Catalase and Peroxiredoxin 5 Protect Xenopus Embryos against Alcohol-Induced Ocular Anomalies

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Purpose. To study the molecular mechanisms underlying alcohol-induced ocular anomalies in *Xenopus* embryos.

Methods. *Xenopus* embryos were exposed to various concentrations (0.1%–0.5%) of alcohol, and the subsequent effects in eye development and in eye marker gene expression were determined. To investigate the role of reactive oxygen species (ROS) and reactive nitrogen species (RNS) in fetal alcohol syndrome (FAS)–associated ocular injury, two antioxidant enzymes, catalase and peroxiredoxin 5, were overexpressed in the two blastomeres of the two-cell stage *Xenopus* embryos.

Results. Exposure of *Xenopus* embryos to alcohol during eye development produced marked gross ocular anomalies, including microphthalmia, incomplete closure of the choroid fissure, and malformation of the retina in 40% of the eyes examined. In parallel, alcohol (0.1%–0.5%) dose dependently and significantly reduced the expression of several eye marker genes, of which *TBX5*, *VAX2*, and *Pax6* were the most vulnerable. Overexpression of catalase and of cytosolic and mitochondrial peroxiredoxin 5 restored the expression of these alcohol-sensitive eye markers and significantly decreased the frequency of ocular malformation from 39% to 21%, 19%, and 13% respectively. All these enzymes reduced alcohol-induced ROS production, but only peroxiredoxin 5 inhibited RNS formation in the alcohol-treated embryos.

Conclusions. The results suggest that oxidative and nitrosative stresses both contribute to alcohol-induced fetal ocular injury. (*Invest Ophthalmol Vis Sci.* 2004;45:23–29) DOI:10.1167/iovs.03-0550

The eye is a sensitive indicator of the adverse effects of environmental agents. Up to 90% of children with prenatal exposure to alcohol may show ocular manifestations. All parts of the eye can be affected, starting from the periocular facial region to the intraocular structures and the optic nerve. Besides microphthalmia, several external eye signs have been described as part of the syndrome, such as small palpebral fissure, microcornea, strabismus, myopia, astigmatism, and optic nerve hypoplasia.[1–4] Optic nerve hypoplasia, due to a defect in the development of retinal ganglion cells, was found in half of the patients diagnosed with fetal alcohol syndrome (FAS).[5] Experimental models of FAS, including mouse,[6–9] chicken,[10] rat,[11] and zebrafish[12–13] embryos, have enhanced our understanding of the detrimental effects of alcohol in the developing visual system. It has been shown that exposure to alcohol often results in FAS-associated ocular anomalies including retinal folding, various degrees of ocular disorganization,[7,8] disturbances in the normal patterns of recruitment, and loss of neural progenitor cells in the developing retina.[9] A single injection of alcohol in the chicken vitelline sac at the beginning of retinal cell differentiation has been shown to retard synaptogenesis in the inner plexiform layer and to produce abundant ganglion cell death and a sharp diminution of myelinated optic axons.[10] Prenatal exposure to alcohol also leads to long-term deficits in retinal sensitivity, amplitude, light and dark adaptation, temporal processing, and excitability in adult rats.[11]

Despite a decade's research, the mechanisms of alcohol damage to the eyes remain poorly understood, partly because of the difficulty in establishing a valid dose–response level for alcohol and the coexistence of unknown environmental factors specific to the experimental animals used. In the present study, we used *Xenopus laevis* as a model to study the molecular basis of alcohol-induced ocular defects. Using this model, we found that exposure to alcohol (0.1%–0.5%) dose dependently produced eye defects and reduced the expression of multiple genes that regulate the eye development,[14] including *TBX5*, *VAX2*, and *Pax6*. The effect of alcohol on eye morphology was most severe during the developmental period when the eye forms (stages 15–25). Alcohol-exposed tadpoles showed defects in optical and retinal structures comparable to those observed in humans and in rodents. In adult mammals, ingested alcohol is rapidly absorbed through the digestive tract into the bloodstream and readily passes from the mother to the fetus. In our *Xenopus* embryo model, alcohol is also rapidly taken up from the medium, and the level in the embryo may reach approximately 80% of that in the medium (Peng Y, et al., unpublished results, 2003). Therefore, the concentrations of alcohol (0.1%–0.5%) used in this study are similar to those used in the mouse and rat models and to those exposed to human fetuses with FAS.

Alcohol has been shown to induce oxidative stress with the formation of free radicals and derangement of antioxidant defense in the cells of the developing eye.[15,16] However, whether oxidative stress directly contributes to ocular defects has not been demonstrated. In this study, we showed that overexpression of the antioxidant enzyme catalase and both human cytosolic/peroxisomal and mitochondrial peroxiredoxin 5 (cpPRDX5 and mtPRDX5, respectively) offers significant protection against alcohol-induced ocular anomalies. Of these enzymes, mtPRDX5 was the most effective. We further demonstrated that catalase and both forms of PRDX5 attenuate formation of reactive oxygen species (ROS); however, only PRDX5 was found to inhibit formation of reactive nitrogen species (RNS) in alcohol-treated *Xenopus* embryos. The stronger protection provided by PRDX5 over catalase suggests that,

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in addition to oxidative stress, nitrosative stress also plays a key role in ocular malformation in FAS. Therefore, combined antioxidative and antinitrosative therapy may become an effective method to prevent ocular anomalies and other developmental defects in FAS.

