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Original Article

Zebrafish methanol exposure causes patterning defects and suppressive cell proliferation in retina

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Abstract: Purpose: Methanol exposure have been shown to produce retinal abnormalities and visual dysfunctions in rodents and other mammals developing *in utero*. In this study, we characterized how methanol affects the retinal development in an *ex utero* embryonic system, the zebrafish. Methods: Zebrafish embryos were raised for 24 hours in fish water supplemented with various concentrations of methanol at 6 hours after fertilisation. The effects of methanol on retinal morphology were assessed by histologic and immunohistochemical analyses. Results: Zebrafish embryos exposed to moderate (3%) and high (4%) levels of methanol during early embryonic development had a small eye phenotype. Embryos exposed to high (4%) level of methanol had morphological abnormalities of the retinal pigment epithelia and the photoreceptors. Methanol exposure also caused inhibition of cell differentiation and proliferation in the retina at the early developmental stage. Conclusions: Low concentrations of methanol affect photoreceptor function but do not disturb retinal morphology. Higher levels of methanol exposure cause retinal patterning defects and a small eye phenotype.

Keywords: Zebrafish, retina, methanol, retinal differentiation, cell proliferation, small eye

Introduction

Methanol poisoning with its associated severe ocular and central nervous system toxicity is an important public health hazard and environmental concern worldwide. Acute and chronic methanol exposure have been shown to produce retinal dysfunction and optic nerve damage, both clinically in humans [1-3] and in experimental animal models [4-6]. Methanol is commonly used as an industrial organic solvent and is available to the public in a variety of products. It is also being developed as an alternative fuel and energy source [2]. The expanded use and availability of methanol increases the probability of accidental acute or chronic methanol exposure and underscores the importance of understanding the mechanisms responsible for its toxicity. Humans and nonhuman primates are uniquely sensitive to the toxic effects of methanol [2, 3]. Methanol poisoning in humans and monkeys is characterized by an initial mild central nervous system depression, followed by an asymptomatic latent period lasting about 12-24 h. The latent period is followed by a syndrome consisting of formic acidemia, uncompensated metabolic acidosis, visual toxicity, coma and, in extreme cases, death. Initial signs of visual toxicity include misty or cloudy vision, and ophthal moscopic examination typically reveals retinal and optic disc edema.

A rodent model of methanol toxicity was used to evaluate retinal dysfunction in methanol poisoning [5]. Seme and coworkers [7] examined the effects of exposure to methanol on rat electroretinograms (ERGs). Zebrafish has emerged as an important model organism for vertebrate development due to its easy maintenance, rapid extracorporeal development, transparent embryo, and availability of gene markers [8]. Therefore, we designed experiments to expose zebrafish embryoto methanol at varying concentrations 6 hours post fertilization (hpf) to 24 hpf. The 6-24 hpf exposure was used because

