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Original Article

The effects of embryonic exposure to methanol on zebrafish growth, locomotor activity, and photoreceptor development

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Abstract: Background: Methanol toxicity can lead to potentially fatal poisoning. We characterized how methanol affects zebrafish growth, external morphology, and locomotion, paying particular attention to photoreceptor development. Methods: Zebrafish embryos were divided into six groups and placed in hatching liquid for 6 hours. The embryos from five of the six groups were treated with varying concentrations (1%, 2%, 3%, 4%, and 5% by volume) of methanol from 6 to 24 hours post-fertilization. The toxic effects of methanol on zebrafish embryonic development were assessed based on the mortality rate, the hatching rate, and the morphological deformity rate. The effects of methanol on the locomotor activity of zebrafish embryo/larvae were assessed using autonomous motion and swimming behavior analyses. The effects of methanol on photoreceptor development were assessed using electron microscopy analyses. Results: Methanol increases mortality and induces growth retardation in zebrafish embryos. Zebrafish embryos exposed to 3% and 4% methanol during their early embryonic development displayed dramatic decreases in spontaneous tail movement at 24 hours post-fertilization and in the speed of their movement at 120 hours post-fertilization compared with the control group. Embryos exposed to a high (4%) concentration of methanol displayed morphological abnormalities in their photoreceptors. Conclusion: These findings suggest that methanol affects zebrafish growth and external morphology, and higher levels of methanol exposure can cause defects in locomotor activity and photoreceptor development.

Keywords: Zebrafish, methanol, toxicity, locomotion, photoreceptors

Introduction

Methanol is a colorless, toxic, volatile liquid, with an odor similar to alcohol, which can be absorbed into the blood through the respiratory tract, gastrointestinal tract, and skin, leading to poisoning. Methanol toxicity can lead to potentially fatal poisoning that requires specific treatment [1, 2]. The latent period of methanol poisoning is approximately 12-24 hours, but in rare cases it can be as long as 2-3 days [3].

Acute methanol poisoning is very dangerous. If the treatment is not timely, the death rate can exceed 40% [4]. The toxic effect of methanol is caused by the methanol itself and its metabolites, formaldehyde and formic acid [5]. The main clinical manifestations are metabolic acidosis, visual disturbances, and nervous system symptoms [6, 7]. The initial visual and ocular manifestations include dark shadows, flashes, blurred vision, eye pain, photophobia, and diplopia. In severe cases, permanent binocular blindness can be caused by atrophy of the optic nerve, which is related to the special selective toxic effect of methanol on the optic nerve and retina [8, 9].

In recent years, rodent models have been used to validate methanol's impact on the embryo [10]. However, the structure of the zebrafish retina is very similar to that of the human retina, and zebrafish are highly sensitive to environmental pollutants. Our previous research showed that zebrafish methanol exposure causes patterning defects and suppressive cell proliferation in the retina [11]. In this study, zebrafish embryos were treated with different concentrations of methanol from 6 hours post-fertilization (hpf) to 24 hpf, at which time the

eyecups are well-formed [12]. We characterized how embryonic exposure to methanol influences zebrafish growth, locomotion, and photoreceptor development. The methanol-induced ultrastructural and morphological changes in the photoreceptor cells were observed to elucidate the physiological basis of visual impairment caused by methanol poisoning so as to provide a theoretical basis for the effective treatment of methanol poisoning.

Materials and methods

Animal source and breeding

AB wild-type zebrafish were provided by the Translational Medicine Institute of Zhejiang University. The zebrafish were placed in the same tank. The male to female ratio was 2:1, and the light/dark cycle ratio was 14:10. The fertilized eggs were collected and washed according to Schulte's and Nagel's methods [13], and the normal, divided fertilized eggs were selected under a stereoscopic zoom microscope (Leica Microsystems, Illinois, USA) for the toxicity testing. The embryos were raised in hatching liquid at 28.5°C for 6 hours.

Animal grouping and methanol treatment

Before the methanol treatment, the embryos were divided into six groups and put into a beaker sealed with parafilm. The embryos from five of the groups were treated with varying concentrations (1%, 2%, 3%, 4%, and 5% by volume) of methanol (Sigma, Missouri, USA) beginning at 6 hpf. After the treatment, the embryos from each group were put into an incubator to continue to develop for 18 hours. At the completion of the methanol treatment, the embryos were put into the hatching liquid for further development to 120 hpf. All the animal experimental procedures in this study were approved by the Animal Care and Use Committee of Zhejiang University. These protocols were devised in accordance with the *Guide for the Care and Use of Laboratory Animals* (8th edition, National Academies Press).