Materials and Methods

Plasmids Construction

The mammalian vectors expressing full-length cDNAs of human catalase, mtPRDX5, and cpPRDX5 (kind gift of Bernard Knoops, Université Catholique de Louvain, Belgium) were constructed from the pCMS-EGFP vector (Clontech, Palo Alto, CA), with the target genes expressed under the control of the CMV promoter and EGFP expressed under the control of a separate SV40-promoter. The human PRDX5 cDNA[17] (GenBank accession number: NM_012094; http://www.ncbi.nlm.nih.gov/Genbank; provided in the public domain by the National Center for Biotechnology Information, Bethesda, MD) was PCR amplified with forward primer 5′-GGCCGT*GAATTC*GGTATGGGACTAGCTGGC-3′ (*Eco*RI site in italic) for mtPRDX5 or 5′-AGAGCC*GAATTC*GCCATGGCCCCAATCAAG-3′ (*Eco*RI site in italic) for cpPRDX5 and reverse primer 5′-GTGGCC*GCGGCCGC*GGTATGGGACTAGCTGGC-3′ (*Not*I site in italic). The PCR products were digested with *Eco*RI and *Not*I and ligated into pCMS-EGFP. The insert coding for catalase, the mitochondrial form of human PRDX5 (long form of the protein with its mitochondrial presequence) or the cytosolic/peroxisomal form (short form without the mitochondrial presequence) was sequenced on an automatic DNA sequencer (model 377; Applied Biosystems, Foster City, CA). The resultant vectors were used to transform the *Escherichia coli* TOP10 strain (Invitrogen, San Diego, CA). The expression plasmid vector containing human catalase or human PRDX5 cDNA was purified from transformed bacteria and used for injection into blastomeres. The empty vector pCMS-EGFP was used as the control plasmid.

Embryo Collection and Experimental Conditions

Xenopus laevis embryos were obtained by in vitro fertilization, as previously described.[18] Developmental stages of embryos were determined using the criteria of Nieuwkoop and Faber.[19] All procedures relating to the animal use were performed according to the ARVO Statement for the Use of Animals in Ophthalmic and Vision Research. In each experiment, embryos at the two-cell stage were collected, and each of the two blastomeres was microinjected with 50 or 100 pg of the expression plasmid for catalase, cpPRDX5 or mtPRDX5. After reaching stage 15, the embryos were divided into groups, each consisting of 20 to 25 embryos, which were then incubated in 10 mL of 30% modified Marc's Ringer (MMR), in the absence or presence of varied concentrations of alcohol until the control group reached stage 25. Because alcohol is volatile, the experimental media were replaced with fresh media every 4 hours during the course of the alcohol treatment, to minimize fluctuations in alcohol concentration. At the end of the alcohol treatment, embryos were transferred to 10 mL of 30% MMR and allowed to grow until the control group reached the tadpole stage (stage 45). The tadpoles were then fixed and examined. Each experiment was repeated at least once with reproducible results.

Morphology of the Defective Eyes

The stage-42 or -45 tadpoles were fixed in 2% paraformaldehyde in 30% MMR. The morphology of the eyes was examined by light microscopy. Photographs were taken at a fixed magnification for the measurement of eye diameter and the examination of eye morphology. A defective eye is defined as one that has an incomplete closure of the choroid fissure.

Animal Cap Explant Assay for the Detection of Molecular Markers

Animal caps were dissected from embryos at stage 8.5 and cultured in 67% Leibovitz's L-15 medium (pH 7.5), in the presence or absence of various concentrations of alcohol until stage 25. Total RNA was isolated from animal caps or whole embryos using extraction reagent (TRIzol; Invitrogen) in accordance with the manufacturer's recommended procedures. The total RNA was reverse transcribed using a commercial system (Superscript; Invitrogen), and the mRNA of the target gene was amplified by PCR. Primers used for PCR were: xPax6 (forward [F]: 5′-CAGAACATCTTTTACCCAGGA-3′ and reverse [R]: 5′-ACT-ACTAGGCGA-3′); xBMP-4 (F: 5′-GCATGTACGGATAAGTCGATC-3′ and R: 5′-GATCTCAGACTCAACGGCAC-3′); xVent-1 (F: 5′-TTCCCTTCAGCATGGTTCAAC-3′ and R: 5′-GCATCTCCTTGGCATATTTGG-3′); xTBX3 (F: 5′-GTCCAAAGTAGTGAACTTCCAT-3′ and R: 5′-ACTGCAATGAATTCTGTCTCA-3′); xTBX5 (F: 5′-AACAAATGGTCAGTCACAGG-3′ and R: 5′-TGGACATCCGGTGTAGCTC-3′); xVAX2 (F: 5′-TAGCAGCCTCATCTTCTCC-3′ and R: 5′-CAGATTTAGACAGACACCTTCC-3′); and EF-1α (F: 5′-CAGATTGGTGCTGGATATGC-3′ and R: 5′-ACTGCCTTGATGACTCCTAG-3′).

Histologic Analysis

For histologic analysis, the control and alcohol-treated embryos were fixed in 2% paraformaldehyde, dehydrated, and embedded in paraffin. The embryos were then sectioned coronally through the head and stained with hematoxylin and eosin.

