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Ethanol-Induced Microphthalmia is Not Mediated by Changes in Retinoic Acid or Sonic Hedgehog Signaling During Retinal Neurogenesis

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Abstract

Background—Microphthalmia (reduced eye size), generally accompanied by vision defects, is a hallmark of fetal alcohol spectrum disorder (FASD) in humans. In zebrafish, embryonic ethanol exposure over the time of retinal neurogenesis also results in microphthalmia. This microphthalmia is in part the consequence of reduced retinal cell differentiation, including photoreceptors. Here we pursue 2 signaling pathways implicated in other aspects of FASD pathogenesis: retinoic acid (RA) and Sonic hedgehog (Shh).

Methods—We evaluated markers for RA and Shh signaling within the eyes of embryos treated with ethanol during the period of retinal neurogenesis. We also perrormed rescue experiments using administration of exogenous RA and microinjection of cholesterol, which augments Shh signaling.

Results—Using sequential or co-treatments, RA did not rescue ethanol-induced microphthalmia at any concentration tested. In addition, RA itself caused microphthalmia, although the underlying mechanisms were distinct from those or ethanol. Interestingly, RA treatment appeared to recover photoreceptor differentiation in a concentration-dependent manner. This may be an independent effect or exogenous RA, as ethanol treatment alone did not alter RA signaling in the eye. Cholesterol injection also did not rescue ethanol-induced microphthalmia at any concentration tested, and ethanol treatments did not alter expression of *shh*, or of *ptc-2*, which is normally regulated by Shh signaling.

Conclusions—Together these findings indicate that, during the time of retinal neurogenesis, effects of ethanol on eye development are likely independent of the RA and Shh signaling pathways. These studies suggest that FASD intervention strategies based upon augmentation or RA or Shh signaling may not prevent ethanol-induced microphthalmia.

Keywords

Ethanol; Zebrafish; Microphthalmia; Retinoic Acid; Sonic Hedgehog; Photoreceptor

ETHANOL CONSUMPTION DURING pregnancy results in a spectrum of irreversible disorders in the fetus ranging from minor birth defects to a severe syndrome involving the

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brain, eyes, heart, and limbs referred to as fetal alcohol spectrum disorder (FASD; Stratton et al., 1996) Ophthalmic involvement is very common in FASD. Eye defects range from microphthalmia (reduced eye size), optic nerve hypoplasia, and coloboma of the iris and retina, as well as more subtle effects including strabismus and epicanthus (Stromland and Pinazo-Duran, 2002). Microphthalmia is a diagnostic criterion for FASD (Rosett, 1980) and ocular manifestations are seen in 90% of the children with FASD (Stromland and Pinazo-Duran, 2002). The mechanism(s) through which embryonic and fetal ethanol exposure results in visual system defects are not known.

The zebrafish has recently achieved credibility as an animal model appropriate for the pursuit of the understanding of the mechanisms underlying FASD in general, and the effects of ethanol on the developing visual system in particular. The vertebrate retina is highly conserved, such that anatomical, physiological, and developmental characteristics of the zebrafish retina resemble those of mammals. During the second and third day of embryonic development, the zebrafish retina undergoes a proliferative phase that overlaps with the generation of ganglion cells, then the cells of the inner nuclear layer, and finally the photoreceptor cells of the outer nuclear layer (Hu and Easter, 1999; Schmitt and Dowling, 1999). The zebrafish embryo develops externally, and therefore can be easily manipulated pharmacologically. A host of molecular and genetic tools are available to evaluate effects on the developing visual system (Easter and Malicki, 2002; Kashyap et al., 2007; Stenkamp, 2007).

The zebrafish embryo shows developmental abnormalities at doses of ethanol predicted to match those estimated to occur in human embryos as a consequence of maternal social drinking (Li et al., 2007). These developmental abnormalities resemble the phenotype seen in human FASD, including specific defects in the eye. These defects depend upon the timing of ethanol administration and include cyclopia (Blader and Strahle, 1998; Loucks and Ahlgren, 2009; Loucks et al., 2007), microphthalmia (Bilotta et al., 2002; Kashyap et al., 2007; Matsui et al., 2006), reduced retinal lamination (Arenzana et al., 2006), reduced photoreceptor differentiation (Dlugos and Rabin, 2007; Kashyap et al., 2007; Matsui et al., 2006), and optic nerve hypoplasia (Matsui et al., 2006). Our earlier study (Kashyap et al., 2007) revealed that exposure to ethanol over the period of retinal proliferation and neurogenesis (24 to 48 hours postfertilization [hpf]) results in significant microphthalmia, and that reduced eye size is a consequence of overall developmental delay, increased cell death in the lens, and reduced retinal cell differentiation.

The molecular mechanism(s) underlying FASD are not completely known. Various mechanisms have been proposed, including effects via reduced retinoic acid (RA) synthesis (Duester, 1991 ; Marrs et al., 2010; Yelin et al., 2005), and impairment of Sonic hedgehog (Shh) signaling (Ahlgren et al., 2002; Blader and Strahle, 1998; Li et al., 2007). RA, a Vitamin A metabolite, is known to be essential in the normal development of the central nervous system, eyes, limbs, and heart (Zachman and Grummer, 1998). The systems involved in FASD are similar to those seen in Hypovitaminosis A (decreased vitamin A), supporting the hypothesis that the spectrum of manifestations seen in FASD is due to decreased RA signaling (Deltour et al., 1996; Duester, 1991). Ethanol is thought to act by inhibiting the enzymes catalyzing the synthesis of retinaldehyde and RA (Duester, 1998; Duester et al., 1997; Kot-Leibovich and Fainsod, 2009; Fig. 1A). In support of this mechanism, studies in *Xenopus* (Yelin et al., 2005, 2007), chick (Satiroglu-Tufan and Tufan, 2004), mouse (Johnson et al., 2007), quail (Twal and Zile, 1997), and zebrafish embryos (Marrs et al., 2010) have shown reduced RA levels due to ethanol exposure and/ or were able to rescue, or partially rescue, phenotypes caused by ethanol exposure by treating with RA. Of particular relevance to our current studies, RA plays a vital role in retinal development. An excess of RA during eye morphogenesis results in a double retina

phenotype (Hyatt et al., 1996a), whereas inhibition of RA synthesis results in the lack of a ventral retina (Marsh-Armstrong et al., 1994). Later in development, RA promotes the expression of some photoreceptor-specific genes and inhibits the expression of others (Hyatt et al., 1996b; Prabhudesai et al., 2005). However, roles for RA in mediating the effects of ethanol on retinal development have not been tested.

Shh is another important morphogen involved in the development of the vertebrate eye. Shh signaling is essential for eye morphogenesis (Chiang et al., 1996; Ekker et al., 1995; Macdonald et al., 1995; Roessler et al., 1996; Stenkamp and Frey, 2003), retinal lamination (Wang et al., 2002), and the development of ganglion cells (Neumann and Nuesslein-Volhard, 2000; Zhang and Yang, 2001) and photoreceptors (Shkumatava et al., 2004; Stenkamp et al., 2000, 2002). Shh has been implicated in pathogenesis of FASD because phenotypic features such as holoprosencephaly and cyclopia are commonly seen as a consequence of defective Shh signaling (Blader and Strahle, 1998; Loucks et al., 2007; Maity et al., 2005; Sulik and Johnston, 1982). In support of this mechanism, studies in mouse (Yamada et al., 2005), chick (Ahlgren et al., 2002), and zebrafish (Li et al., 2007; Loucks and Ahlgren, 2009; Loucks et al., 2007) embryos have shown reduction in Shh signaling after ethanol treatment, and were able to rescue specific phenotypes associated with FASD. The proposed mechanisms for the reduced Shh signaling are reduction in *Shh* transcripts (Loucks and Ahlgren, 2009; Loucks et al., 2007), or the disruption of cholesterol homeostasis and/ or synthesis (Aguilera et al., 2004; Guizzetti and Costa, 2007; Li et al., 2007), resulting in impaired post translational modification of Shh (Fig. 1B). As with RA, roles for Shh in mediating the effects of ethanol specifically on retinal development have not been pursued.

In this study, we test the hypothesis that the microphthalmic effects of embryonic ethanol exposure are mediated by effects on the RA or Shh signaling systems. We use rescue strategies involving supplementation with exogenous RA or cholesterol to ethanol-treated zebrafish embryos. Surprisingly, we find no evidence of rescue of microphthalmia using either RA or cholesterol, suggesting that alternative mechanisms mediate the effects of ethanol on eye development. In support of this interpretation, molecular indicators of RA and Shh signaling within the eye were not affected by ethanol treatments. These studies also revealed that RA itself can cause microphthalmia by reducing retinal cell proliferation. Finally, we report that RA can recover photoreceptor differentiation in ethanol-treated embryos, but likely due to an independent mechanism.

MATERIALS AND METHODS

Zebrafish Maintenance

All adult zebrafish were maintained in an aquatic housing facility on a 14:10 hour light/dark cycle at 28.5°C according to Westerfield (2000). The experiments conducted were in accordance with an approved animal care and use protocol. Two different strains of zebrafish were used: a wild-type strain purchased from Aquatica Habitats (Plant City, FL; formerly available from Scientific Hatcheries of Huntington, CA), which we refer to as SciH; and RGnY transgenic fish (a gift of Elwood Linney, Duke University). Our previous studies of the effects of ethanol on zebrafish embryos were performed using the SciH strain (Kashyap et al., 2007). In the RGnY line, the transgene consists of cis-regulatory elements (3 copies of RA response elements [RAREs]) coupled to a GATA-2 basal promoter driving the expression of enhanced yellow fluorescent protein (eYFP; Perz-Edwards et al., 2001) and we refer to this line as RARE-YPF.