Toxicity testing and morphological deformity assessment

The mortality and hatching rates of the larvae at 120 hpf were assessed in more than 100 embryos for each concentration. The live larvae were observed and photographed at 120 hpf through a stereoscopic zoom microscope (Leica Microsystems, Illinois, USA), and the body length and eye diameter of the zebrafish larvae were measured and analyzed with ImageJ software (NIH, Maryland, USA). The body length is defined as the distance from the center of the retina to the end of the tail, and the eye diameter is defined as the distance along the anterior-posterior (AP) axis.

Locomotor activity analysis

Fifteen embryos at 24 hpf from each group were used for the autonomous motion detection. The embryos were placed in a Petri dish, and the frequencies of alternating movements of the tail spontaneously from one side to the other within 60 s were observed through a stereoscopic zoom microscope (Leica Microsystems, Illinois, USA). Fifteen larvae at 120 hpf from the control or methanol-treated groups were used for a swimming behavior analysis.

Transmission electron microscopy

Fifteen larvae at 120 hpf from each group were fixed with 2.5% glutaraldehyde after PBS washing, and refrigerated at 4°C overnight. The larvae were then fixed with 1% osmium tetroxide for 2 hours. The larvae were dehydrated through acetone series, embedded directionally, and ultrathin sections (60 to 70 nm) were prepared. The ultrastructural changes of photoreceptor cells were observed using a Hitachi H600 transmission electron microscope (Hitachi, Hitachi, Japan).

Statistical analysis

All experiments were repeated independently at least three times. All the data were analyzed using Origin 6.0 statistical software. All of the results were presented as the mean ± S.E.M. Independent samples t tests were used for the comparisons between two groups, and multiple groups of data were compared using one-way ANOVA. A p-value < 0.05 was considered statistically significant.

Results

The effect of methanol toxicity on the survival and hatching ability of zebrafish larvae

Larvae exposed to 1% or 2% methanol showed low mortalities of 0 and 3%, respectively (**Figure 1**) at 120 hpf. However, when treated with

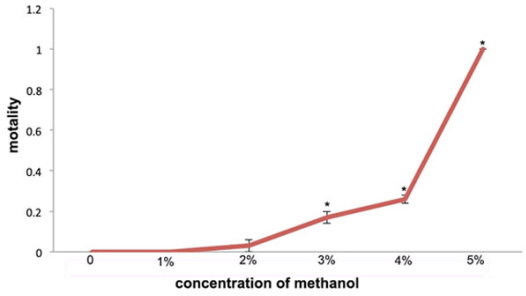

Figure 1. The effects of methanol on embryo mortality. The embryos were left to develop for 6 h and were then incubated in methanol solutions at different concentrations (0% [control], 1%, 2%, 3%, 4%, and 5% by volume) for 18 h. The embryos were then examined at 120 hpf (n=100, 20 embryos in five replicates each. *P < 0.05).

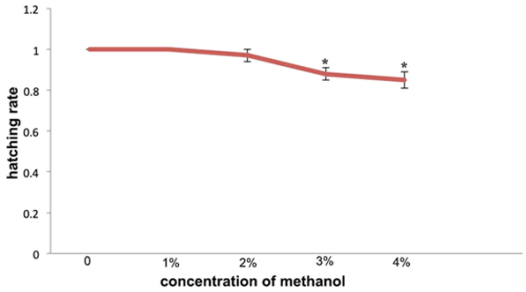

Figure 2. The effects of methanol on the embryo hatching rate. The embryos were left to develop for 6 h and were then incubated in methanol solutions at different concentrations (0% [control], 1%, 2%, 3%, and 4% by volume) for 18 h. The embryos were then examined at 120 hpf (n=100, 20 embryos in five replicates each. *P < 0.05).

higher concentrations of methanol (3% and 4%), the zebrafish larvae showed mortalities of 17% and 26%, respectively (**Figure 1**). The mortalities increased as the concentration of methanol increased. In the 5% methanol-treated group, the mortality reached 100% (**Figure 1**). The hatching rates of the zebrafish larvae at 120 hpf decreased with increasing concentrations of methanol from 1% to 4%. The hatching rates were 100%, 96.8%, 88.1%, and 85.2%, respectively (**Figure 2**).