Western Blot Analysis

Stage-25 embryos were lysed with cold PBS with 2 mM phenylmethylsulfonyl fluoride by pipetting. Lysates were resolved on 12.5% SDS-PAGE gels and transferred to polyvinylidene difluoride membranes (Immobilon; Millipore, Bedford, MA). Membranes were probed with a polyclonal antibody against human catalase (Calbiochem, La Jolla, CA) or a polyclonal anti-PRDX5 antibody[20] and visualized by chemiluminescence reagent (ECL+Plus; Amersham, Arlington Heights, IL).

ROS and RNS Measurements

The hydrogen peroxide content was determined by the colorimetric quantitation of hydrogen peroxide (Bioxytech H_2O_2-560 kit; Oxis International, Portland, OR). The nitric oxide production was determined by the spectrophotometric measurement of total nitrite produced in the in vitro experimental system using the nitric oxide assay kit (Colorimetric; Calbiochem). The assays were conducted in accordance with the protocols provided by the manufacturers.

Statistical Analysis

Significant differences were determined by using an unpaired Student's *t*-test. For multiple comparisons, one-way ANOVA with multiple comparisons was used. $P < 0.05$ was considered statistically significant.

Results

Effect of Exposure to Alcohol during Eye Development in Tadpoles

Studies of zebrafish have shown that the damaging effects of alcohol on visual structure and function are most pronounced when exposure occurs during eye development.[12,13] We therefore investigated the window of time when eye development in *Xenopus* embryos is susceptible to alcohol. In *Xenopus*, the presumptive eye anlagen begins in stage 15. At stage 22, primary eye vesicles make contact with the epidermis; at stage 25, primary eye vesicles are fully developed.[16] Therefore, we exposed embryos to 0.5% alcohol during stages 6 to 14, 15 to 25, and 26 to 35. The tadpoles were harvested and fixed when the control group reached stage 45, and the morphology of the eye was examined. Consistent with the results from zebrafish studies, the most severe defect was observed when *Xenopus* embryos were exposed to alcohol during stages 15 to 25, as determined by the higher frequency of defective eyes (Table 1). The effect of alcohol was also dose dependent and at 0.5% alcohol concentration, exposure to alcohol from stages 15 to

TABLE 1. Effects of Ethanol Dose on the Vitality and Morphology of the Tadpoles

Ethanol Concentration (%)	Exposure Time	Number of Embryos (Live/Dead)	Number of eyes Defective/Total* (% of Defective Eyes)
0.00	NA	38/2	0/76 (0)
0.50	Stage 6–14	36/4	4/72 (6)
0.50	Stage 15–25	38/2	30/76 (40)
0.50	Stage 26–35	38/2	4/76 (5)
0.00	NA	38/2	0/76 (0)
0.25	Stage 15–25	36/4	8/72 (11)
0.50	Stage 15–25	35/5	26/70 (37)

Data are combined from two separate experiments.
* Eye defects of the stage-45 live tadpoles were determined visually.

25 produced marked gross eye defects in 37% to 40% of the surviving tadpoles (Table 1).

A 0.5% exposure to alcohol produced ocular abnormalities that were easily identified under the microscope. Morphologically, the most prominent eye defect is the disappearance of the pigmented layer at the ventral site, as shown by an incomplete closure of the choroid fissure (Figs. 1A, 1B). Compared with the normal eye (Figs. 1A, 1B, left panels), an increase in the alcohol concentration from 0.3% to 0.5% produced tadpoles with ocular anomalies of increasing severity (Figs. 1A, 1B, middle and right panels). Histologic analysis of the eye sections revealed that the tadpoles with morphologically normal eyes had complete closure of the choroid fissure and distinct retinal layers (Fig. 1B, bottom left). In contrast, the histology of the defective eyes exhibited an absence of the ventral quadrant of the choroid and a severely distorted retinal structure. In 0.5% alcohol–treated embryos, the retinal layers were not clearly formed, the neural retina was detached from the eye wall, and multiple folds were observed (Fig. 1B, bottom right). All these congenital eye defects would severely impair visual functions.

Effect of Exposure to Alcohol on the Expression of Several Ocular Developmental Genes

To gain a better understanding of how alcohol affects the development of the eye, we also used an animal cap assay to further examine the effect of alcohol on the expression of genes that regulate eye development. The animal cap comprises pluripotent cells that can be induced to form endodermal, mesodermal, or ectodermal cell types and is a useful tissue to assess the activity of various inducing factors. Consistent with the eye morphologic data, alcohol dose-dependently and specifically inhibited the expression of several genes, such as *TBX5*, *VAX2*, and *Pax6*, which are essential for eye development (Fig. 2). At a concentration as low as 0.1% (21 mM), alcohol reduced *TBX5*, *VAX2*, and *Pax6* gene expression by 25%, 20%, and 10%, respectively. At 0.3% alcohol, *Pax6*, *TBX5*, and *VAX2* gene expression was reduced by 90%, 65%, and 45%, respectively. Finally, at 0.5% alcohol, *Pax6*, *TBX3*, *TBX5*, and *VAX2* gene expression was almost completely suppressed. In contrast, the expression of *TBX3* was only modestly affected and that of *Vent1* and *BMP4* was not affected. The range of alcohol concentrations (0.1%–0.5%) used was comparable to the levels found in the blood of modest and heavy drinkers. These results support the notion that alcohol mediates ocular defects by suppressing the expression of multiple and specific key ocular developmental genes.