Ethanol, RA and Cholesterol Treatments

Adult zebrafish were bred and embryos were manually dechorionated using forceps before treatments (Prabhudesai et al., 2005). Embryos were treated with 1.5% ethanol as in Kashyap and colleagues (2007). Briefly, 100% ethanol was diluted into system water in glass beakers. Embryos were added to the glass beakers and loosely covered with parafilm to minimize evaporation. All ethanol treatments took place from 24 to 48 hpf.

All-*trans* RA (Sigma, St. Louis, MO) and diethylaminobenzaldehyde (DEAB; Sigma) stocks were prepared using dimethylsulfoxide (DMSO; Sigma) and were stored at –20°C under nitrogen in the dark (Perz-Edwards et al., 2001; Prabhudesai et al., 2005). Co- and sequential rescue treatments (as shown in Fig. *1C*) were performed by addition of exogenous RA, and embryos were either fixed at 48 hpf, or were washed and transferred to fresh system water until 72 hpf. Controls for these rescue experiments included DMSO treatments, untreated embryos, and those treated with RA only. Controls for the DEAB experiments included DMSO treatments and untreated embryos.

Stock solutions of cholesterol (Sigma) were prepared in DMSO. Cholesterol (0.5, 1.0, or 2.0 μg/μl; Li et al., 2007) was microinjected in 3 nl volumes into the yolk of dechorionated embryos (Nelson et al., 2009) at 24 hpf. Microinjection was immediately followed by ethanol treatments as described above (Fig. *1C*).

Tissue Processing

Whole embryos were fixed at 36, 48, or 72 hpf using 4% paraformaldehyde with 5% sucrose in a pH = 7.4 phosphate buffer (Barthel and Raymond, 1993). Additional washes with increasing concentrations of sucrose were performed, and then embryos were cryoprotected overnight in 20% sucrose at 4°C before embedding in a 1:2 solution of OCT embedding medium (Sakura Finetek, Torrance, CA) and 20% buffered sucrose medium. Tissue blocks were sectioned at 5 μm using a cryostat.

Eye Measurements and Statistics

Embryonic eye circumferences were measured as in Kashyap and colleagues (2007). A Nikon stereomicroscope (Meridian Instrument, Freeland, WA) fitted with a CCD camera was used to photograph the fixed embryos (prior to histological processing), which were laid on their sides in a petri dish. Usually the left eye was photographed, using a plane of focus appropriate for clearly viewing the lens and eye boundaries. Images were imported into Scion Image software/Image J (http://imagejdocu.tudor.lu/), where the free transform measure tool was used to measure the eye boundaries. Measurements were analyzed using Student's *t*-tests or analyses of variance (ANOVAs) followed by post hoc analyses, as appropriate, in the R statistical environment (R Core Development Team, 2006).

Cell Death and Cell Specific Markers

Acridine Orange—Live embryos were assessed for cell death using Acridine orange (Sigma) at 48 hpf. Briefly, embryos were dechorionated and exposed to 5 μg/ml Acridine orange for 10 min (Nelson et al., 2009). Embryos were washed extensively with phosphate buffered saline (PBS) to remove excess Acridine orange. Embryos were anesthetized using buffered MS-222 (0.003%) and the fluorescent, dead cells were visualized using a Leica DMR microscope (Bartels and Stout, Issaquah, WA) and photographed using a SPOT digital camera and associated software (Diagnostic Instruments, Sterling Heights, MI). Eyes of treated versus untreated embryos were scored as to whether Acridine orange positive cells were present in the lens and/or retina.

Live Imaging of Transgene Expression—Embryos expressing the RARE-YFP transgene at 32 hpf were treated with 1.5% ethanol or 10 μM DEAB for 24 hours. Following the treatments, live embryos were anesthetized using buffered MS-222 (0.003%), and transferred to a slide. YFP expression was visualized using the FITC filter on a Leica DMR microscope. Images were collected using a SPOT digital camera using the associated software.

Immunocytochemistry—Immunocytochemistry was performed to detect expression of various different cell specific markers as described previously (Kashyap et al., 2007; Stenkamp et al., 2000). Briefly, sections were blocked with blocking buffer (20% goat serum, 0.5% TritonX-100 in PBS) for 30 minutes and incubated with primary antibodies overnight at 4°C. The following antibodies (dilutions) were used: zpr-1 (1:200) and zpr-3 (1:200) were purchased from the University of Oregon monoclonal facility (Eugene, OR); α-islet-1 (1:100) was purchased from the University of Iowa monoclonal facility (Ames, IA); α-phosphohistone H3 (1:1,000) came from Upstate Cell Signaling Solutions (Lake Placid, NY); and α-GFP (1:1,000) from Chemicon (Temecula, CA). Labeling was detected using Cy3- or FITC-conjugated α-mouse or α-rabbit IgG (1:200) purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). Sections were washed in PBS with 0.05% TritonX-100 (Sigma) for 30 minutes and were mounted using an anti-fade medium (Prolong Gold; Molecular Probes, Invitrogen, Carlsbad, CA).

Cell Counts and Scoring of Expression Patterns

Photoreceptors—Expression patterns of the photoreceptors were assessed using the zpr-l and zpr-3 antibodies on cryosections. Two methods were employed: 1 form of scoring was based on the extent of labeling present throughout the retina, and the other was based on the qualitative distribution of the expression of markers (Fig. 4). The first method included the following 3 categories: *None* = no labeled cells; *Few* = 1 to 10 labeled cells; *Many* > 10 labeled cells (see Nelson et al., 2009; Stenkamp et al., 2002). The second method, which was applied only to embryos scored by the first method as *Few* or *Many*, included the following 3 categories: *Sporadic* = large gaps between the labeled cells; *Discontinuous* = 1 to 2 unlabeled cells in between (zpr-l), or 3 to 4 unlabeled cells in between (zpr-3) labeled cells; *Dense* = no unlabeled gaps between the labeled cells (zpr-1), or 1 to 2 unlabeled cells between the labeled cells (zpr-3).

Mitotic Cells—Counts of cells labeled with anti-H3 were performed on digital images collected by a SPOT camera (Kashyap et al., 2007). Three sections per embryo, for at least 3 embryos per treatment were averaged. Double counting of labeled cells was avoided in 2 ways: (i) by using alternate sections, and (ii) by not using sections that showed labeled cells in identical positions to those of a previously counted section. R Statistical software (R Core Development Team, 2006) was used for all the statistical analysis, including ANOVAs, post hoc analysis, or as appropriate. All figures were assembled using Adobe PhotoShopCS (Mountain View, CA).

Quantitative RT-PCR

Zebrafish embryos were exposed to 1.5% ethanol between 24 and 48 hpf, and control and ethanol-treated embryos were collected at 24, 27, 30, 36, and 48 hpf. Embryos were fixed using a liquid nitrogen-cooled methanol fixation procedure that preserves nucleic acids and hardens tissues for dissection (Stenkamp et al., 2000). Eyes were dissected from the rest of the body using fine forceps. The bodies and eyes were refrozen separately. Total, eye-specific RNA was extracted from 20 embryos (each group) using the RNeasy micro kit (Qiagen, Valencia, CA). The cDNA template for qRT-PCR was synthesized using High

Capacity cDNA Reverse Transcription kit with random primers (Applied Biosystems, Inc. [ABI], Foster City, CA).

Expression levels of *Nr2f5, shha*, and *ptc-2* mRNA in control and ethanol-treated eyes were quantified by qRT-PCR. Three technical replicates were performed for each group, with *β-actin* as the endogenous reference gene. Primers used were *β-actin* 5′-CCAGCTG TCTTCCCATCCA (forward), 5′-TCACCAACGTAGCTGTCT TTCTG (reverse), 86 bp amplicon (Lin et al., 2009); *Nr2f5* 5′-GAC AGAATGTTGCCATGCC (forward), 5′-TCCTGGGCCAAAT TAGCA (reverse), 167 bp amplicon (Chen et al., 2008); *ptc-2* 5′-TG GCGCTAGCGTAGCTTTG (forward), 5′ -GGGCGGCCATAAA AAACG (reverse), 58 bp amplicon; *shha* 5′-GGTGTTCAG CGACTTCATCA (forward), 5′-AACGGGTTCTTGCGTTTCTA (reverse), 84 bp amplicon (Stenkamp et al., 2008); all designed using Primer Express 3 (ABI). Amplification was performed on a model 7900HT Fast Real-Time PCR System using SYBR-Green PCR Master Mix (ABI). Prior to normalization, threshold cycle values were averaged among the technical replicates (Stenkamp et al., 2008).

Measurement of Tissue Ethanol Concentrations

Ethanol-treated embryos were collected at designated times after treatment, washed briefly in system water, followed by a rinse in assay buffer, and then were homogenized into a ti ssue suspension. After a 30-second centrifugation at 15,000×*g* to remove any remaining solids, the supernatant was pipetted into a fresh 1.5 ml tube and placed on ice. Tissue ethanol concentrations in the homogenate were determined using an Ethanol Assay Kit (BioVision Research Products, Mountain View, CA).

RESULTS

RA Does Not Rescue Ethanol-Induced Microphthalmia

Zebrafish embryos treated with 1.5% ethanol at 24 to 48 hpf, during a major period of retinal neurogenesis (Li et al., 2000) show significant microphthalmia (Fig. 2; Kashyap et al., 2007). One of the major hypotheses regarding the mechanisms through which ethanol influences embryonic development, is that ethanol inhibits RA synthesis and that the effects of ethanol are thereby mediated by reduced RA signaling (Dejonge and Zachman, 1995; Deltour et al., 1996; Duester, 1991; Marrs et al., 2010; Yelin et al., 2005; Fig. *1A*). We tested this hypothesis, specifically for the effects of ethanol during retinal neurogenesis, by performing rescue expeliments in which both ethanol and RA were administered. We performed a series of experiments with different concentrations of all-*trans* RA under various rescue protocols as shown in Fig. *1C*.