The effect of methanol toxicity on the morphological properties of zebrafish larvae

As shown in **Figure 3**, larvae exposed to 1% or 2% methanol appeared morphologically normal (**Figure 3B** and **3C**). However, at 3% or 4% concentrations of methanol exposure, the zebrafish larvae were smaller than the controls and exhibited a high incidence of external morphological malformations (**Figure 3D** and **3E**).

The larvae exposed to 1% or 2% methanol showed low malformation rates of 0 and 4.9%, respectively (**Figure 4**). However, when treated with higher concentrations of methanol (3% and 4%), 87.2% and 95.5% of the zebrafish larvae exhibited an abnormal external appearance (**Figure 4**). The severity and frequency of the external appearance of the defects increased with the increasing concentrations of methanol.

The effect of methanol toxicity on the eye size and body size of the zebrafish larvae

The effects of methanol on the total body length and eye diameter of the zebrafish larvae are shown in **Figure 3**. At 120 hpf, the eye diameter and body length of the 3% methanol-treated larvae were about 75% and 66% of their wild-type siblings, respectively. The eye diameter and body length of the 4% methanol-treated larvae were about 66% and 55% of their control siblings.

The effect of methanol toxicity on the locomotor activity of zebrafish embryos and larvae

The effects of methanol on the locomotor activity of zebrafish embryo/larvae and on the autonomous motion of zebrafish embryos at 24 hpf are shown in **Figure 5**. The data indicate that the zebrafish embryos exposed to 3% and 4% methanol displayed a significant decrease in spontaneous tail movement at 24 hpf compared with the control group.

At 120 hpf, no erratic movement from the control group was observed. The larvae exposed to 1% and 2% methanol were active; they swam normally around the Petri dish. However, the larvae treated with 3% and 4% methanol were stalled as they displayed significantly less movement.

Methanol toxicity on the structure of photoreceptors of zebrafish larvae

As shown in **Figure 6**, the overall structure of the photoreceptors in the methanol-treated larvae at 120 hpf was disrupted and the nuclei were shrunk (**Figure 6B**). The membrane discs

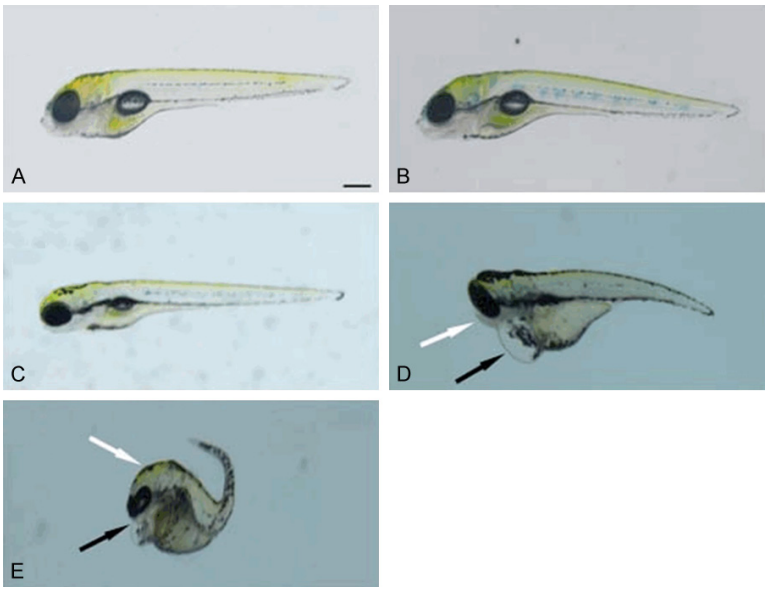

Figure 3. Gross morphological changes after methanol treatment. (A-E) The embryos were exposed to 0%-4% methanol, respectively, from 6 to 24 hpf and then allowed to develop in normal methanol-free egg water. Images of living zebrafish were acquired at 120 hpf. (D, E) The embryos that were exposed to higher methanol concentrations exhibited a curved body axis, swollen hearts (black arrow, D), swollen intestines (black arrow, E), irregular jaws (white arrow, D) and rounded forebrains (white arrow, E). Scale bar: 20 μm.

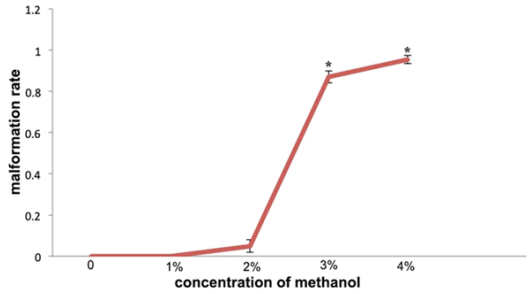

Figure 4. The effects of methanol on the embryo malformation rate. The embryos were left to develop for 6 h and were then incubated in methanol solutions at different concentrations (0% [control], 1%, 2%, 3%, and 4% by volume) for 18 h. The embryos were then examined at 120 hpf (n=100, 20 embryos in five replicates each. *P < 0.05).