Effect of Overexpression of Catalase- or PRDX5-Protected Embryos on Alcohol-Induced Ocular Abnormalities

We chose to overexpress antioxidant enzymes to investigate the role of ROS and RNS in FAS-associated ocular injury. We injected 50 pg of plasmids encoding catalase or the cytosolic-peroxisomal and mitochondrial forms of PRDX5 into the two blastomeres of the two-cell stage embryos. The embryos were

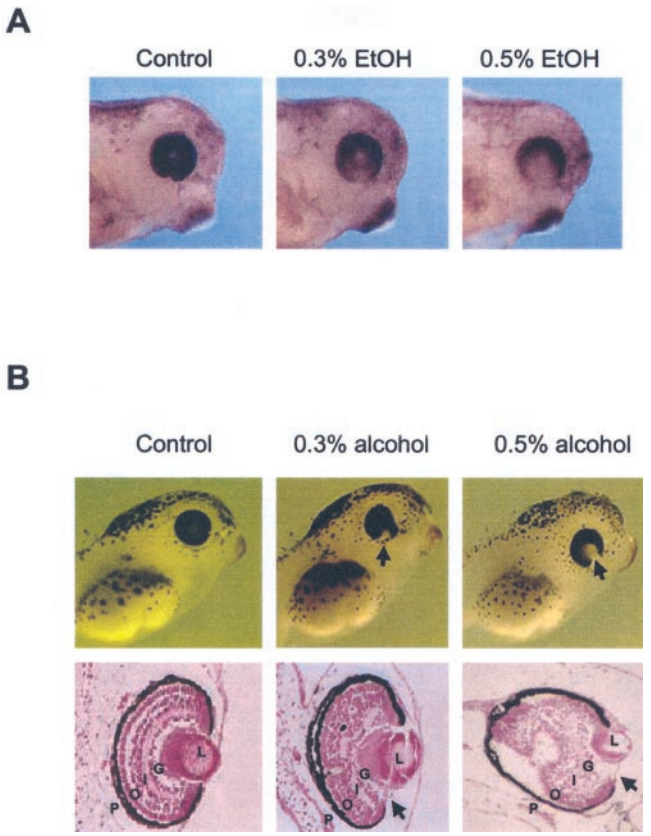

FIGURE 1. Alcohol-induced ocular anomalies. (**A**) The eye morphology of stage-42 *Xenopus* embryos which had been incubated with either 0.3% or 0.5% alcohol, as indicated. Alcohol dose dependently produced severe eye defects in the treated embryos as shown by an incomplete closure of the choroid fissure. (**B**) *Top*: representative pictures showing the eye morphology of the control (*left*), 0.3% alcohol-treated (*middle*), and 0.5% alcohol-treated tadpoles (*right*) at stage 45. *Arrow*: incomplete closure of the choroid fissure. *Bottom*: the corresponding hematoxylin and eosin-stained sections from the eyes of the control (*left*), 0.3% alcohol-treated (*middle*), and 0.5% alcohol-treated tadpoles (*right*). For the control tadpole, the eye had distinct retinal pigment layers and a fully developed choroid tissue. In contrast, the alcohol-treated tadpoles had moderate (*middle*) to severe (*right*) damages in the different layers of eye tissues manifested by an incomplete closure of the choroid fissure at the ventral site (*arrow*) and a multiple folding in the retinal layer. G, retinal ganglion cells; I, inner plexiform layer; L, lens; O, outer plexiform layer; P, retinal pigment epithelia.