An initial concentration of 0.3 μM RA was chosen as this concentration of RA is biologically active during eye development in zebrafish (Hyatt et al., 1992, 1996b; Marsh Armstrong et al., 1994; Prabhudesai et al., 2005). Embryos were treated with 1.5% ethanol, 0.3 μM RA, or the combination (Fig. *1C*), at 24 hpf, and were either fixed for eye measurements at 48 hpf (Fig. 2*A*-2*D*), or were transferred to system water and fixed at 72 hpf (Fig. 2*E*–2*H*). As shown in our previous study (Kashyap et al., 2007), ethanol-treated embryos displayed reduced eye size when compared with untreated control embryos (Fig. 2*A*, 2*B*, 2*E*, and 2*F*). When ethanol and 0.3 μM RA were co-administered between 24 and 48 hpf, embryos also displayed reduced eye sizes, which were significantly smaller than those of both untreated and ethanoltreated embryos at 48 and 72 hpf (Fig. 2*D*, 2*H*, 2*I*, and 2*J*; $p < 0.01$). The administration of exogenous 0.3 μM RA in the absence of ethanol also produced significantly red uced eye size at both 48 and 72 hpf (Fig. 2*C*, 2*G*, 2*I*, and 2*J*; $p < 0.01$). The effects of RA on eye size were more persistent than those of ethanol; eyes

exposed to RA from 24 to 48 hpf did not grow following the treatment, while control and ethanol-treated eyes did grow (Fig. 2*I* and 2*J*). Additionally, we observed additive effects of ethanol and RA on heart development and on pigmentation (Fig. 2*A*–2*H*, and data not shown).

As we observed deleterious effects by administering 0.3 μM RA at 24 hpf, we performed experiments using lower concentra tions of RA (0.1 μM RA, 30 nM RA, and 1.0 nM RA), co-administered with 1.5% ethanol from 24 to 48 hpf, as well as alternative rescue protocols involving delayed treatment with RA (Fig. *1C*). Figure 3 shows eye circumferences of representative clutches for each co-treatment with RA (Fig. 3*A* and 3*B*, 0.1 μM RA; Fig. 3*C* and 3*D*, 30 nM RA; Fig. 3*E*, 1.0 nM RA) and for the sequential treatments with 0.3 μM RA (Fig. 3*F*, 48 to 72 hpf; Fig. 2*G* and 2*H*, 36 to 48 hpf; Fig. 3*I* and 3*J*, 26 to 48 hpf). Under these protocols, we did not observe rescue of ethanol-induced microphthalmia. Moreover, the effects of exogenous RA on eye size were both dose- and time-dependent, as we observed no significant microphthalmia with 1.0 nM RA (Fig. 3*E*), and a slight effect with 30 nM RA (Fig. 3*C* and 3*D*) when compared with severe microphthalmia with 0.1 or 0.3 μM (Fig. 3*A*–3*D*), and no significant microphthalmia with RA treatment beginning at 48 hpf (Fig. 3*F*). Analysis of the measurements was performed on a clutch by clutch basis, with 2 to 6 clutches tested for each protocol. In each of the clutches, we performed an ANOVA followed by post hoc analysis (Fisher's LSD and HSD) using R statistical software (R Core Development Team, 2006) and results were highly reproducible from clutch to clutch.

Effects on Retinal Cell Differentiation in Rescue Experiments

In zebrafish, ethanol-induced microphthalmia is mediated through several mechanisms, including overall developmental delay, cell death in the lens, and reduced differentiation of retinal cells, including photoreceptors (Kashyap et al., 2007; Matsui et al., 2006). Expression of photoreceptor markers such as zpr-1 (red and green cone photoreceptors) and zpr-3 (rod photoreceptors) is limited or nonexistent in ethanol-treated embryos (Kashyap et al., 2007). Because RA is known to influence photoreceptor development in zebrafish (Hyatt et al., 1992, 1996b; Prabhudesai et al., 2005), we wished to determine whether RA could selectively rescue photoreceptor development in the microphthalmic eyes. Embryos were treated with 1.5% ethanol, or were co-treated with RA and ethanol between 24 and 48 hpf and transferred to system water until 72 hpf. Sections were processed for indirect immunofluorescence with the zpr-1 and zpr-3 antibodies. Untreated embryos, and those treated with DMSO (vehicle for RA; not shown) showed extensive labeling for both markers in the outer nuclear layer, with distributions that appeared uniform and continuous (Fig. 4*A* and 4*E*). In contrast, ethanol-treated embryos showed no or few cells positive for zpr-1 or zpr-3 (Fig. 4*B* and 4*F*; Kashyap et al., 2007)). Treatment with 0.3 μM RA alone resulted in a qualitative change in the distribution of labeled cone and rod photoreceptors when compared with the controls, with less continuous labeling of the outer nuclear layer by zpr1 (Fig. 4*C*), and with the appearance of ectopic zpr3-positive cells in the inner nuclear layer (Fig. 4*G*). Rescue experiments involving co-treatment with RA and ethanol (24 to 48 hpf; 0.3 μM RA) resulted in recovery of expression of both photoreceptor markers (Fig. 4*D* and 4*H*). Lower concentrations of RA, and alternative (sequential) rescue protocols (RA at 36 hpf; Fig. 1) did not result in recovery of photoreceptor differentiation (data not shown).

We quantified the photoreceptor marker labeling in 2 different ways, based on the extent of labeling in the retina (*None, Few*, or *Many* photoreceptors labeled; Fig. 4*I*), and qualitative features of the distribution of the markers (*Sporadic, Discontinuous*, or *Dense* labeling; Fig. 4*J*), as described in the Materials and Methods section. Untreated, or DMSO-treated embryos typically showed *Many* labeled photoreceptors, with a *Dense* distribution (Fig. 4*K*-4*N*), while ethanol-treated embryos were frequently scored as *None* for extent of label (Fig. 4*K* and 4*L*), and those with label present were frequently scored as having a *Sporadic*

distribution of labeled cones and rods (Fig. 4*M* and 4*N*). RA treatment alone did not substantially alter the extent of labeling of cones or rods (Fig. 4*K* and 4*L*), but did influence the distribution, particularly of cone photoreceptors (Fig. 4*M* and 4*N*). The administration of RA along with ethanol resulted in the recovery of the extent of zpr-1 and zpr-3 labeling (Fig. 4*K* and 4*L*), but did not result in a normal distribution of labeled cells (Fig. 4*M* and 4*N*). Collectively these data suggest that RA may counteract some of the effects of embryonic ethanol exposure, specifically for photoreceptor development, but these effects do not result in complete recovery of the untreated photoreceptor phenotype. Additionally, this finding indicates that RA, when administered during retinal neurogenesis, can influence the later differentiation of photoreceptors.

Next we examined the differentiation of 3 other retinal cell types: ganglion cells, amacrine cells, and Müller glia, as embryonic ethanol exposure also influences their development (Kashyap et al., 2007). We used indirect immunofluorescence with antibodies directed at islet-l (LIM homeobox protein that labels ganglion cells and amacrine cells (Galli-Resta et al., 1997; Korzh et al., 1998) and those directed at glutamine synthetase (Peterson et al., 2001), respectively. The cotreated embryos did not show recovery of these cell types (data not shown), consistent with selective effects of RA for recovery of photoreceptor differentiation.

Ethanol and RA Cause Microphthalmia via Distinct Mechanisms

Because both ethanol and RA independently result in microphthalmia, and these effects appeared to be additive, we hypothesized that they influenced embryonic eye growth through different mechanisms. We tested this hypothesis by evaluating 2 likely mechanisms for reduced eye growth: changes in the rate of cell proliferation, and changes in the rate of cell death (Kashyap et al., 2007). Retinal cell proliferation was assayed using indirect immunofluorescence with anti-phospho histone (αH-3), an M-phase (mitotic) marker, on sectioned eyes from embryos treated with 1.5% ethanol and/or 0.3 μM RA, and collected at 48 hpf. Control and ethanol-treated embryo eyes showed numerous αH-3 positive cells in the outer nuclear layer (Fig. 5*A* and 5*B*; Kashyap et al., 2007) and showed no significant differences ($p = 0.95$) in the average number of αH-3 positive cells (Fig. 5*E*; Kashyap et al., 2007). Immunopositive cells were also regularly observed in the lens, at the retinal margin, and in the inner nuclear layer (Fig. 5*A*–5*D*). Embryos treated with RA, as well as those co-treated with ethanol and RA showed fewer αH-3 cells, although the locations of the αH-3 positive cells were similar to those of control eyes (Fig. 5*C* and 5*D*). A statistical analysis (one-way ANOVA, followed by a Fisher's post hoc analysis) confirmed that the numbers of mitotic cells were significantly reduced in the RA-treated, and ethanol + RA-treated embryos (Fig. 5*E*; $p < 0.05$). These results are consistent with RA causing microphthalmia at least in part by decreasing the rate of retinal proliferation, while ethanol has its microphthalmic effects through other mechanisms (Kashyap et al., 2007).

Cell death within the eye was assayed at 48 hpf by in vivo staining with Acridine orange, of untreated embryos, and of those treated at 24 hpf with 1.5% ethanol, 0.3 μM RA, or the combination. Consistent with Kashyap and colleagues (2007), we found that ethanol exposure results in a significantly higher number of embryos showing Acridine staining in the lens ($p < 0.001$; Fisher Exact Test), but no significant difference in the number of embryos showing cell death in the retina, when compared with untreated embryos (Fig. 6*A* , 6*B*, and 6*E*). Treatment with RA or ethanol alone resulted in few embryos displaying cell death in either lens or retina (Fig. 6*C* and 6*E*), supporting the hypothesis that RA and ethanol influence eye size/growth by independent mechanisms. However, a significantly higher proportion of embryos showed cell death in the retina when treated with both ethanol and RA ($p < 0.05$; Fisher Exact Test; Fig. 6*D* and 6*E*). This synergistic effect of ethanol and RA on retinal cell death indicates potential interactions between the 2 treatments.