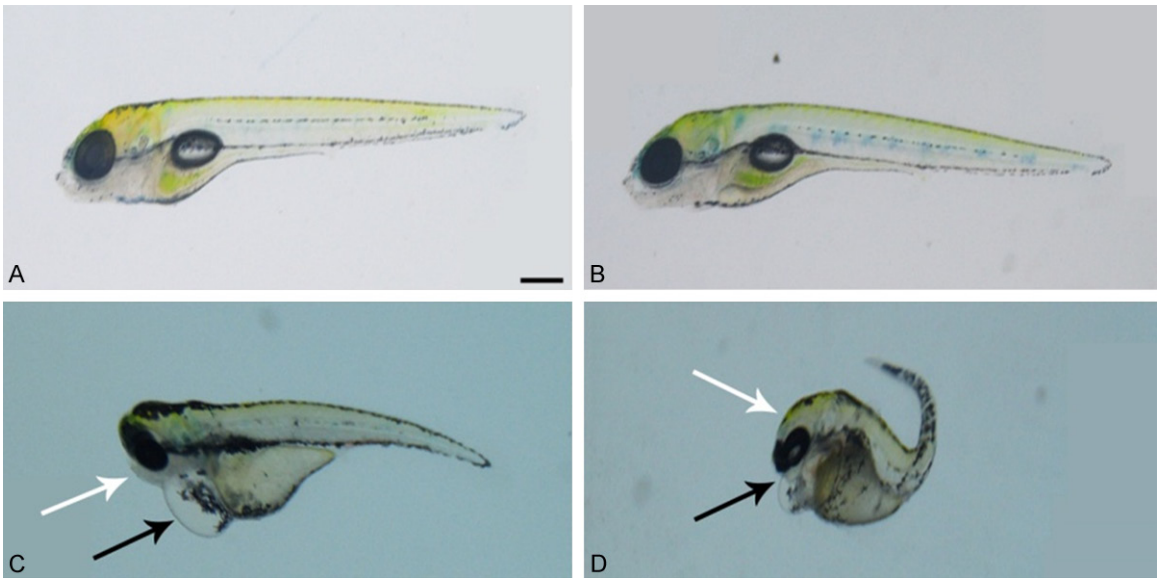

Figure 1. The effect of methanol treatment on the eye size of zebrafish. (A-D) Zebrafish embryos were raised in fish water (A) or fish water supplemented with 2% methanol (B), 3% methanol (C), or 4% methanol by volume from 6 hours postfertilization (hpf) through 24 hpf. (A, B) Treatment with low concentrations of methanol (2%) resulted in no observable morphological differences compared with untreated controls at 120 hpf. (C, D) Increasing the concentration of methanol treatment resulted in smaller eye size and body size, swollen hearts (black arrow, C), swollen guts (black arrow, D), rounded forebrain (white arrow, D) and irregular jaw (white arrow, C). Scale bar: (A-D) 20 μm.

this is the time period when zebrafish eye develops, and it was shown to have significant effects on eye diameter and the presence of abnormal morphological characteristics in zebrafish [9]. By 24 hpf, the eyecups are well-formed [10]. Moreover, in this study, we choose to emphasize how embryonic exposure to methanol influences zebrafish patterning, with particular regard to histological and immunohistochemical changes of retinas. These experiments were performed to explore the possible causes underlying the developmental toxicity of methanol on the visual function.

Materials and methods

Fish breeding and methanol treatment

The AB wild-type zebrafish were maintained in a 14-hr light and 10-hr dark cycle. All experimental procedures conformed to Zhejiang University standards for use and care of animals in research. Fertilized eggs were collected and placed in Petri dishes containing fish water (30% Danieau buffer) as an incubation medium, and left to develop for 6 hours post-fertilization at 28.5°C before adding methanol (Sigma) at varying concentrations (2%, 3% and 4% by volume). Embryos were raised in methanol-supplemented water from 6 to 24 hpf. At the completion of methanol treatment, the treated embryos were transferred to fresh methanol-free water. The fish water was changed on a daily basis. Dead embryos were discarded immediately whenever detected.

Histology

Fish larvae were fixed in 4% paraformaldehyde. For hematoxylin and eosin (HE) staining at 120 hpf, zebrafish were embedded in paraffin, and 3 μm thick transverse sections were prepared. Sections were deparaffinized, rehydrated through graded ethanol, and stained using standard protocols [11, 12]. In each group, ten animals were processed.

Immunohistochemistry

Larvae were fixed in 4% paraformaldehyde. For immunofluorescence examination at 36 hpf and 120 hpf, the embryos/larvae were cryoprotected with 20% sucrose in 0.1 mol/L phosphate-buffered saline (pH 7.2) and frozen in optimal cutting temperature compound (Sakura Finetek). Serial transverse cryosectioning at 8 μm thickness was performed, and immunohistochemistry analysis was performed using standard protocols [13]. The following cell type-specific markers were used: zpr1 antibody for