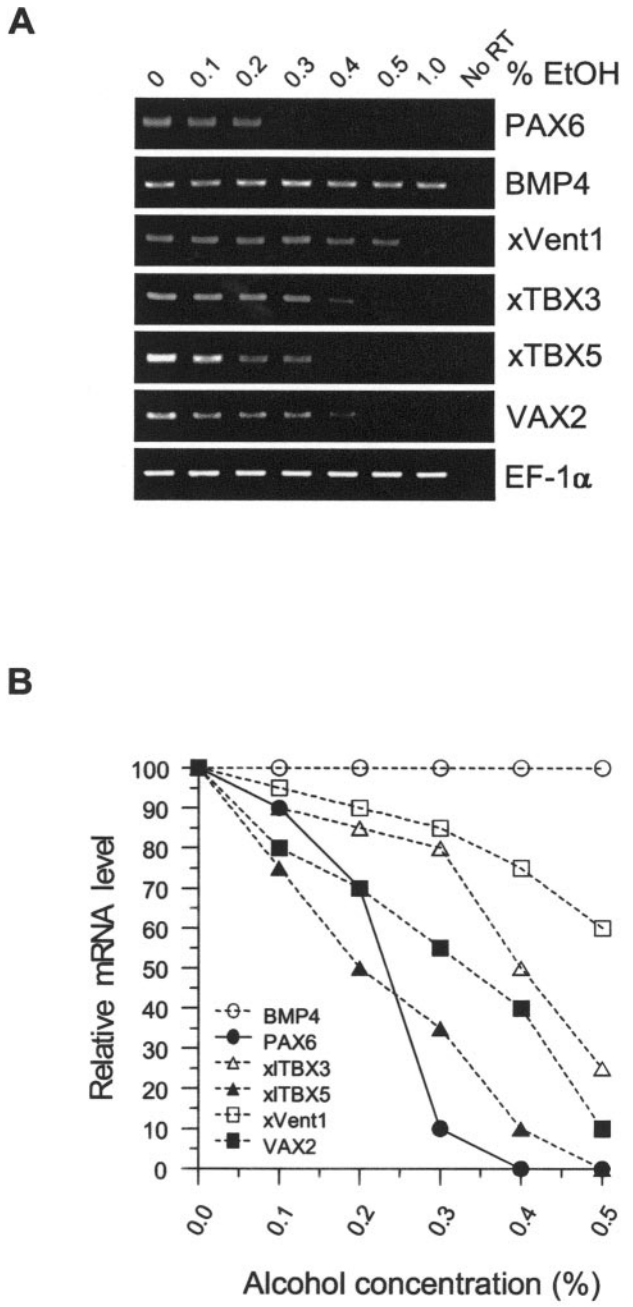

FIGURE 2. Alcohol downregulates the expression of several key ocular development genes. (**A**) RT-PCR analysis of the eye molecular markers in animal caps treated with 0%, 0.1%, 0.2%, 0.3%, 0.4%, and 0.5% alcohol. (**B**) The relative mRNA levels of the ocular molecular markers were determined by comparing the quantity of the amplified RT-PCR products using densitometry.

treated with either the control medium or 0.5% alcohol–containing medium from stage 15 to 25. After that, embryos were maintained in normal medium until the control tadpoles reached stage 45, and the eye morphology was examined. To confirm the expressions of catalase and peroxiredoxins, embryos were harvested at stage 25 and the expression of catalase, cpPRDX5, and mtPRDX5 were confirmed by fluorescence microscopy (Fig. 3A) and Western blot analysis (Figs. 3B, 3C). The in vivo production of H_2O_2 and nitrite was also measured (Fig. 3D). Our results showed that alcohol significantly increased in vivo H_2O_2 and nitrite production. Catalase effectively abrogated the increase in H_2O_2, but only PRDX5 inhibited alcohol-induced nitrite formation. Among the two forms of PRDX5, mtPRDX5 was more potent and reduced the alcohol-induced nitrite production by approximately 50%, reaching normal levels without alcohol treatment.