Endogenous RA Signaling is Not Reduced by Ethanol Treatment

To elucidate further whether ethanol-induced microphthalmia is related to reduced RA signaling specifically within the eye, we utilized transgenic zebrafish that express a YFP transgene under control of a series of RA receptor elements (RAREs). In these RARE-YFP transgenic fish, YFP is expressed in the retina in a restricted manner, confined to ventral retina and a smaller expression domain in dorsal retina (Perz-Edwards et al., 2001; Prabhudesai et al., 2005). These transgenic fish have been verified as responding to embryonic ethanol exposure in a manner similar to that of the SciH strain used in all other experiments (Kashyap et al., 2007). Transgenic embryos (from a heterozygous cross) were identified at 30 hpf (when transgene expression becomes obvious in the eye; Perz-Edwards et al., 2001), and were exposed to 1.5% ethanol for 24 hours. Control and ethanol-treated embryos were imaged live for YFP expression (Fig. 7*A* and 7*B*). Embryos showed highly similar transgene expression patterns, suggesting that ethanol had not influenced RA signaling within the eye. To determine whether there were differences in the cell populations engaged in RA signaling as a result of ethanol treatment, control and ethanol-treated embryos were sectioned and subjected to indirect immunofluorescence using an anti-GFP antibody. Both untreated and ethanol-treated embryos showed expression of transgene predominantly in neuroepithelial cells of the ventral retinal margin (Fig. 7*C* and 7*D*; Prabhudesai et al., 2005). As a positive control, we treated RARE-YFP embryos with the aldehyde dehydrogenase inhibitor DEAB, at 10 μM, a concentration known to decrease RA signaling in the neural tube of zebrafish embryos (Perz-Edwards et al., 2001). DEAB-treated embryos showed no transgene expression within the eye after 24 hours of treatment (Fig. 7*E* and 7*F*), confirming the RARE-YFP transgenic as a valid tool for the reporting of RA signaling in the eye during retinal neurogenesis.

We also performed experiments to test whether a known regulatory target of RA signaling was affected in eyes of ethanol-treated embryos. The *Nr2f5* gene encodes a COUP-TF transcriptional repressor that is positively regulated by RA, and mediates the toxic effects of RA upon heart development in zebrafish (Chen et al., 2008). We quantified expression of *Nr2f5* mRNA in eyes of control versus ethanol-treated embryos collected at 24, 27, and 48 hpf. Levels of *Nr2f5* mRNA did not differ significantly as a function of cthanol treatment (Fig. S1), consistent with no effect of ethanol on RA signaling within the eye.

Finally, we note that 24 to 48 hpf treatments with the aldehyde dehydrogenase inhibitor DEAB (10 μM) did not fully phenocopy the treatments with 1.5% ethanol. DEAB-treated embryos were microphthalmic (Fig. S2*A* and S2*B*), although not as severely as ethanol-treated embryos (DEAB vs. control at 48 hpf, $p = 0.003$; ethanol vs. control at 48 hpf, $p < 0.00001$, Kashyap et al., 2007; and the present study). In addition, ganglion cell differentiation took place during exposure to DEAB (Fig. S1), but was impaired by exposure to ethanol (Kashyap et al., 2007). These data indicate that the mechanisms through which ethanol and DEAB cause microphthalmia are likely distinct.

Taken together, these findings indicate that ethanol treatment during retinal neurogenesis does not result in changes in endogenous RA signaling within the embryonic zebrafish eye, and therefore our studies do not support a role for RA signaling in ethanol-induced microphthalmia. In addition, these findings are consistent with the hypothesis that the apparent rescue effect of RA on ethanol-induced defects in photoreceptor differentiation is due to an independent effect of RA on photoreceptors.

Cholesterol Does Not Rescue Ethanol-Induced Microphthalmia or Defects in Photoreceptor Development

Recently, cholesterol has gained credibility as a rescue agent for the effects of embryonic ethanol exposure (Li et al., 2007; Loucks and Ahlgren, 2009). Altered Shh signaling has been postulated to be one of the causes for spectrum of symptoms associated with fetal alcohol exposure, because the signaling function of Shh is associated with a posttranslational modification with a cholesterol molecule (Li et al., 2007; Mao et al., 2009; Porter et al., 1996), and because cholesterol homeostasis can be altered by ethanol (Guizzetti et al., 2007; Guizzetti and Costa, 2007; Li et al., 2007; Polo et al., 2003; Fig. 1*B*). In zebrafish, ethanol exposure prior to gastrulation (4 hpt) results in profound abnormalities that can be rescued with microinjection of cholesterol (Li et al., 2007). Because Shh is also an important signaling molecule for retinal neurogenesis and for differentiation of photoreceptors (Neumann and Nuesslein-Volhard, 2000; Stenkamp et al., 2000, 2002), we pursued this as a potential alternative mechanism through which ethanol may influence eye growth and photoreceptor development.

Embryos were microinjected with 3 nl of 0.5, 1.0, or 2.0 μ/μl of cholesterol, or of the DMSO vehicle, immediately prior to exposure to 1.5% ethanol (24 to 48 hpf; see Fig. 1*C*). Embryos were either fixed at 48 hpf or were transferred to system water until 72 hpf. Significant rescue of microphthalmia was not observed using any of the rescue protocols (Fig. 8*A*–8*F*).

We next examined the possibility of cholesterol rescuing the reduced photoreceptor development phenotype seen in ethanol-exposed embryos. Sectioned embryos were processed for indirect immunofluorescence using the photoreceptor markers zpr-1 (Fig. 9*A*-9*D*) and zpr-3 (Fig. 10*E*-10*H*) to label cones and rods, respectively. Cholesterol-treated embryos showed no appreciable changes in photoreceptor quantity, distribution, spacing, or morphology (Fig. 9*C*) as compared with their untreated counterparts (Fig. 9*A*). Expression patterns of zpr-1 and zpr-3 were also unchanged following co-treatment with cholesterol and ethanol, when compared with treatment with ethanol alone (Fig. 9*B* and 9*F*). We again quantified the photoreceptor marker expression pattern, based on the extent of labeling in the retina (*None vs. Few vs. Many* labeled photoreceptors; Fig. 9*E* and 9*F*). In ethanol-treated embryos, with or without the cholesterol treatment, the predominant scoring categories were *None* and *Few* for both the markers (Fig. 9*I* and 9*J*), while in control embryos, and those treated with cholesterol alone, the most frequent scoring category was *Many*, suggesting no rescue of ethanol effects on photoreceptors. These data suggest that Shh signaling is not involved in mediating the effects of ethanol exposure on photoreceptor development.

Exposure to Ethanol Does Not Alter Shh Signaling Within the Developing Eye

We also performed experiments to test whether Shh signaling was impaired in ethanol-treated embryos. The *patched (ptc*) genes encode transmembrane proteins that form part of the Shh receptor complex (Riobo and Manning, 2007), and these genes are positively regulated by Shh signaling (Bergeron et al., 2008; Xu et al., 2006). *Ptc2* is expressed within the zebrafish eye during the period of retinal neurogenesis (Bibliowicz and Gross, 2009; Stenkamp et al., 2000), and its expression is reduced within the retinas of zebrafish heterozygous for a deletion mutation (syu^{t4} +/-) of the *Shh* gene (Stenkamp et al., 2008), suggesting that expression of this gene is a reliable readout of Shh signaling within the zebrafish eye. Expression of *ptc-2* was quantified in a time series manner during the period of retinal neurogenesis using qRT-PCR. Levels of *ptc2* mRNA did not change significantly over time, and did not differ significantly as a function of ethanol treatment (Fig. 10*A*). Similarly, levels of *shha* mRNA did not change over time and as a function of ethanol

treatment during the period of retinal neurogenesis (Fig. 10*B*). Taken together with the results of the cholesterol rescue experiments, these findings indicate that altered Shh signaling is not involved in mediating the microphthalmic effects of ethanol exposure during retinal neurogenesis.

Tissue Ethanol Levels in the Zebrafish Model are Relevant for Human FASD

The effects of embryonic ethanol exposure during retinal neurogenesis do not appear to be mediated by changes in the RA or Shh signaling systems, in contrast to the findings reported for earlier developmental periods (Li et al., 2007; Marrs et al., 2010). Therefore we wished to verify that our ethanol treatments, between 24 and 48 hpf, resulted in tissue ethanol concentrations that are relevant for human FASD. Embryos treated with 1.5% ethanol at 24 hpf were harvested 1 and 24 hours after treatment (at 25 and 48 hpf). Ethanol levels in embryonic tissues immediately following treatment were approximately 35 mM, and declined to 21.4 mM by the end of the treatment period (Table 1). These levels correspond to approximately 0.1 g/dl, levels that can be reached in human tissues after 2 to 3 alcoholic drinks, suggesting that our findings in the zebrafish, over the 24 to 48 hpf exposure period, are relevant for human embryonic ethanol exposure and FASD. Embryos assayed after ethanol treatments matching those of Li and colleagues (2007, 1.5% ethanol at 4 to 10 hpf) showed tissue ethanol concentrations of approximately 20 mM (data not shown), suggesting that the slightly higher tissue ethanol concentrations measured in our experiments, when compared with Li and colleagues (2007, 4 mM), were not due to developmental changes in embryonic capacity to metabolize ethanol. It is possible that the slight differences in our measurements are related to strain-specific differences in embryonic responses to ethanol exposure (Loucks and Carvan, 2004).