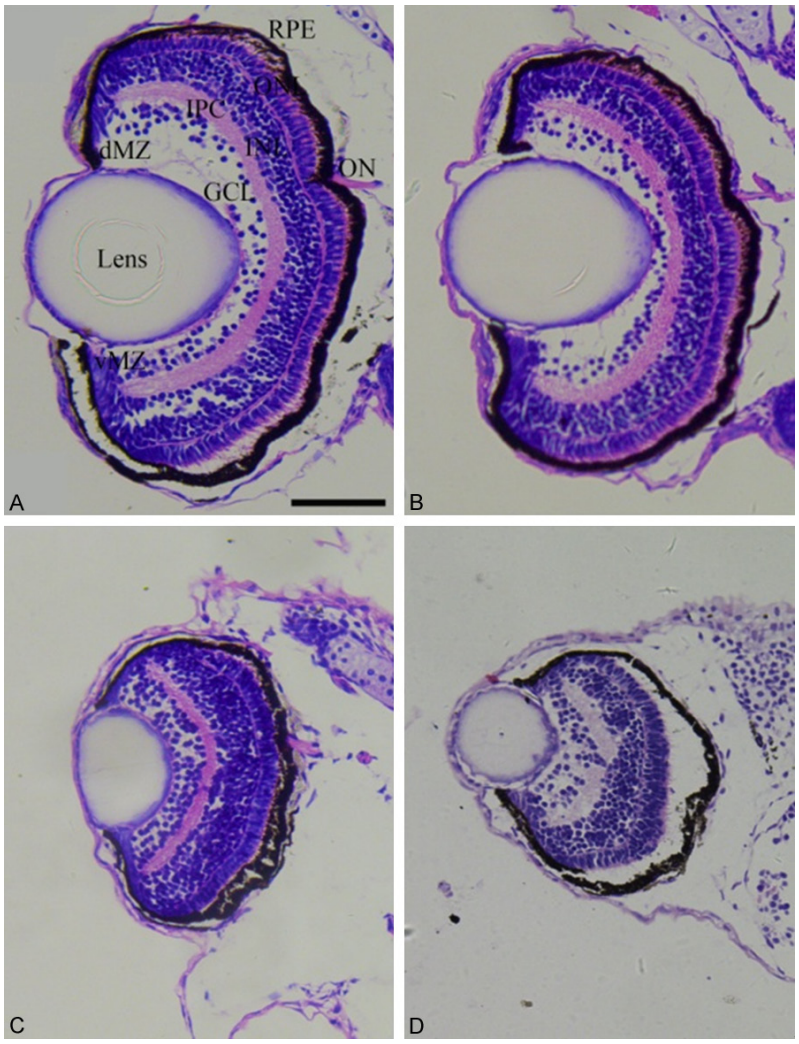

Figure 2. The effect of methanol treatment on the architecture and size of retina. (A-D) Zebrafish embryos were raised in fish water (A) or fish water supplemented with 2% methanol (B), 3% methanol (C), or 4% methanol (D) by volume from 6 hpf through 24 hpf. Increasing the concentration of methanol treatment resulted in smaller retinas, but lamination and cellular components were similar at 120 hpf. (D) At 120 hpf, the RPE had withdrawn completely from neural retina in 4% methanol-treated retinae. Labeled are the lens (Lens), optic nerve (ON), retinal pigment epithelia (RPE), outer nuclear layer (ONL), inner nuclear layer (INL), inner plexiform layer (IPL), dorsal marginal zone (dMZ), ventral marginal zone (vMZ), and ganglion cell layer (GCL). Scale bar: (A-D) 50 μm.

the Zpr1 antigen, which is specifically expressed in red/green double cones (ZIRC, 1:200 dilution); zn8 antibody against the Zn8 antigen that is expressed in retinal ganglion cells (ZIRC, 1:200 dilution); anti-phosphorylated-Histone H3 antibody for M-phase nuclei (Sigma, 1:200 dilution); anti-HuC/D for ganglion cells and amacrine cells (Invitrogen, 1:200 dilution); anti-Crb2a antibody (gift from JianZou, 1:200 dilution); rabbit anti-rhodopsin (gift from JianZou, 1:200 dilution) for rhodopsin; rabbit anti-red opsin (gift from JianZou, 1:200 dilution) for red opsin. The nuclei were stained with Dapi (Sigma, 1:200 dilution). Actin was visualized with Alexa Fluor 488-conjugated phalloidin (Invitrogen, 1:200 dilution). ZO-1 was visualized with monoclonal mouse anti-ZO-1 antibody (Invitrogen, 1:200 dilution).

Quantification of mitotic cells

Phospho-Histone H3 (pH3) is a marker of M-phase mitotic cells [14]. The number of pH3-positive cells in retinal area in wild-type and methanol-treated retinas were normalized. In brief, pH3-positive retinal cells in five sections from five larvae each at 120 hpf were counted. The retinal regions counted were contoured and measured with MetaMorph v6.1, and the average number of pH3-positive cells per 3,600 μm^2 of retinal area was calculated. Statistical significance of the differences between methanol-treated and wild-type was assessed with paired t-test.

Statistical analysis

All experiments were repeated at least thrice independently. All of the data are presented as the mean ± standard error of the mean. A paired t-test was performed to examine the difference between groups. A p-value <0.05 was considered statistically significant.