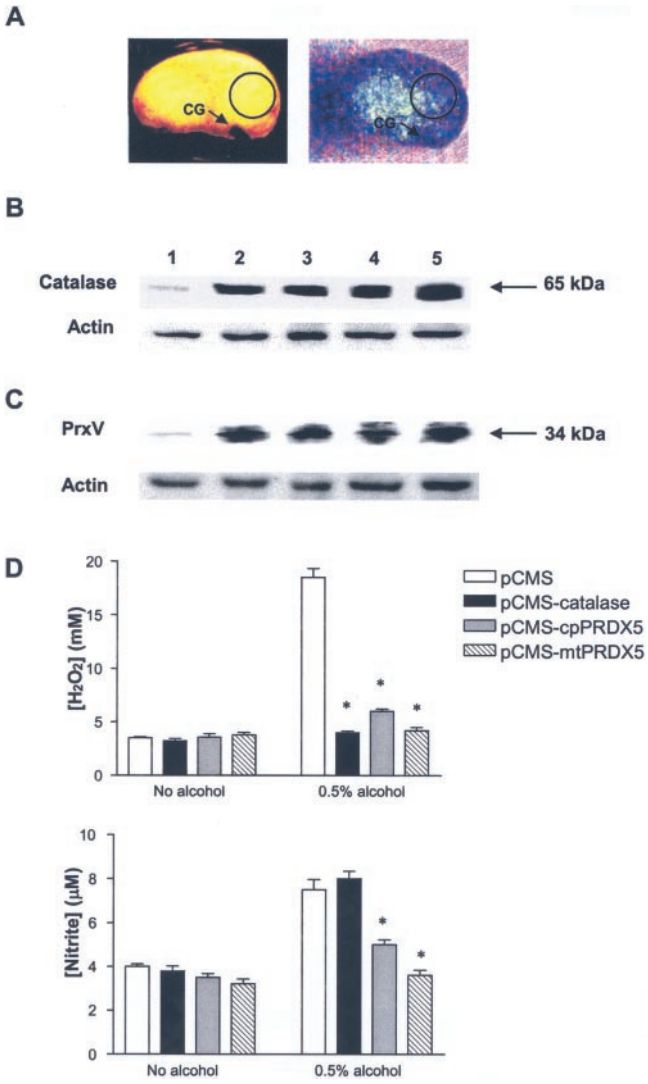

FIGURE 3. Overexpression of human catalase and PRDX5 in *Xenopus* embryos. In each experiment, embryos were microinjected with 50 or 100 pg of pCMS, pCMS-catalase, pCMS-cpPRDX5, or pCMS-mtPRDX5 expression plasmid at the two-cell stage and treated with 0.5% alcohol from stage 15 until stage 25. At stage 25, embryos were harvested for detection of enhanced green fluorescent protein (EGFP), Western immunoblot analysis, and the measurement of ROS and RNS. (**A**) Distribution of the injected pCMS-catalase plasmid, pCMS-cpPRDX5, or pCMS-mtPRDX5 as indicated by EGFP fluorescence. *Left*: light micrograph; *right*: fluorescence micrograph. *Arrows*: position of the cement gland (CG); *circles*: region that is destined to be the eye. (**B**) Expression of human catalase in *Xenopus* embryos. *Lane 1*: embryos injected with the control pCMS vector; *lanes 2* and *3*: embryos injected with 50 pg of pCMS-catalase plasmid; *lanes 4* and *5*: embryos injected with 100 pg of pCMS-catalase plasmid. (**C**) Expression of cpPRDX5 and mtPRDX5 in *Xenopus* embryos. *Lane 1*: embryos injected with the control pCMS vector; *lanes 2* and *3*: embryos injected with 50 and 100 pg of pCMS-cpPRDX5 plasmid, respectively; *lanes 4* and *5*: embryos injected with 50 and 100 pg of pCMS-mtPRDX5 plasmid respectively. (**D**) Quantitation of ROS (*top*) and RNS (*bottom*) in the control (no alcohol) and 0.5% alcohol-treated *Xenopus* embryos. The embryos were first injected with pCMS, pCMS-catalase, pCMS-cpPRDX5, or pCMS-mtPRDX5 expression plasmid, and the subsequent effects on H_2O_2 and NO formation in vivo were determined. *Significant differences ($P < 0.05$) from the control pCMS vector–injected group.

TABLE 2. Effect of Catalase and PRDX5 on Alcohol-Induced Eye Deformation

Injection	0.5% Alcohol	Number of Embryos (Live/dead)	Number of Eyes Defective/Total (% of Defective Eyes)
Control plasmid	+	46/4	36/92 (39)
cpPRDX5	+	45/5	17/90 (19)
mtPRDX5	+	43/7	11/86 (13)
Catalase	+	42/8	18/84 (21)
Control plasmid	−	46/4	4/92 (4)

Data are combined from two independent experiments, each consisting of 25 embryos.

The eye morphology of both catalase and PRDX5-injected embryos was also analyzed in stage-45 tadpoles and the data from two separate experiments are combined and presented in Table 2. The injection of catalase and both cpPRDX5 and mtPRDX5 significantly reduced the rate of eye defect from 39% to 21%, 19%, and 13%, respectively. The morphology of normal eyes resembled that shown in Figure 1A (left) or Figure 1B (top left). The histology of normal eyes displayed a normal eye wall, complete closure of the choroid fissure, and distinct retinal layers similar to those shown in Figure 1B (bottom left). Tadpoles with defective eyes had morphology similar to that illustrated in Figure 1A (right) or Figure 1B (top right), and histology similar to Figure 1B (bottom right).

To demonstrate that it is the local concentration of catalase or PRDX5 that protects the embryos from alcohol-induced eye defects, we performed another experiment in which one blastomere of the two-cell stage embryos was injected with the expression plasmid for catalase or cpPRDX5, whereas the remaining blastomere was injected with the expression plasmid for β-gal as a control. As a result, the expression of either catalase or cpPRDX5 would be targeted to only one side of the injected embryos. After injection, these embryos were exposed to 0.5% alcohol from stages 15 to 25, and the resultant phenotypes were analyzed at stage 45. In one representative tadpole (Fig. 4), the side injected with the expression plasmid for catalase or cpPRDX5 appeared normal (Fig. 4A), whereas the β-gal–injected side showed defective eye formation (Fig. 4B). The frequency of defective eye formation in the catalase- or cpPRDX5-injected side was reduced from approximately 40% to 20% (Table 3). The expression of catalase or cpPRDX5 in one side of the embryo was confirmed by the EGFP fluorescence (Fig. 4C). Together, these results suggest that a local increase in the level of catalase or PRDX5 protects the alcohol-treated embryos from ocular defects by reducing the levels of ROS and RNS production.

Effect of Expression of Catalase or PRDX5 on Alcohol-Induced Downregulation of Key Ocular Development Genes

To test whether expression of catalase or PRDX5 is able to restore the expression of the alcohol-sensitive eye markers, we microinjected *Xenopus* embryos at the two-cell stage with the expression plasmid for catalase or mtPRDX5, and then used an animal cap assay to analyze the effect on the expression of various ocular developmental markers (Fig. 5). We found that expression of catalase or mtPRDX5 prevented the alcohol-induced downregulation of *Pax6*, *TBX3*, *TBX5*, and *VAX2*. Thus, our data imply that catalase and PRDX5 offer protection to alcohol-induced eye defects by restoring the expression of specific ocular developmental genes.