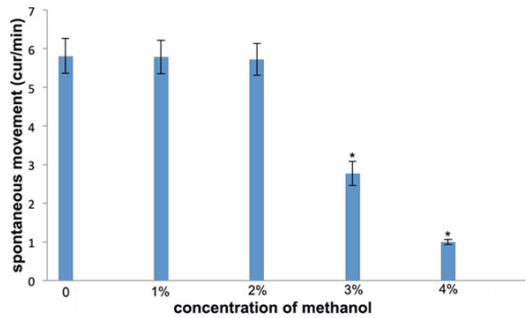

Figure 5. The spontaneous movement per minute in the zebrafish embryos of 24 hpf after exposure to different concentrations (0% [control], 1%, 2%, 3%, and 4% by volume) of methanol from 6 to 24 hpf. Compared with the control embryos, the spontaneous movement of the embryos significantly decreased as the concentration of methanol increased (n=100, 20 embryos in five replicates each. *P < 0.05).

of the photoreceptor outer segments were most significantly affected by the methanol treatment. The gaps between the membranous discs were noticeably enlarged, and there were autophagic vacuoles present (**Figure 6D**). The membranous discs in the local area lost their lamellar structure completely and presented a disordered medullary structure (**Figure 6D**). In the methanol-treated retinas, the nuclei of the photoreceptors lacked their typical elongated morphology, and the mitochondria were scattered throughout the cytoplasms (**Figure 6B**).

Discussion

Embryonic exposure to methanol affects zebrafish survival and development

Zebrafish are a highly sensitive model for assessing environmental pollutants. In this experiment, we established a zebrafish model of methanol poisoning. Our results demonstrated that methanol exposure increased the mortality rate and induced growth retardation in zebrafish embryos. The results also showed that methanol's toxicity becomes more discernible at higher concentrations. In addition to an apparent reduction of eye and body size, the zebrafish embryonic deformities induced by methanol were mainly manifested as a curved body axis, paracardiac edema, abdominal edema, rounded forebrain, and irregular jaw.

The zebrafish hatching rate is an important index of toxicity [14]. Our results showed that methanol inhibits zebrafish embryonic

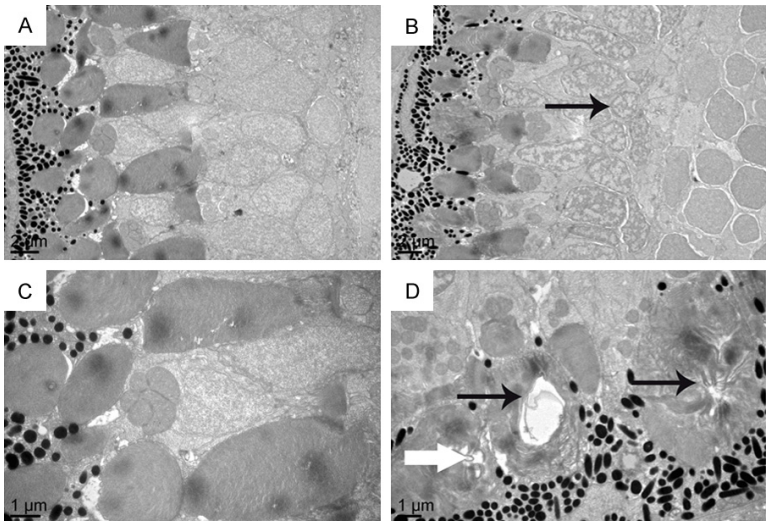

Figure 6. Methanol treatment affects photoreceptor development. (A-D) The embryos were untreated (A, C) or received 4% methanol (B, D) from 6 hpf through 24 hpf. The photoreceptor arrangement was disrupted, and the nuclei were shrunk in methanol-treated larvae (black arrow, B). Severely disrupted outer segments showing tubulation and vesiculation (black arrow, D) of the disc structures were observed in the methanol-treated larvae. Many vacuoles and holes (white arrow, D) were found between the outer segments. In the methanol-treated retinas, the nuclei of the photoreceptor cells lacked their typical elongated morphology, and the mitochondria were scattered throughout the cytoplasms (black arrow, B).

hatching and delays the incubation time. Zebrafish egg stripping requires a close combination of two processes. The first process is the softening of the egg film through the embryonic release of the hatching enzymes. The second process is the rupture of the egg membrane leading to hatching through embryonic autonomous movement [15, 16]. The zebrafish embryonic autonomous movement dramatically decreased after the methanol treatment. The slowing of the autonomous movement can explain why methanol inhibits embryonic hatching. Methanol slows down the autonomous movement, leading to a delay in the rupture of the egg membrane and the subsequent hatching process. We speculate that prolonged incubation is the leading cause of morphological deformities. The methanol toxicity mechanism that leads to embryonic developmental malformations needs to be further studied.