DISCUSSION

This study tested 2 hypotheses regarding the signaling mechanisms that may be involved in mediating the deleterious effects of embryonic ethanol exposure upon eye development. We used as our model system, the embryonic zebrafish, which responds to ethanol treatment during the period of retinal neurogenesis by a significant slowing of eye growth due to a combination of overall developmental delay, cell death within the lens, and reduced retinal cell differentiation (Kashyap et al., 2007). The first hypothesis, that the effects of ethanol on eye development are mediated by changes in RA signaling (DeJonge and Zachman, 1995; Johnson et al., 2007; Marrs et al., 2010; Satiroglu-Tufan and Tufan, 2004; Twal and Zile, 1997; Yelin et al., 2005, 2007), was not supported by the results of several different rescue experiments, and by the failure of ethanol to alter expression of a transgene that reports RA signaling. However, one of the rescue protocols was successful in recovering some aspects of photoreceptor differentiation, and our experiments revealed novel functions for RA signaling in regulating retinal cell proliferation. The second hypothesis, that the effects of ethanol on eye development are mediated by changes in Shh signaling (Ahlgren et al., 2002; Blader and Strahle, 1998; Li et al., 2007; Maity et al., 2005), was similarly not supported by the results of rescue experiments, and by the failure of ethanol to alter expression of a gene that reliably reports changes in Shh signaling.

Ethanol-Induced Microphthalmia is Not Rescued by RA or Cholesterol

Reduction in RA has been postulated as one of the mechanisms underlying the phenotypes observed in human FASD (DeJonge and Zachman, 1995; Deltour et al., 1996; Zachman and Grummer, 1998), where ethanol may competitively inhibit the dehydrogenase enzymes that catalyze the synthesis of RA (Kot-Leibovich and Fainsod, 2009; Molotkov and Duester, 2002). For example, Twal and Zile (1997) rescued the cardiovascular related effects of ethanol on cultured quail embryos using RA treatments, and Johnson and colleagues (2007)

similarly rescued the cell death induced by ethanol during limb development in mice through the administration of RA. Marrs and colleagues (2010) showed significant, partial rescue of ethanol-induced anterior–posterior axis defects and neural axis formation in zebrafish by co-treatment with RA. In a *Xenopus* model for FASD, treatment with 2% ethanol at midblast ula transition reduced. RA signaling, and could rescue teratogenic effects of retinoids (Yelin et al., 2005). Dietary deficiency of vitamin A (the precursor to RA) results in microphthalmia or anophthalmia, along with other ocular defects, in a number of animal models (reviewed by Dräger and McCaffery, 1997), and this apparent phenocopy of FASD has been considered as further evidence for a role for RA in FASD (Zachman and Grummer, 1998). We tested this hypothesis explicitly for the microphthalmic consequences of embryonic ethanol exposure in the zebrafish. We observed no rescue of eye size when RA was co- or sequentially administered with ethanol, even when RA was administered at levels that did not result in deleterious effects (1.0 nM; see also Marrs et al., 2010). These results suggest that reduced RA levels may not mediate some effects of ethanol on eye development. We undertook an additional test of the effects of ethanol on RA signaling by evaluating RA signaling specifically within the eye using the transgenic line RARE-YFP. The RARE-YFP fish express YFP under the control of a series of RAREs, therefore serving as reporters of endogenous RA signaling. Ethanol exposure during retinal neurogenesis did not result in altered RA signaling in the eyes of these embryos, and did not alter the expression of a known transcriptional target of RA signaling. Together, our findings do not support a role for reduced RA signaling in mediating the effects of embryonic ethanol exposure during the period of retinal neurogenesis. The regulation of RA synthesis and degradation within the developing eye may be different from its regulation during embryonic gastrulation, making RA signaling less responsive to ethanol or its metabolites.

Another mechanism postulated for mediating the effects of embryonic ethanol exposure is reduced Shh signaling. The most severe form of human FASD displays a phenotype that matches that seen in cases of mutations in *Shh*: holoprosencephaly and cyclopia (Maity et al., 2005). This apparent phenocopy has led to the hypothesis that Shh signaling may be impaired in FASD. Mechanistically, ethanol has been shown to inhibit a key enzyme involved in the biosynthesis of cholesterol (Polo et al., 2003), which in turn is involved in posttranslational modification of the Shh protein (Porter et al., 1996). In zebrafish, this hypothesis has been tested for the severe effects of ethanol treatment during gastrulation, by supplementing cholesterol via microinjection into the yolk of treated embryos. (Li et al., 2007; Loucks and Ahlgren, 2009). In these experiments, FASD-like phenotypes including growth retardation and cyclopia were rescued by cholesterol (Li et al., 2007). Additionally, axial defects including holoprosencephaly and cyclopia were rescued using *Shh* mRNA injection in zebrafish embryos (Loucks and Ahlgren, 2009). In the present study, we tested this hypothesis explicitly for the microphthalmic consequences of embryonic ethanol exposure in the zebrafish, as well as for effects on photoreceptor differentiation. We found that cholesterol supplementation was entirely ineffective at rescuing microphthalmia, and similarly ineffective at rescuing defects in photoreceptor development. These results were somewhat surprising because of the clear rescue of axial defects observed by Li and colleagues (2007) in the same animal model, and beca use Shh signaling has multiple roles in eye development in the zebrafish, including regulating retinal proliferation, ganglion cell neurogenesis, photoreceptor differentiation, and retinal cell survival (Neumann and Nuesslein-Volhard, 2000; Stenkamp and Frey, 2003; Stenkamp et al., 2000, 2002), many of which are affected by ethanol treatment (Kashyap et al., 2007). Furthermore, the *Shh* deletion mutant *syu*−/− has a small eye phenotype (Stenkamp et al., 2002), and the *ptc-2* mutant *lep*, in which Shh signaling is amplified, has an enlarged retina (Bibliowicz and Gross, 2009). Failure of cholesterol supplementation to rescue eye development, when similar strategies successfully rescue major axial defects due to early ethanol treatment, may be due to ethanol acting through distinct mechanisms in a developmental time- or tissue-

specific manner. Consistent with this idea is the absence of any quantitative effect on eye-specific expression of *Shh* or on one of the target genes downstream of Shh signaling, *ptc-2*, as a consequence of ethanol treatment. These findings do not support a role for altered Shh signaling in mediating the effects of embryonic ethanol exposure during retinal neurogenesis.

RA Treatment Results in Recovery of Photoreceptors in Ethanol-Treated Embryos

Ethanol induces altered differentiation of all retinal cell types including ganglion cells, Müller glia and photoreceptors (Chmielewski et al., 1997; Dlugos and Rabin, 2007; Kashyap et al., 2007; Matsui et al., 2006; Tufan et al., 2007). A number of different approaches have been used to pursue the mechanisms of these effects in zebrafish, including the use of prolonged ethanol treatment (Dlugos and Rabin, 2007), treatment over the period of retinal neurogenesis but prior to photoreceptor differentiation (Kashyap et al., 2007), and treatment during photoreceptor differentiation (Matsui et al., 2006). Deleterious outcomes for photoreceptors include failed terminal differentiation of rods and cones (Kashyap et al., 2007), reduced volume of the photoreceptor layer and failure to form rod outer segments (Dlugos and Rabin, 2007; Matsui et al., 2006), and physiological deficits related to photoreceptor function (Matsui et al., 2006). Functional deficits related to photoreceptors are observed in human cases of FASD (Hug et al., 2000) indicating that photoreceptor development is a teratogenic target of ethanol in both species. Similarly, roles for RA in regulating a variety of aspects of photoreceptor fate, differentiation, and survival have been reported from the use of a number of different vertebrate model systems including zebrafish (Hyatt et al., 1996a; Prabhudesai et al., 2005), chick retinal cultures (Stenkamp et al., 1993), and rodent retinal cultures (Kelley et al., 1994; Wallace and Jensen, 1999), and one of the retinoid receptors, RXRγ, regulates the expression of S-opsin in the mouse (Roberts et al., 2005). It is therefore of great interest that RA treatment was successful at restoring the differentiation of rod and cone photoreceptors in ethanol-treated zebrafish.

We believe the effects of RA on restoring photoreceptor differentiation did not constitute a true rescue for the following reasons: (i) ethanol did not alter RA signaling within the developing eye, based upon our studies with the RARE-YFP transgenic zebrafish; (ii) ethanol and RA each had additional effects on eye development that appear to be independent of each other (see next section of Discussion); and (iii) the pattern and morphology of the “rescued” photoreceptors in the co-treated embryos did not match that of control embryos. Together, these findings suggest that RA was able to recover photoreceptors in a manner that is not directly related to the mechanism through which ethanol inhibited their differentiation. We note that, in ethanol-treated embryos, a photoreceptor layer forms that expresses a number of photoreceptor-specific transcription factors, including *NeuroD* and *crx* (Kashyap et al., 2007), suggesting that only the final steps of photoreceptor differentiation are inhibited by ethanol. It is possible that the addition of RA was sufficient to independently engage the remaining components of any photoreceptor differentiation program, either through a direct influence on retinoid response elements in photoreceptor genes themselves (Khanna et al., 2006; Li et al., 2002), or by influencing the activities of other cell types, such as the retinal pigmented epithelium. The capacity of RA to affect photoreceptor development also indicates that ethanol treatment did not affect other aspects of RA signaling, such as the expression of RA receptors and co-activators. If the effects of RA and ethanol truly are independent, then these results imply that RA is a very potent agent for regulating photoreceptor development, even in the presence of a pathological response.

An alternative interpretation is that the RA co-treatment experiments did achieve a true rescue for photoreceptors (albeit not for eye size). Under this interpretation, the effects of ethanol on photoreceptors are related to the inhibition of RA signaling, but at a level that is

not detectable by our assay with the RARE-YFP transgenics or by our measurements of expression of a transcriptional target of RA signaling. Also under this interpretation, the unusual photoreceptor patterns and morphologies must be the consequence of additional effects of RA itself, beyond the rescue effect.