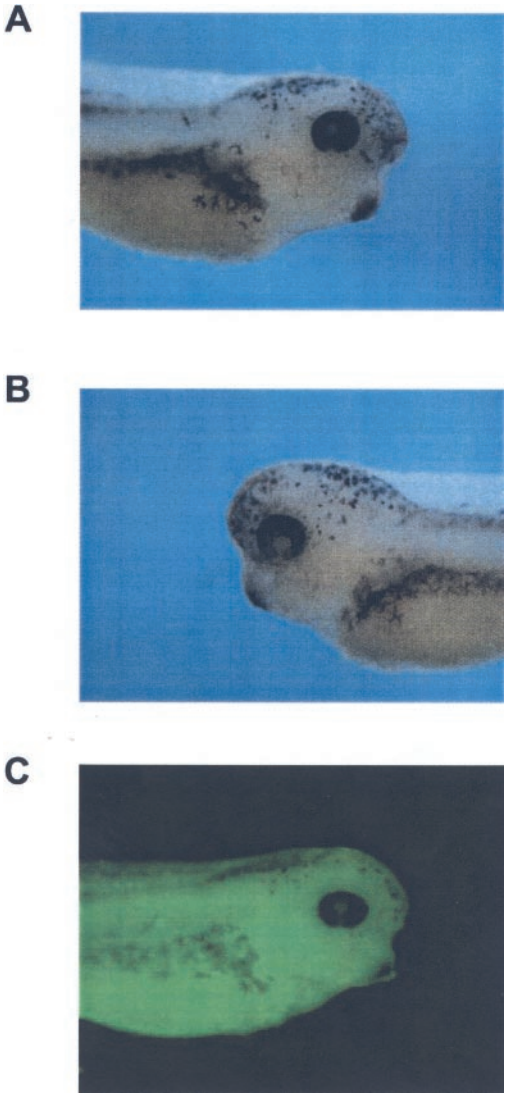

FIGURE 4. Protection offered by expressing PRDX5 in one side of the *Xenopus* embryos. A representative alcohol-treated tadpole showing (**A**) the pCMS-PRDX5–injected side with a normal eye and (**B**) the pCMS-β-gal injected side, which displayed a defective eye with incomplete closure of choroid fissure. (**C**) Expression of enhanced green fluorescent protein (EGFP) on the side of the embryo injected with the pCMS-cpPRDX5 plasmid.

DISCUSSION

In this report, we describe a *Xenopus* model of alcohol-associated ocular abnormalities. The relevance and applicability of this model to human FAS are suggested by the concentrations

TABLE 3. Effect of Local Expression of Catalase and PRDX5 on Alcohol-Induced Eye Deformation

Injection	Living Embryos (*n*)	No. of Eyes (Defective/Total) Cat or PRDX Side; β-gal Injected Side (%)
Catalase/β-gal	44	8/44 (20.5); 19/44 (43.2)
cpPRDX5/β-gal	40	7/40 (17.5); 16/40 (40)

Embryos were injected with β-gal in one cell and catalase or PRDX5 in the other cell of the two-cell stage embryos, exposed to 0.5% alcohol from stage 15 to stage 25, and grew until stage 45.

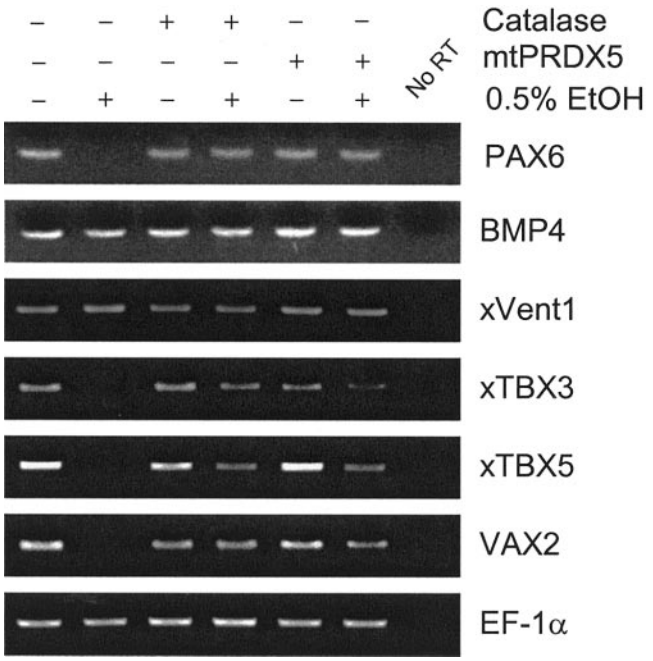

FIGURE 5. Catalase and PRDX5 restored alcohol-induced downregulation of eye markers. *Xenopus* embryos at the two-cell stage were microinjected with the pCMS-catalase or pCMS-mtPRDX5 expression plasmid, and RT-PCR analysis of various eye marker genes was performed in animal caps, with or without 0.5% alcohol treatment.

of alcohol (0.1%–0.5%) that can produce the ocular injuries. In addition to the observed eye defects, other FAS-associated phenotypes, such as abnormal smallness of the head (microcephaly) and growth retardation were observed in the 0.5% alcohol-treated embryos (data not shown). In fact, FAS-like features including craniofacial malformations, reduced body size, and hypoplasticity of the anterior end of the body around the mouth have been documented in an early study on *Xenopus* embryos by Nakatsuji,[21] but higher concentrations (1%–2%) of alcohol were used, and no description on any alcohol-induced eye defect was reported at that time. In our study, we showed for the first time that oxidative and nitrosative stresses contribute to the ophthalmic abnormalities. The causative roles of ROS and RNS in the alcohol-induced eye defects were supported by the observations that (1) 0.5% alcohol significantly augmented the levels of H_2O_2 and nitrite in vivo; (2) expression of catalase or PRDX5 prevented the alcohol-induced downregulation of various genes essential in eye development; and (3) overexpression of antioxidant enzymes, catalase, and PRDX5, reduced the frequency of defective eyes with deformed retina and incomplete closure of the choroid fissure.

