of the teratogenic effects of ethanol exposure during development [1,4,9]. Further studies with cyclopic larvae of EK strain, targeting the expression pattern of different molecules involved in the morphogenesis of the visual system will provide valuable neuroanatomical and mechanistic insight into the nature of the teratogenic and neurotoxic effects of ethanol.

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