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Original Article

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Folic acid supplement rescues ethanol-induced developmental defects in the zebrafish embryos

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Abstract

Fetal alcohol syndrome (FASD) describes a range of birth defects. Mechanisms of FASD-associated defects are not well understood. It has great significance to investigate whether nutrient supplements like folic acid (FA) can effectively rescue ethanol-induced defects. Moreover, it is very important to determine the optimal time for FA supplementation when it can most effectively antagonize the teratogenic effects of ethanol during embryonic development. Our results indicated that ethanol exposure interrupted the development of zebrafish embryos and induced multiple defects in cardiac function, pharyngeal arch arteries, vessel, craniofacial cartilage, pharyngeal arches, brain, somite and hemoglobin formation. The expressions of critical genes that play important roles in above organs such as *tbx1*, *flk-1*, *hand2*, *ngn1*, *huc*, *titin*, *gata-1* and *c-myb* were reduced, and the apoptosis was increased in ethanol-treated group. FA supplementation could reverse ethanol-induced defects, improve the decreased expressions of above genes and reduce the apoptosis. We also found that giving FA at 6–12 h post-fertilization (hpf), which is at the gastrula period (5.25–10 hpf), can obviously prevent the teratogenicity of ethanol. This research provides clues for elucidating the mechanism of fetal abnormalities caused by alcohol intake and for preventing FASD.

Key words: folic acid, ethanol, developmental defects, zebrafish

Introduction

Fetal alcohol syndrome (FASD) is associated with maternal alcohol consumption. Clinically, it is characterized by cardiovascular malformation, craniofacial abnormalities, central nervous system damage and body growth defects. Epidemiological studies showed that FASD incidence is higher in infants born to lower socioeconomic status alcoholic mothers compared to infants born to middle class alcoholics [1–3]. A lower socioeconomic population is at a greater risk for poor nutrition such as folic acid (FA). FASD is the most frequent preventable birth defect syndrome [4]. Epidemiologic studies showed that severe congenital heart defect prevalence was significantly reduced by prenatal FA fortification [5,6].

FA is an essential vitamin that participates in nucleic acid synthesis and repair. FA also plays a crucial role as a co-factor in 1-carbon metabolism as tetrahydrofolate, which is needed in DNA and histone methyl transfer. FA deficiency and inhibition have been found to induce embryonic malformations [7–10]. Prenatal FA supplementation significantly reduces the risk of neural tube defects, congenital heart defects and cleft lip/palate [11], and thus, FA is a recommended dietary supplement for pregnant mothers [12]. Studies

showed that FA transport to the fetus is impaired in pregnancies with chronic alcohol exposure. Embryonic ethanol exposure affects FA metabolism, including reduced maternal-to-fetal folate transfer and reduced expression of folate-metabolizing enzymes. Alcohol ingestion can also inhibit folate-dependent DNA methylation [13].

A better understanding of the effects of ethanol in embryonic development in animal model may provide important clues for the study of the relationship between alcohol consumption and fetal anomaly in human. Exploring the best time and possible mechanism of FA antagonizing ethanol using animal model is of great significance to effectively prevent ethanol-induced abnormal human embryonic development.

Zebrafish shares high evolutionary conservation with human, and zebrafish offers several distinct advantages for genetic and embryological studies because of its external fertilization, rapid development, easy genetic manipulability, high fecundity and embryo optical clarity. Zebrafish embryo experiments showed that ethanol exposure interrupted divergent cardiac morphogenetic events causing heart defects [14]. The transgenic zebrafish lines can help to clearly observe the target organ. Using the transgenic zebrafish line *Tg(fli1:neGFP)*^{y7} expressing green fluorescent protein (GFP) in endothelial and endocardial cells [15], we can better visualize the vessel and endothelial cells.

In this study, we used zebrafish to observe the teratogenic effects of ethanol on embryonic development and detect the expression levels of related genes as well as apoptosis. We also explored the best time for FA administration during embryonic development when it can most effectively antagonize the teratogenic effects of ethanol. This research provides clues for elucidating the mechanism of fetal abnormalities caused by alcohol intake in humans. Our investigation is also expected to provide clues for the design of better therapeutics and preventive measures for FASD.

Materials and Methods

Zebrafish strains and maintenance

Wild-type (AB strain) zebrafish, *Tg(fli1:neGFP)*^{y7} transgenic zebrafish lines were raised and housed under standard laboratory conditions [16]. The breeding facility was purchased from Aquatic Habitats Corporation (Apopka, USA). Embryos were obtained from natural spawning, raised at 28.5°C in embryo medium (EM) and staged according to the standard protocol [17]. Embryos used for *in situ* hybridization were treated with 0.003% PTU (phenylthiourea) to prevent pigmentation at 24 h post-fertilization (hpf). Embryos were fixed in 4% paraformaldehyde (PFA)–phosphate-buffered saline (PBS) at 4°C and stored in 100% methanol at −20°C prior to *in situ* hybridization.