It has been suggested that alcohol promotes oxidative and nitrosative stresses to the tissues by increasing the production ROS and RNS.[22–24] ROS encompasses oxygen radicals such as superoxide ($\dot{y}O_2^-$) and hydroxyl ($\dot{y}OH$), and nonradicals, such as hydrogen peroxide (H_2O_2) and ozone (O_3), whereas RNS includes a number of highly reactive nitrogen-containing radicals and nonradicals, such as nitric oxide ($\dot{y}NO$), nitrogen dioxide ($\dot{y}NO_2$), nitrous acid (HNO_2), and peroxynitrite ($ONOO^-$). Both ROS and RNS are powerful oxidizing agents. When present in excess, these reactive species can overwhelm the cellular antioxidant defense mechanisms, leading to cell apoptosis, chromosome aberration, lipid peroxidation, and protein damage.[25] All these detrimental effects could lead to the observed morphologic and histologic abnormalities in the alcohol-treated *Xenopus* embryos.

Our findings open up a new possibility for the use of catalase and PRDX5 as therapeutics to protect embryos against alcohol-induced eye defects. Although both catalase and PRDX5 catalyzed the breakdown of peroxides, it is interesting to note that mtPRDX5 was more effective than catalase and cpPRDX5 in lowering the levels of H_2O_2 and NO, and reducing the frequency of defective eye formation. PRDX5 is a recently cloned member of the PRDX family of peroxidases.[17] It is a thioredoxin peroxidase that reduces hydrogen peroxides and hydroperoxides. Compared with other PRDXs, PRDX5 is unique because it may be targeted intracellularly to mitochondria, peroxisomes, or cytosol. With reference to the phenotypes of the alcohol-treated embryos, mtPRDX5 may offer protection against the eye defects in two ways. First, because ROS such as O_2^- and H_2O_2 are produced by mitochondria during respiration, mtPRDX5 may quench the damaging effects of these ROS by breaking them down quickly once they are formed within the mitochondria. Second, because mitochondria play a central role in cell apoptosis,[26] mtPRDX5 may help to maintain the mitochondrial membrane structure and/or block the signaling pathway leading to the apoptotic cell death of the retina. A recent study demonstrating that the loss of mitochondrial membrane permeability is crucial for the oxidative stress-induced apoptotic death of the retina photoreceptors may support this notion.[27]

Our data also suggest that alcohol may produce ocular injuries by suppressing several key genes important in eye development. Previous studies have shown that genes encoding 14 transcription factors, including specific HOX and PAX genes, are essential for normal eye development.[14] Furthermore, high levels of alcohol during gestation may disturb the general intrinsic mechanisms of gene expression and compromise the dynamics of ocular tissue development. Our results showing that alcohol specifically suppressed the expression of a PAX gene *Pax6*, a HOX gene *VAX2*, and a T-box gene TBX5, but not other ocular developmental genes such as *TBX3*, *Vent1*, *BMP4*, have raised the possibility that alcohol induces ocular injuries by downregulating a subset of ocular developmental genes.

Pax6, *VAX2*, and *TBX5* are key regulators for eye development, but their expression pattern and respective roles in the eye's developmental process are different. *Pax6* is considered as the master gene for eye development in both vertebrates and invertebrates. In the eye, *Pax*6 is primarily expressed in the presumptive retina and lens. Heterozygous loss-of-function mutations of the mouse *Pax6* gene result in the small eye and small lens phenotypes,[28] and Pax6 conditional mouse knockout leads to impaired retinogenesis.[29] *VAX2* is a homeobox transcription factor expressed in the ventral portion of the prospective neural retina in the developing vertebrate eyes.[30] It controls the patterning of the eye dorsoventral axis and is required for the proper closure of the optic fissure.[31] Mice with a null mutation of the *VAX2* gene display incomplete closure of the optic fissure, leading to eye coloboma.[32] Taken together, the phenotypes generated from mutations of *Pax6* or *VAX2* are generally in line with those observed in our *Xenopus* model.

TBX5 is expressed asymmetrically across the embryonic neural retina. As the retina matures, *TBX5* expression is restricted within the ganglion cell layer.[33] Misexpression of *TBX5* induces dorsalization of the ventral side of the eye and altered projections of retinal ganglion cell axons, suggesting that it is involved in eye morphogenesis and is a topographic determinant of the visual projections between retina and tectum.[34] In our morphologic and histologic data, we found no changes in the dorsal-ventral axis of the *Xenopus* eye, implying that the function of TBX5 in *Xenopus* eye development could be different. The establishment of *TBX5* knockout or knockdown mice is instrumental for uncovering the precise function of this gene in eye development.

In summary, we have established a *Xenopus* model to study alcohol-induced eye injury during embryonic development. Our results suggest for the first time that both oxidative and nitrosative stresses are responsible for the injuries. The information obtained from this study provides insights into the causative molecules and important signals controlling alcohol-mediated injury to ocular development. Furthermore, the *Xenopus* embryo, with its relatively short life cycle and easily distinguishable developmental stages, is an excellent model system for investigating the molecular basis of alcohol-induced disorders and for developing effective protective therapies.

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