Results

Methanol treatment causes a small eye and body phenotype

External morphological changes in zebrafish larvae after methanol treatment were observed

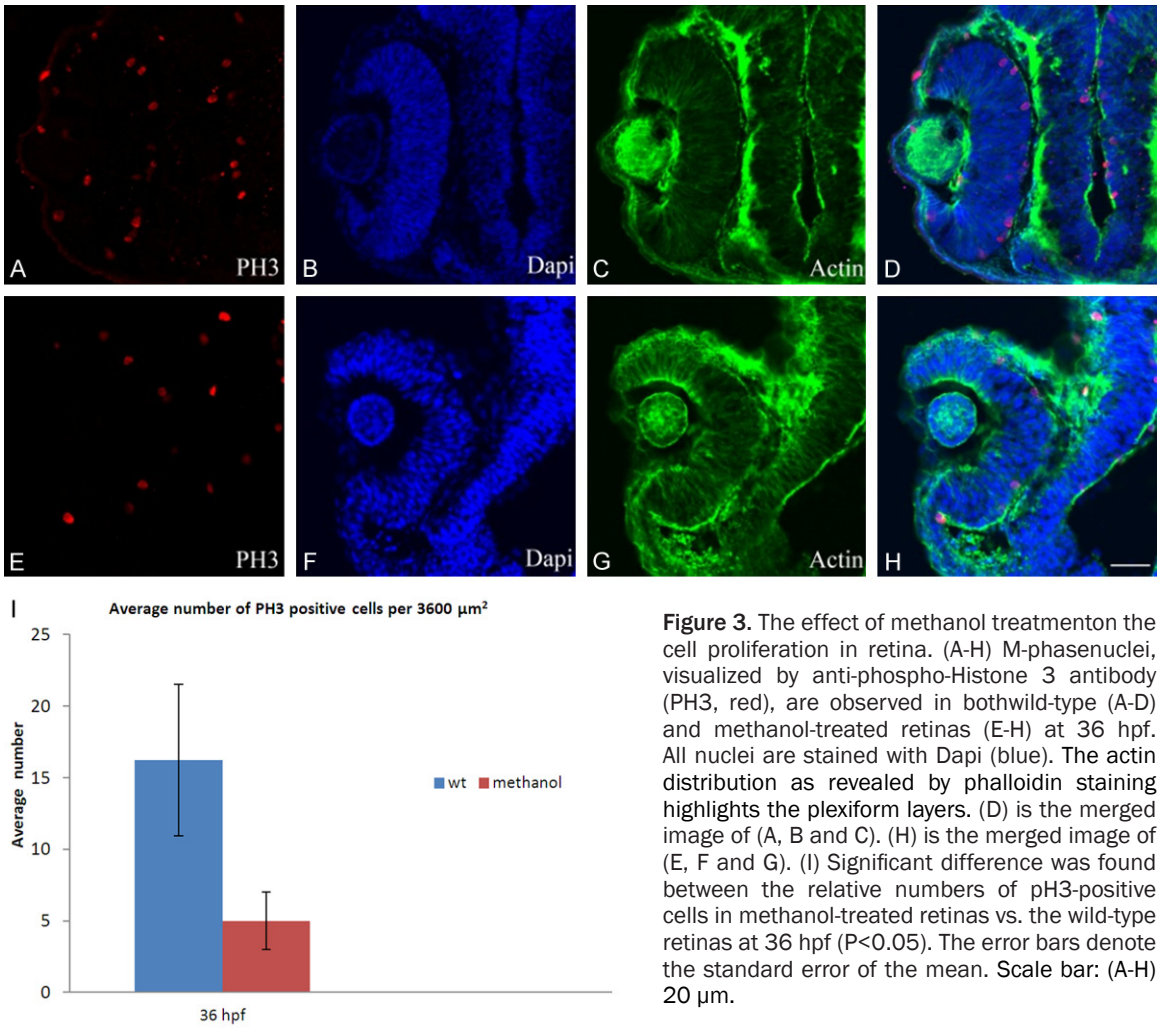

Figure 3. The effect of methanol treatmenton the cell proliferation in retina. (A-H) M-phasenuclei, visualized by anti-phospho-Histone 3 antibody (PH3, red), are observed in bothwild-type (A-D) and methanol-treated retinas (E-H) at 36 hpf. All nuclei are stained with Dapi (blue). The actin distribution as revealed by phalloidin staining highlights the plexiform layers. (D) is the merged image of (A, B and C). (H) is the merged image of (E, F and G). (I) Significant difference was found between the relative numbers of pH3-positive cells in methanol-treated retinas vs. the wild-type retinas at 36 hpf ($P<0.05$). The error bars denote the standard error of the mean. Scale bar: (A-H) 20 μm.