It is intriguing that RA treatment during retinal neurogenesis, whether in the presence of absence of ethanol, resulted in changes to photoreceptor pattern. RA-treated embryos showed a more sporadic or discontinuous distribution of cones, and regularly contained ectopic rod opsin-expressing cells in the inner nuclear layer. We and others have previously described the rod and cone-specific effects of exogenous RA on photoreceptor differentiation: rod and red cone differentiation is promoted, while the differentiation of blue and UV cones is inhibited (Hyatt et al., 1996b; Prabhudesai et al., 2005). However, in these experiments, in which RA treatment took place during photoreceptor differentiation (48 to 72 hpf), rod and cone pattern were not changed, suggesting these were not effects on photoreceptor fate. In contrast, this study involves RA treatment during retinal neurogenesis (24 to 48 hpf). It is possible, therefore, that RA signaling prior to photoreceptor differentiation can influence photoreceptor fate in the zebrafish . A more rigorous analysis of 2-dimensional photoreceptor pattern in RA-treated embryonic eyes (as in Prabhudesai et al., 2005) was not performed because eyes treated with RA at 24 hpf are too small to prepare as whole mounts.

Effects of Exogenous RA on Retinal Neurogenesis

Treatment with 0.3 μM RA during retinal neurogenesis caused significant microphthalmia, through mechanisms distinct from that of ethanol. RA exposure resulted in reduced cell proliferation, while ethanol has no effect on cell proliferation (Kashyap et al., 2007). Experiments in mouse have shown that in cases of vitamin A deficiency, and in retinoic acid receptor (RAR$\beta 2y2$) mutants, there is a thinning of the retina (Maden et al., 2007) and reduced proliferation of neuroepithelial cells (Grondona et al., 1996), consistent with the present findings. Endogenous sources of RA exist within the vertebrate eye during the period of retinal neurogenesis (Duester, 2009; Marsh-Armstrong et al., 1994). These sources of RA are known to be involved in a variety of additional functions related to eye growth such as promoting or pennitting apoptosis of cells in the perioptic mesenchyme (Matt et al., 2005). In the present study, we did not evaluate effects of RA on tissues outside the eye. Within the eye, however, supplemental RA caused only modest increases in apoptosis, suggesting that the effects of RA on eye growth are most likely due to inhibition of retinal proliferation. The endogenous, ocular sources of RA in the zebrafish may therefore be involved in regulation of retinal proliferation.

Implications for Treatment and Prevention of FASD

This study demonstrated that 2 important developmental signaling systems—RA and Shh—are not involved in mediating ethanol-induced microphthalmia during retinal neurogenesis in zebrafish embryos. In addition, one of the rescue strategies tested (RA) was itself a cause of microphthalmia through independent mechanisms. Collectively our experiments suggest that pharmacological intervention strategies based upon these signaling pathways may not counteract all of the embryonic effects of gestational ethanol consumption in humans, and may themselves result in teratogenic consequences. At concentrations that recover photoreceptor differentiation (Fig. 4), RA treatments caused microphthalmia (Fig. 2) due to reduced retinal cell proliferation (Fig. 5), and exacerbated the microphthalmia caused by ethanol by increasing retinal cell death (Fig. 6). This is unfortunate because the RA and Shh signaling systems lend themselves to dietary interventions (vitamin A and cholesterol) that are simple to administer and for which compliance may readily be achieved. In addition, rescue experiments based upon the RA and Shh signaling systems in animal models for

FASD have been successful at counteracting effects of ethanol exposure during embryonic gastrulation and neurulation (Li et al., 2007; Loucks and Ahlgren, 2009; Marrs et al., 2010; Yelin et al., 2005). Our study therefore highlights the need for further evaluation of the effects of embryonic ethanol exposure on multiple developing body systems, and at multiple developmental times, using in vivo models.

The effects of ethanol on early embryonic development likely involve changes in RA and/or Shh signaling; however, our studies suggest that at least some of the effects, and most notably those on eye growth during retinal neurogenesis, must be mediated through alternative mechanisms. A number of different mechanisms have been proposed, including oxidative stress (Reimers et al., 2004, 2006) and deployment of the heat shock response (Lele et al., 1997) due to the presence of ethanol and its metabolites. These may prove to be promising directions for pursuing the mechanisms underlying microphthalmia in response to embryonic ethanol exposure.

The ethanol treatments used here (1.5%; approximately 250 mM) resulted in accumulation of approximately 20 mM ethanol (~0.1 g/d1) in embryonic tissues. In humans this tissue concentration can be achieved by consuming about 3 standard drinks (in pregnant mammals, fetal alcohol concentrations likely match or exceed maternal levels; Fuchs, 1965; Mann et al., 1975), suggesting that our findings in the zebrafish, and those of others utilizing similar treatments, are relevant for human embryonic ethanol exposure and FASD.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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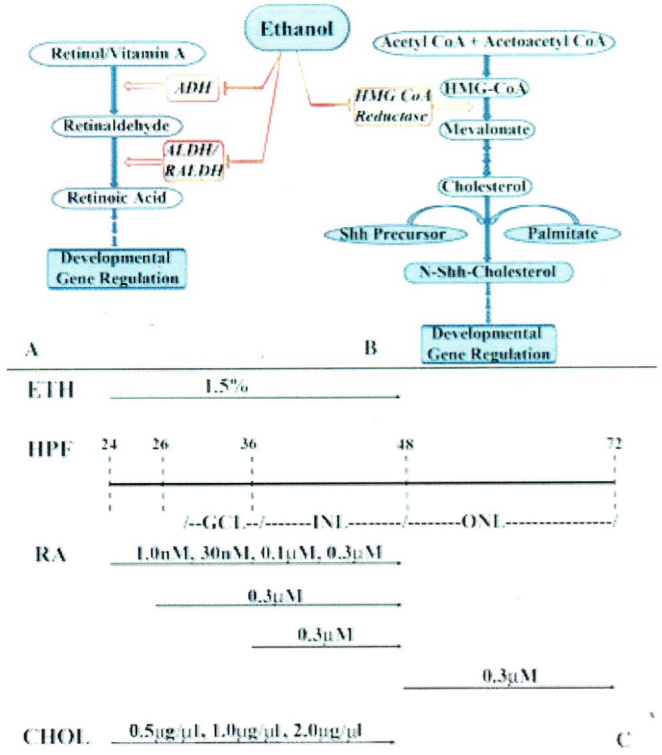

Fig. 1.

Rationale and experimental design. (**A**) Flow chart depicting a mechanism through which ethanol may result in developmental defects by inhibition of enzymes involved in retinoic acid (RA) synthesis. ADH, alcohol dehydrogenase; ALDH, aldehyde dehydrogenase; RALDH, retinaldehyde dehydrogenase. (**B**) Flow chart depicting a mechanism through which ethanol may result in developmental defects by inhibition of cholesterol synthesis and sonic hedgehog (Shh) signaling. HMG-Co-A, 3-hydroxy-3-methylglutaryl-coenzyme A. (**C**) Rescue protocols. Various treatment schemes were employed to probe the capacity of RA or cholesterol to rescue the effects of ethanol (ETH) on eye development. Four different concentrations of RA (1.0, 30 nM, 0.1 and 0.3 μM) and 4 different protocols (co-treatment: 24–48; sequential treatment: 26–48, 36–48, or 48–72 hours postfertilization [hpf]) were employed. Cholesterol (0.5, 1.0, and 2 μg/μl) in DMSO was microinjected in 3 nl volumes immediately prior to treatment with ethanol. Embryos were fixed at 48 or 72 hpf for further evaluation. For experiments in which embryos were fixed at 72 hpf, they were transferred to system water at 48 hpf, following either of the treatments except for the sequential treatment (48 to 72 hpf) with RA. GCL, INL, and ONL depict the period of neurogenesis of the embryonic ganglion cell layer, inner nuclear layer, and outer nuclear layer, respectively (Hu and Easter, 1999).

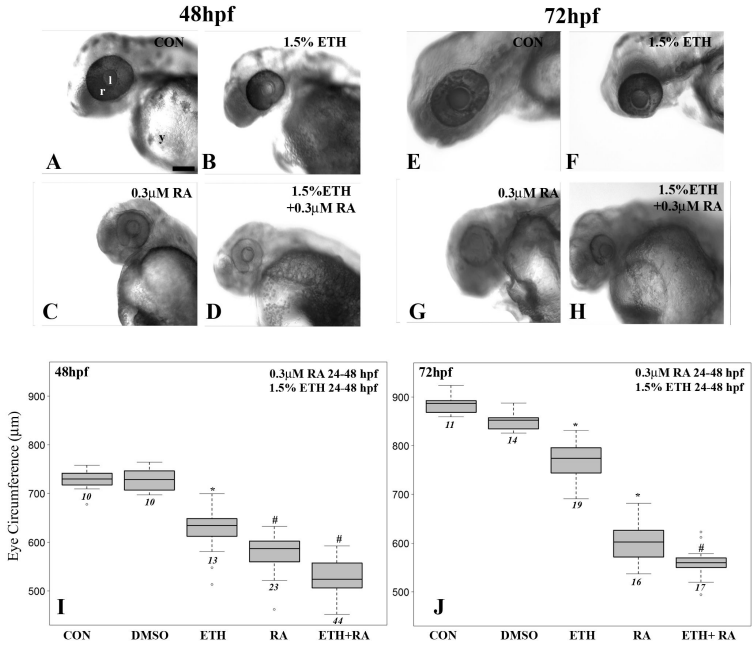

Fig. 2.

Retinoic acid (RA) does not rescue ethanol-induced microphthalmia. Panels *A–H* show representative photos of embryos that were untreated (*A, E*), ethanol-exposed (*B, F*), 0.3 μM RA-treated (**C, G**), and co-treated with ethanol and RA (**D, H**) at 48 (**A**–**D**) and 72 hours postfertilization (hpf) (*E–H*). Bar in A = 100 μm (applies to **A**–**H**); I, lens; r, retina; y, yolk. Panels **I** and **J** show boxplots of eye circumference measurements of representative of co-treatment rescue experiments with 0.3 μM RA (24 to 48 hpf) in embryos assessed at 48 (**I**) and 72 hpf (**J**). Boxplots were generated in R statistical software (R Core Development Team, 2006); the boxes demarcate the 25th and 75th percentiles, dark horizontal lines the median, whiskers represent the upper and lower limits, and the open circles indicate any outliers. Asterisks (*) represent groups that are significantly different from the control and DMSO-treated groups, and the number signs (#) indicate groups different from both control and ethanol-treated groups (p value < 0.05; ANOVA, post hoc analysis). Numbers of embryos in each group (n) are provided below each boxplot.