Embryonic exposure to methanol affects zebrafish locomotor activity

Methanol exhibits strong neurotoxicity and vascular toxicity, which can directly damage the central nervous system [17]. The zebrafish nervous system has many similarities to the mammalian nervous system. Neurological disorders and mental retardation are often associated with exposure to toxic compounds. The necrosis and apoptosis of the neurons in the brain can be used as an index of the neurotoxicity of various compounds, and motor neuron apoptosis is associated with motor defects.

Previous studies have investigated the swimming behavior of zebrafish after exposure to various toxins, such as chronic sublethal dietary selenomethionine, domoic acid, perfluorooctanesulfonate, alcohol, and sodium hypochlorite [18, 19]. Our results showed dramatic decreases in spontaneous tail movement at 24 hpf and movement speed at 120 hpf compared with the control group after methanol treatment at the higher (1.5% and 2.5%) levels. From the aspect of motion behavior, it is shown that methanol has latent neurotoxicity.

Embryonic exposure to ethanol affects zebrafish photoreceptor development

At present, the most common acute methanol poisoning event is taking fake alcohol mixed with methanol. Methanol poisoning is rarely seen in suicide, misuse, or occupational poisoning [20]. There are also cases of methanol poisoning caused by the external application of alcohol foam [21]. The main clinical manifestations of methanol poisoning are liver failure, eye damage, and nervous system damage. The water solubility of methanol is very high, and the water content of aqueous humor and the vitreous body are more than 99%. Therefore, eye injury is one of the typical manifestations of acute methanol poisoning [22]. Clinically, the diagnosis is based on the history of methanol intake, the abnormal concentration of methanol in the blood, the symptoms of liver, eye, and nervous system damage; as well as clinical manifestations such as headache, disturbed

consciousness, sensory impairment, blurred vision, reduced vision, abnormal electroretinogram (ERG), and visual evoked potential (VEP) [23, 24].

Photoreceptor cells are a type of highly polarized cells in the retina, and they are well-known in the vertebrate nervous system and are considered to be the best model for studying neuron specialization and differentiation. Apoptosis of the photoreceptor cells can lead to visual impairment and retinal degeneration [25]. Because photoreceptor cells are the most vulnerable cells in the retina, it is very important to study their development in order to improve the treatment of photoreceptor cell diseases.

The ocular methanol toxicity is mainly reflected in abnormal changes of the fundus and conjunctival congestion or hemorrhage. Our previous studies showed that methanol exposure at the early stages of development caused decreases in retinal size and the inhibition of cell proliferation and differentiation in the retina [11]. In this study, our results showed that the photoreceptor arrangement was perturbed and the nuclei were shrunk in methanol-treated larvae. Severely disrupted membrane discs of photoreceptor outer segments were observed in the methanol-treated larvae. The damage can cause a dysfunction in the light stimulation transmission pathway from the initial point and can seriously affect the production and transmission of visual signals from the retina, which may underly the visual impairment caused by methanol poisoning [26].

Cellular metabolism in the retina, especially in photoreceptors, is very active. Photoreceptors are marked by high oxygen consumption and mitochondrial activity. If the electron transfer and oxidative phosphorylation of mitochondria are blocked, this will lead to a dysfunction of the ion pumps, edema, and the pathological expansion of various layers of tissues and organelles, thus affecting photoreceptor cell function. Methanol's capacity to disrupt vision may be related to its damage to photoreceptor mitochondria.

In summary, our study reveals that embryonic exposure to methanol at the early stages of development leads to decreased survival and hatching rates and an increased frequency of malformations, which might subsequently impede the locomotive activity of zebrafish larvae. Furthermore, our study demonstrated that methanol exposure during early development causes abnormalities of photoreceptor development. However, the potential underlying mechanisms and implications of photoreceptor degeneration need to be further investigated.

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Disclosure of conflict of interest

None.

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