at 120 hpf (**Figure 1**). Larvae exposed to 2% methanol swam normally around the Petri dish. These fish appeared morphologically normal (**Figure 1B**). **Figure 1C** and **1D** showed dramatic reduction in the eye size and body size in larvae treated with 3% and 4% methanol. At 120 hpf, the diameter of the 3% methanol-treated eyes along the anterior-posterior (AP) axis was about 75% of the diameter of wild-type eyes (**Figure 1A** and **1C**). These larvae could swim, but were not as active as the controls or 2% methanol-treated fish. The size difference between methanol-treated and wild-type eyes increased with the exposure to higher methanol concentrations. At 120 hpf, the 4% methanol-treated eyes were only about 66% of the diameter of the wild-type eyes (**Figure 1A** and **1D**). Larvae treated with these higher concentrations of methanol were also listless; they swam little and had abnormal touch responses (failed to swim in response to touch).

A reduction in body size upon methanol exposure is also apparent from an external examination. At 120 hpf, the 3% methanol-treated larvae were 34% shorter than their wild-type siblings (**Figure 1A** and **1C**). The size difference between methanol-treated and wild-type eyes increased with the higher concentration of methanol in media. The body length of the 4% methanol-treated larvae was only about 45% of the wild-type body length (**Figure 1A** and **1D**). In addition, the body axis of the methanol-treated larvae was curled up (**Figure 1D**), and paracardiac edema, abdominal edema, rounded forebrain and irregular jaw developed at 120 hpf (**Figure 1C** and **1D**). Together, a reduction of organ size is apparent in the methanol-treated eyes and body.

Methanol treatment affects retinal structure

Transverse sections of the retina at 120 hpf show the retinal morphology of wild-type (**Figure**