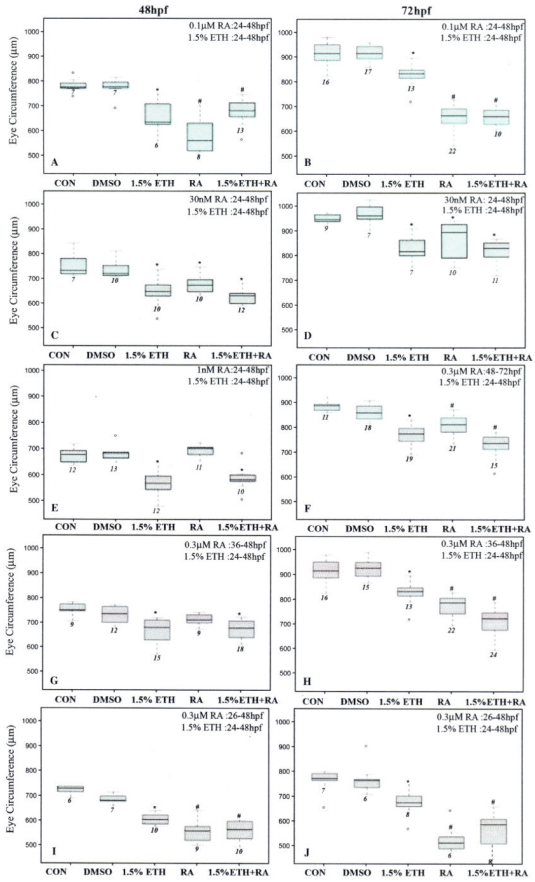

Fig. 3.

Altemative retinoic acid (RA) rescue protocols do not prevent ethanol-induced microphthalmia. The left column of panels (*A, C, E, G, I*) shows eye circumference measurements in μm at 48 hours postfertilization (hpf) and the right column of panels (**B, D, F, H, J**) shows those at 72 hpf, as a consequence of various rescue protocols (shown in Fig. 1*C*). Panels **A**–**E** show representative boxplots following co-treatment with ethanol and 0.1 μM RA (**A, B**), 30 nM RA (**C, D**), or 1.0 nM RA (**E**) between 24 and 48 hpf. Panels **F**–**J** show representative boxplots following sequential treatment protocols: ethanol between 24 and 48 hpf with 0.3 μM RA between 48 and 72 (**F**), 36 and 48 (**G, H**), or 26 and 48 hpf (**I, J**). Boxplots were generated in R statistical software (R Core Development Team, 2006); the boxes demarcate the 25th and 75th percentiles, dark horizontal lines the median, whiskers represent the upper and lower limits, and the open circles indicate any outliers. Asterisks (*) represent groups that are significantly different from the control and DMSO-treated groups, and the number signs (#) indicate groups different from both control and ethanol-treated groups ($p < 0.05$; ANOVA; post hoc analysis). Numbers of embryos in each group (n) are provided below each boxplot.

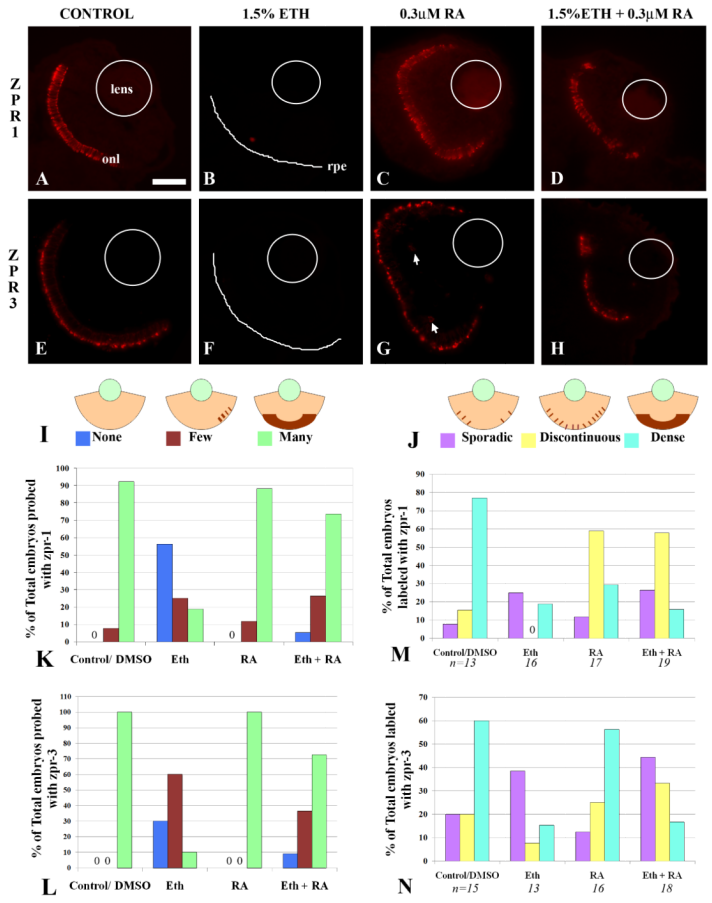

Fig. 4.

Recovery of photoreceptor makers in ethanol-treated embryos by retinoic acid (RA). Panels **A–H** show representative photographs of retinas of embryos that were untreated (**A, E**), ethanol exposed (**B, F**), RA-treated (**C, G**), and co-treated with RA and ethanol (**D, H**), processed for zpr-1(**A–D**) and zpr-3 (**E–H**) indirect immunofluorescence at 72 hours postfertilization (hpf). White open circles indicate lenses in the sections and white semicircles indicate the position of retinal pigmented epithelium (rpe). White arrows indicate ectopic expression of zpr-3 markers. Scale bar in **A** (applies to **A–H**) = 50 μm. Panels I and **J** show the extent of expression of zpr-1 (**I**) and zpr-3 (**J**) at 72 hpf. Panels K (zpr-1) and L (zpr-3) show the distribution of cells expressing each of the 2 photoreceptor markers. Numbers of embryos in each group (*n*) are provided at the bottom of panels **K** and **L**. Methods for scoring the extent and distribution of zpr-1 and zpr-3 labeling are illustrated in panels **M** and **N** and are described in Materials and Methods. onl, outer nuclear layer.

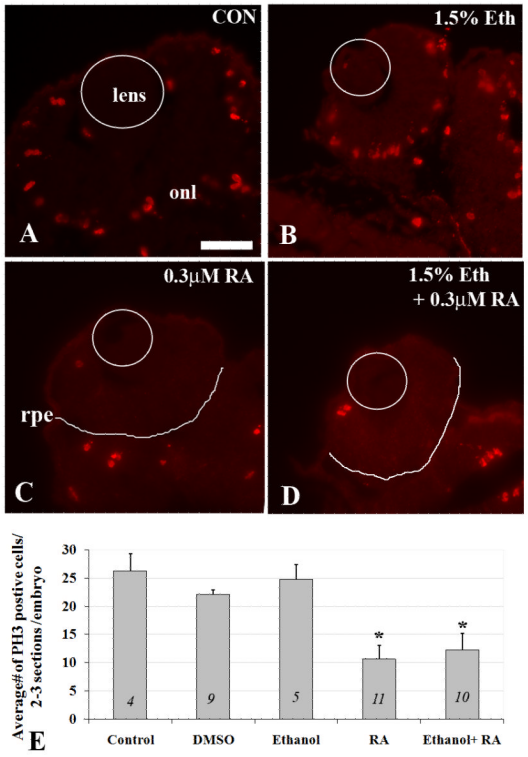

Fig. 5.

Cell proliferation in retinoic acid (RA) and RA ± ethanol co-treated embryos is reduced. Panels **A–D** show representative photographs of retinas of embryos that were untreated (**A**), ethanol-treated (**B**), 0.3 μM RAtreated (**C**), and co-treated with RA and ethanol at 24 hours postfertilization (hpf) (**D**) processed for proliferative cells using anti-phosphohistone 3 using indirect immunofluorescence. White open circles represent the positions of lenses on sections and semicircles represent the positions of the retinal pigmented epithelium (rpe). Scale bar in **A** (applies to **A–D**) = 50 μm. Panel **E** shows average number of α-PH3 positive cells per section ± SEM at 48 hpf. Asterisks (*) represent groups significantly different from control and DMSOtreated groups ($p < 0.05$; ANOVA; post hoc analysis). Numbers of embryos in each group (n) are provided below panel **E**. onl, outer nuclear layer.

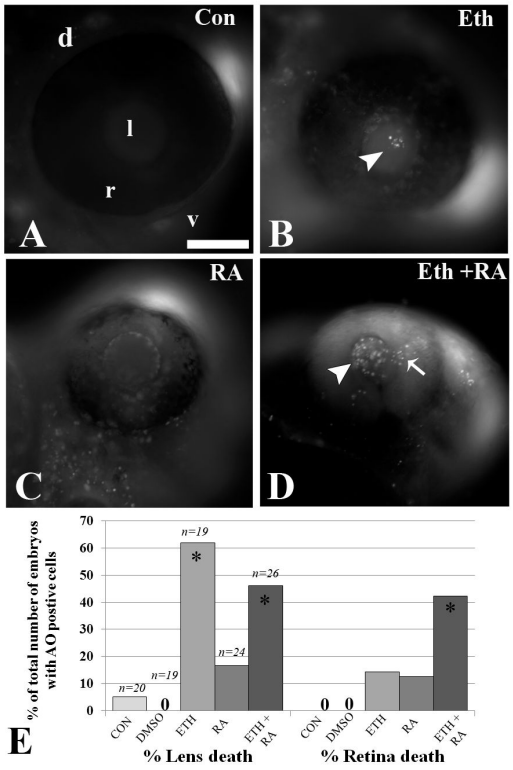

Fig. 6.