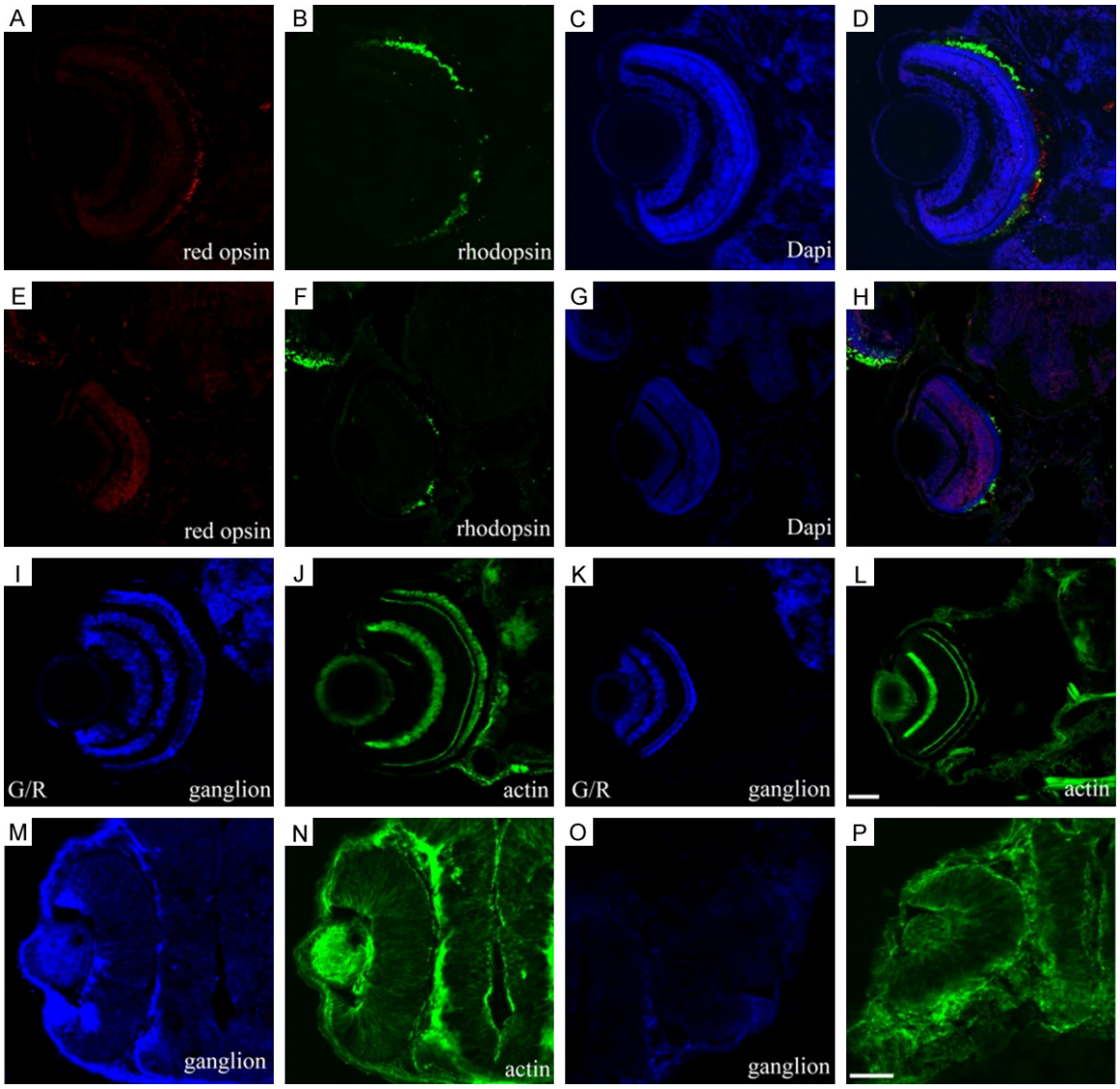

Figure 4. The effect of methanol treatment on retinal cell specification. (A-H) At 120 hpf, rhodopsin (green) and red opsin (red) are observed in both methanol-treated (E-H) and wild-type retinas (A-D). All nuclei are stained with Dapi (blue, G and K). (D and H) are merged images. (I-L) At 120 hpf, green/red double cones (G/R, blue) and amacrine cells (blue) are observed in both methanol-treated (K and L) and wild-type retinas (I and J). Zn8 (blue) expressing ganglion cells are specified in both wild-type and mutant retinas. The actin distribution as revealed by phalloidin staining highlights the plexiform layers. (M-P) The ganglion cells stained with zn8 antibody (blue) are present in wild-type retinas (M and N) but not in the methanol-treated retina (O and P) at 36 hpf. Scale bar: (A-L) 20 μm; (M-P) 20 μm.

2A) and methanol-treated zebrafish (**Figure 2B-D**). In the wild-type zebrafish, as the retinas differentiate, the retinal neurons are segregated into three distinct nuclear laminae according to their identity, and the plexiform layers containing neuronal processes separate the layers containing cell bodies. At 120 hpf, the five principal laminae in the retina could be easily identified, and the photoreceptors in untreated larvae were differentiated with well-defined inner and outer segments (**Figure 2A**). All retinas from the methanol-treated larvae maintained proper lamination and had normally differentiated lenses (**Figure 2B-D**), but they were noticeably smaller than those of the untreated controls. The size of the retina negatively correlated with the amount of methanol to which the larvae were exposed. Larvae treated with medium to high concentrations of methanol (3-4%) also had thinner ganglion cell layers. These larvae had smaller ciliary marginal zones (CMZs) (**Figure 2C** and **2D**), the area of prolifer-

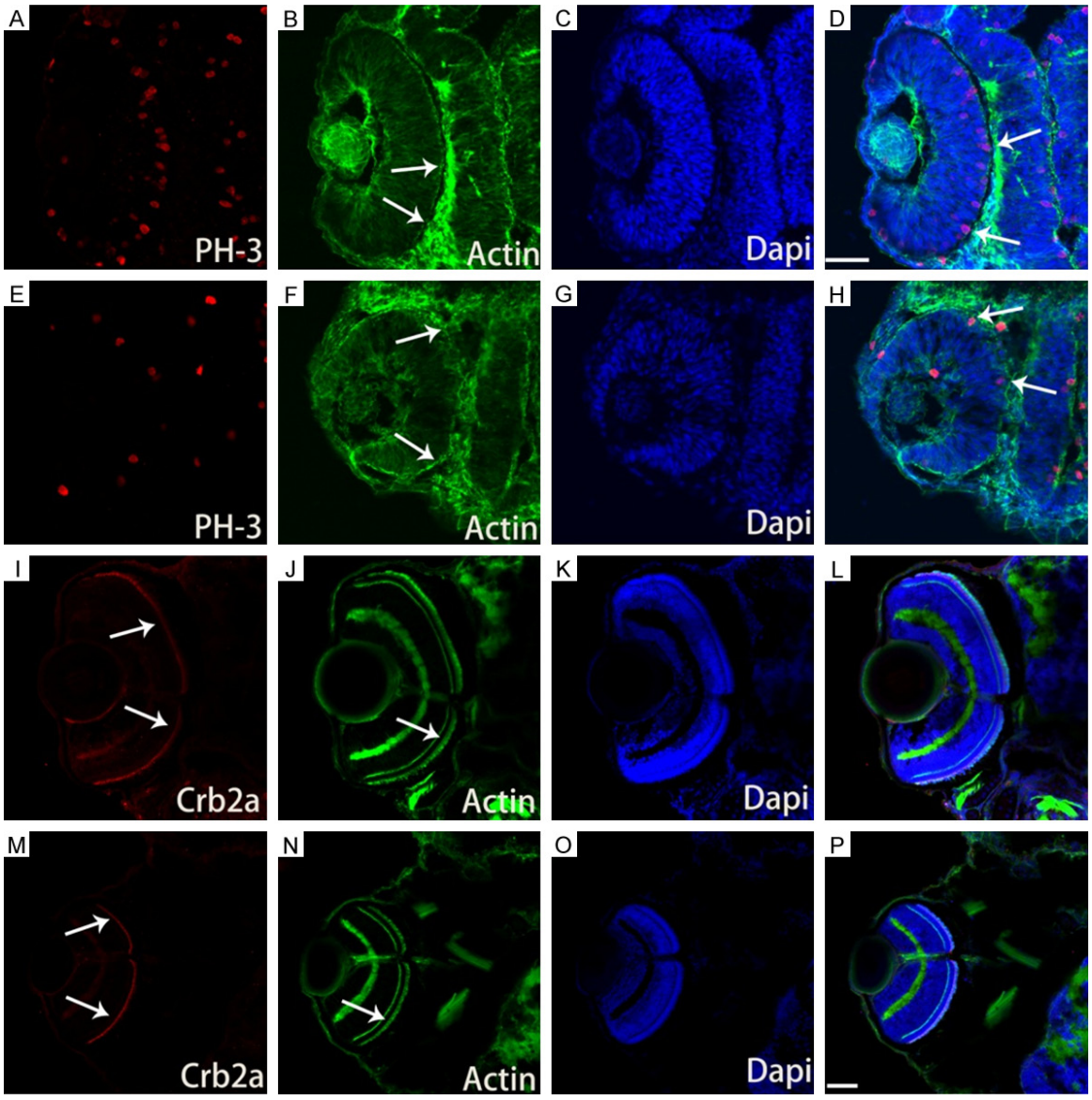

Figure 5. The effect of methanol treatment on the apical epithelial polarity. (A-H) In 36 hpf retinas, the M-phasenuclei as visualized with anti-phosphorylated-Histone H3 antibody (PH3, red) localize to the apical regions of the retinas of methanol treatment (E-H) and wild-type (A-D) embryos. Apical localization of adherens junctions in the retina (arrows) of the 36 hpf wild-type and methanol-treated embryos is visualized by the staining patterns of adherens junction-associated actin bundles (green). Cell nuclei were labeled with Dapi (blue). (I-L) Apical localization of adherens junctions in the retina (arrows) of the 120 hpf wild-type larvae is visualized by the staining patterns of Crb2a (red) and adherens junction-associated actin bundles (green). (M-P) Crb2a (red) and adherens junction-associated actin bundles (green) localize properly to the apical surface of the retina (arrows) in methanol-treated larvae at 120 hpf. Cell nuclei were labeled with Dapi (blue). Scale bar: (A-H) 20 μm; (I-P) 20 μm.

ating cells that mediates the continuous growth of the eye in the cold-blooded vertebrates [15, 16] and populates the retina with all its neuronal cell types [17]. The retinal pigment epithelia (RPE) had withdrawn completely from neural retina in retinae treated with 4% methanol (**Figure 2D**). Taken together, our observations suggest that embryonic exposure to methanol is harmful for maintaining the proper structure of the retina during early development.

Methanol treatment causes inhibition of cell proliferation in retina

To determine whether or not cell proliferation was affected by methanol and consequently

contributes to the small eye phenotype, the level of active cell proliferation was analyzed at 36 hpf. The retinas were stained with anti-phospho-Histone H3 (pH3) antibody, which specifically stains cell nuclei in M-phase (**Figure 3A-H**). The average number of positive cells per 3,600 μm^2 of retinal section was calculated, and significant difference was found between the relative numbers of pH3-positive cells in 4% methanol-treated retinas vs. the wild-type retinas (**Figure 3I**, $P<0.05$). Thus, the methanol treatment affects cell proliferation dynamics in the zebrafish retina at early stages of development.