Cell death in lens and retina of embryos with rescue treatment. Panels **A–D** show representative photographs of eyes of 48 hours postfertilization (hpf) embryos subjected to the following conditions: untreated (**A**), ethanol-exposed (**B**), 0.3 μM RA-treated (**C**), and RA plus ethanol co-treatment (**D**), stained with Acridine orange (AO) to visualize dead or dying cells. Scale bar in **A** (applies to **A–D**) = 50 μm ; d, dorsal; v, ventral; I, lens; r, retina. White arrows indicate AO-positive cells in the retina, arrowheads indicate AO-positive cells in lens. Panel **E** is a column graph showing percentage of the number of embryos positive for AO in lens and retina. Asterisks (*) represent groups significantly different from control and DMSOtreated groups ($p < 0.05$; Fisher Exact Test).

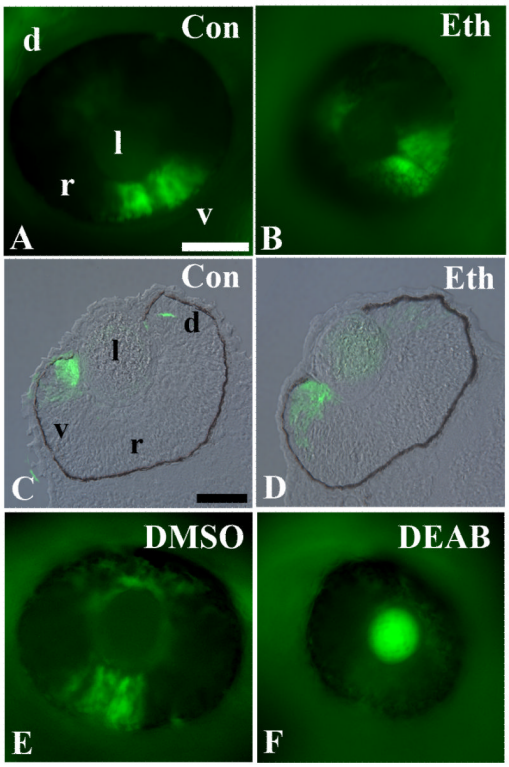

Fig. 7.

No change in retinoic acid (RA) signaling in the eye following ethanol treatment. Panels **A** and **B** show representative fluorescence images of eyes of live, RARE-YFP embryos indicating the presence of active RA signaling in embryos that were untreated (**A**; *n = 4*) or ethanol-exposed (**B**; *n = 6*) for 24 hours. Panels **C** and **D** show representative images of retinal sections of untreated (C; *n = 4*) and ethanol-exposed (**D**; *n = 6*) for indirect immunofluorescence of YFP. Panels **E** and **F** show representative fluorescence images of eyes of live, RARE-YFP embryos indicated active RA signaling in eyes of DMSO-treated embryos (**E**; *n = 5*), and absence of RA signaling in eyes of DEAB-treated embryos (**F**; *n = 5*). Scale bars in **A** (applies **A, B, E, F**) and **C** (applies to **C, D**) = 50 μm; d, dorsal; v, ventral; I, lens; r, retina.

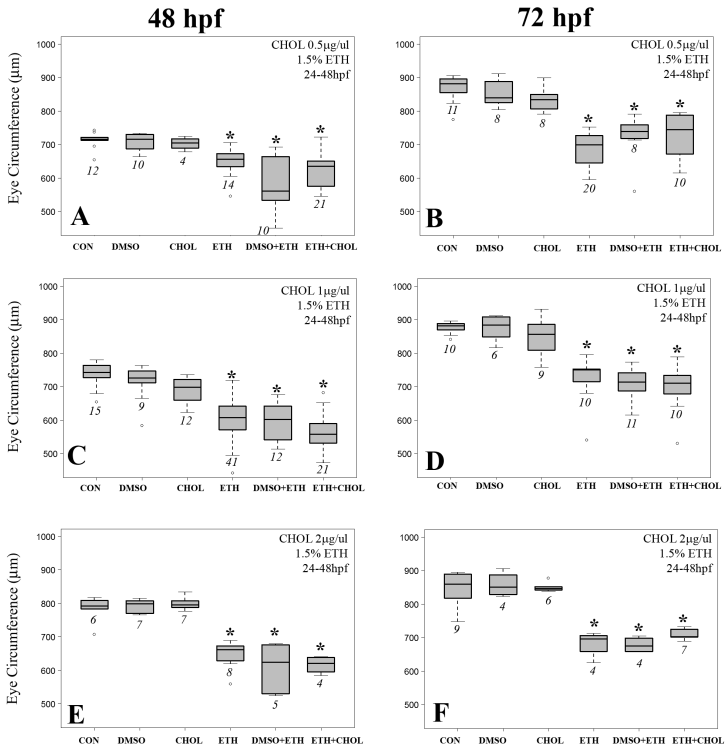

Fig. 8.

Cholesterol does not rescue ethanol-induced microphthalmia. Panels **A–D** show boxplots of eye circumferences of rescue experiments with cholesterol (**A** and **B**: 0.5 μg/μ*l*; **C** and **D**: 1.0 μg/μl **E** and **F**: 2.0 μg/μl) co-treated with ethanol between 24 and 48 hours postfertilization (hpf) and assessed at 48 (**A, C, E**) and 72 hpf (**B, D, F**). Boxplots were generated in R statistical software (R Core Development Team, 2006); the boxes demarcate the 25th and 75th percentiles, dark horizontal lines the median, whiskers represent the upper and lower limits, and the open circles indicate any outliers. Asterisks (*) represent groups that are significantly different from the control and DMSO-treated groups (p value < 0.05; ANOVA, post hoc analysis). Numbers of embryos in each group (n) are provided below each boxplot.

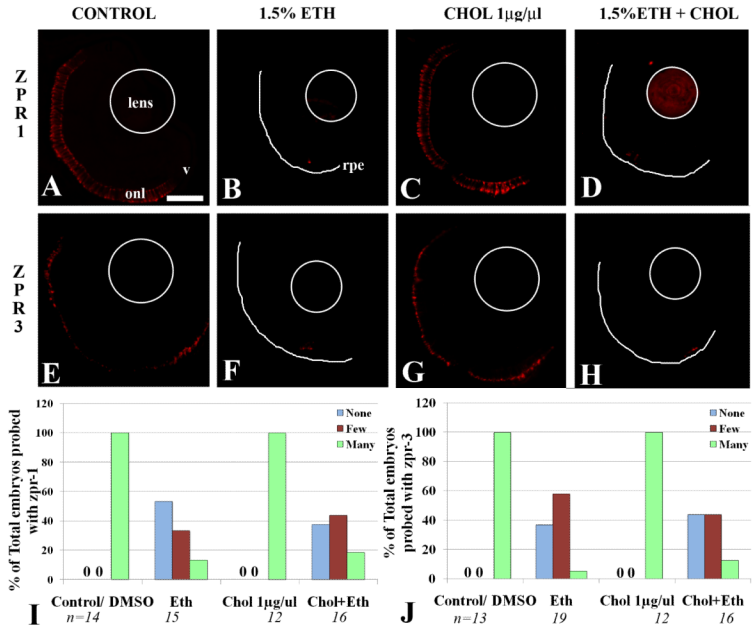

Fig. 9.

No recovery/rescue of photoreceptors with cholesterol. Panels **A–H** show representative photographs of retinas of embryos that were untreated (**A, E**), ethanol exposed (**B, F**), cholesterol treated (**C, G**), and co-treated with cholesterol and ethanol (**D, H**), processed for zpr-1 (**A–D**) and zpr-3 (**E–H**) indio rect immunofluorescence at 72 hours postfertilization (hpf). White open circles indicate lenses in the sections and white semicircles indicate the position of retinal pigmented epithelium (rpe). Scale bar in **A** (applied to **A–H**) = 50 μm. Panels **I** and **J** show the extent of zpr-1 (**I**) and zpr-3 (**J**) expression at 72 hpf. Methods for scoring are as in Fig. 4 (see Materials and Methods). onl, outer nuclear layer; v, ventral.

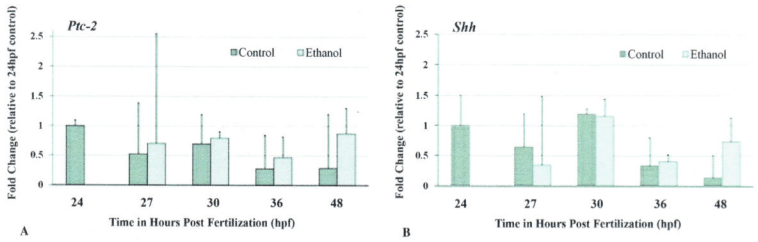

Fig. 10.

No change in *ptc-2* and shh gene expression in ethanol-treated eyes. Relative expression of *ptc-2* (**A**) and shh (**B**) was determined using qRT-PCR with *β*-actin as the endogenous control in eye-specific RNA of embryos at different stages of development (24–48 hpf), in both control and ethanol-treated embryos. Fold changes in expression level are relative to the level at 24 hpf, error bars represent SEM.

Table 1

Tissue Ethanol Concentrations in Embryos Exposed to 1.5% Ethanol at 24 hpf

	Tissue ethanol concentration	
Time of assay (hpf)	mM	g/ dl
25	35.6 ± 16.4	0.16 ± 0.075
48	21.4 ± 4.0	0.098 ± 0.018

hpf, hours postfertilization.