Methanol treatment affects cell specification in retina

To determine whether or not retinal cell specification was affected by methanol, immunohistochemical analysis with cell type-specific markers was performed [13]. The analysis helps to determine whether red/green double cones, amacrine cells, rods and ganglion cells differentiated in the methanol-treated retinas. We found that all of these cell-specific markers appeared in 4% methanol-treated retinas, although the labeled cells were greatly reduced in number compared to the wild-type retinas at 120 hpf (**Figure 4A-L**). We did not find any cells that simultaneously expressed two markers that are normally expressed in different retinal cell classes. Rhodopsin and red opsin were expressed in the outer segments of fish treated with 4% methanol, but the morphologies of photoreceptors were markedly different from their wild-type counterparts. Photoreceptors in the 4% methanol-treated retinas did not have the typical elongated shape (**Figure 4K** and **4L**).

We performed a similar analysis with embryos at 36 hpf. We found that a considerable number of inner nuclear (INL) cells were specified in the 4% methanol-treated embryos at 36 hpf. However, the ganglion cells were not specified at this stage (**Figure 4M-P**). These results suggest that the cell differentiation process is delayed in the methanol-treated retinas.

Methanol treatment does not affect retinal epithelial polarity

To determine if retinal epithelial polarity was affected by methanol, we compared the distribution pattern of the apical polarity markers Crb2a, adherens junction-associating actin bundles, and M-phase nuclei in wild-type and methanol-treated retinas. As shown in **Figure 5**, wild-type and methanol-treated retinas did not differ in terms of the localization of these apical markers at 36 hpf and 120 hpf. Thus, the methanol treatment does not appear to affect the polarity of the retinal epithelia at early and later stages of development.

Discussion

In recent years, methanol poisoning events occurred frequently because of adulterated wine and occupational exposure. Methanol poisoning severely damages the retina and optic nerve resulting in the impairment of vision and visual field [18].

Although methanol optic nerve toxicity was described more than three decades ago [1], very little is known about the retinal patterning mechanisms that underlie the teratogenic effects of methanol in vertebrates. Most of the works aimed at analyzing the effects and mechanisms of methanol teratogenesis have used rodents as animal models [5]. An obvious disadvantage of using systems in which the embryos develops in utero is that it is difficult to assess the role of maternal metabolism in the process; thus, it is difficult to establish the direct effect of methanol on vertebrate development. Therefore, studying of methanol poisoning using mammals as model systems can be complemented by studying the effects of methanol on the development of vertebrate embryos that develop ex utero, such as zebrafish.

Zebrafish treated with moderate to high levels of methanol during the early developmental period had morphological abnormalities in the eye, as assessed by light microscopy. The primary phenotypic characteristic of methanol treatment is the failure of the eyes to grow after the onset of retinal neurogenesis. Methanol treatment also disrupts retinal structure and causes inhibition of cell proliferation at this early stage.

In zebrafish, early morphogenesis of the eyecup is completed before 24 hpf, and the first differentiated retinal ganglion cells appear at approximately 28 hpf [10, 19]. Photoreceptors begin to differentiate in zebrafish at approxi-

mately 43 hpf, shortly after they become postmitotic [19, 20]. Morphological differentiation of these cells progresses quickly thereafter, with outer segments first becoming visible by 60 hpf [19]. Since the small eye phenotype after methanol treatment becomes evident after the onset of retinal differentiation, we suggest that methanol exposure is harmful for the differentiation of retinal cells. This is consistent with our finding that ganglion cells are not specified at 36 hpf in methanol-treated embryos.

In addition to a small eye, the methanol treatment also causes a defect in retinal structure. The size of the retina was negatively correlated with the amount of methanol to which the larvae were exposed. Photoreceptors in the methanol-treated retinas did not have the typical elongated shape. RPE in the methanol-treated retinas had withdrawn completely from neural retina.

While the methanol treatment causes retinal patterning defects, it does not disrupt retinal epithelial polarity. Developmental defects in these methanol-treated zebrafish are not restricted to the retina, as manifested by a curled body axis, circulation defects and malformation of the digestive system. Larvae treated with methanol exhibit an abnormal touch response.

The retinal patterning defect in methanol-treated zebrafish might be explained by suppressive retinal cell proliferation during the early development, which could affect retinal size and result in inconsistent migration and/or differentiation signals to the newly emerged retinal cells. This is consistent with the finding that the germinal zone at the ciliary margin of the methanol-treated retina appears abnormal.

In conclusion, exposure of zebrafish to methanol affects cell proliferation and differentiation in the retina at the early stages of development, thus causing retinal patterning defects and a small eye phenotype.

Acknowledgements

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Disclosure of conflict of interest

None.

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