Ethanol was diluted into egg water, and zebrafish embryos were treated with different concentrations of ethanol at 2–48 hpf. Ethanol with a concentration of 400 mM was chosen as the optimal concentration for treatment because under this concentration the majority of embryos developed obvious malformations and the embryos could survive up to 96 h after fertilization. FA (Sigma, St Louis, USA) stock solution was freshly prepared prior to use and diluted with embryo medium. Different concentrations of FA were tested, and 75 μM was chosen as the optimal concentration for FA treatment to minimize the toxic effects of ethanol (400 mM) and achieve maximum rescue phenotypes.

In the ethanol treatment experiment, embryos were transferred to EM containing 400 mM ethanol at 2–48 hpf. Eggs were covered during incubation to prevent evaporation of ethanol. In the FA rescue experiment, the ethanol-treated embryos were divided into eight groups. FA was added to the ethanol-treated group with a final concentration of 75 μM at different stages: 0–6 hpf, 6–12 hpf, 12–18 hpf, 18–24 hpf, 24–30 hpf, 30–36 hpf, 36–42 hpf and 42–48 hpf.

At 48 hpf, the ethanol-treated embryos and the FA + ethanol-treated embryos were transferred back to EM. Embryos treated with 75 μM FA at 6–12 hpf were defined as FA-treated group and were transferred back to EM at 12 hpf. Each group contained 100 embryos.

Microangiography

To monitor the development of the pharyngeal arch arteries and vessels, fluorescein (2–5 nl) was microinjected into the hearts of living zebrafish embryos at 60 hpf [9,10]. About 2–3 min later, images of the pharyngeal arch arteries and vessels were observed under a BX61 fluorescent microscope (Olympus, Tokyo, Japan) and captured with a DP70 digital camera (Olympus).

Measurement of heart rate and analyses of ventricular contractility

The hearts of zebrafish embryos were easily observed with a microscope. Heart rate was measured for 1 min. Cardiac contractions were recorded with a video camera (TK-C1381; JVC, Yokohama, Japan) as previously described [9,10]. The diastolic and systolic lengths of ventricles were measured to calculate the ventricular shortening fraction (VSF) using the following formula: VSF=(ventricular length at diastole−ventricular length at systole)/ventricular length at diastole.

Histological analysis

At 60 hpf, controls and ethanol-treated larvae were fixed with 4% paraformaldehyde in 0.1 M phosphate buffer (pH=7.4) overnight. Larvae were then washed in phosphate-buffered saline (pH=7.4), dehydrated through a graded series of ethanol and embedded in JB-4 embedding medium. Serial sections were made, each containing 10 larvae. Sections were mounted and dried on glass slides and stained with hematoxylin and eosin (H&E; Sangon Biotech, Shanghai, China) according to the manufacturer's instruction.

Cartilage staining

Embryos at 96 hpf were cartilage stained with Alcian Blue (Sangon Biotech) as described previously [18]. Briefly, embryos were fixed in 4% paraformaldehyde overnight at 4°C. After several washes with PBS, embryos were rinsed with acid alcohol (70% ethanol and 0.37% HCl). Embryos were then stained with 0.1% Alcian Blue in acid alcohol for 4 h at room temperature and destained in acid alcohol overnight. After rehydration with PBS, embryos were digested with 1% trypsin for 1 h at 37°C. Embryos were then washed with PBS several times and stored in 70% glycerol.

O-dianisidine staining for globin

At 48 hpf, embryos were dechorionated and fixed in 4% paraformaldehyde overnight at 4°C, then rinsed with PBS before O-dianisidine staining. Fixed embryos were incubated in the staining buffer for 15 min in the dark. The staining buffer consisted of 0.6 mg/ml O-dianisidine (Sigma), 10 mM sodium acetate, 0.65% hydrogen peroxide and 40% (v/v) ethanol [19].

Whole-mount terminal deoxynucleotide TUNEL staining

The transferase-mediated dUTP nick-end labeling (TUNEL) staining was performed using an *in situ* Cell Death Detection kit (Roche, Indianapolis, USA) according to the manufacturer's instructions. Briefly, for whole-mount TUNEL staining, embryos at 10 hpf and 20 hpf were fixed with 4% paraformaldehyde overnight at 48°C, rinsed with PBS, incubated with a 1:10 TUNEL working solution in a dark environment at 37°C for 1 h and washed three times with PBS. Then, embryos were stained in the dark at room temperature for 5 min and then washed three times with PBS.

Whole-mount *in situ* antisense RNA hybridization

Whole-mount *in situ* hybridization of zebrafish embryos was performed as previously described [20]. Plasmids encoding zebrafish *tbx1*, *flk-1*, *hand2*, *ngn1*, *huc*, *titin*, *gata-1*and *c-myb* were linearized by restriction enzymes before transcription with T7 or SP6 RNA polymerase (Roche). Digoxigenin-labeled probes were synthesized using DIG RNA Labeling Kit (Roche). Whole-mount RNA *in situ* hybridization was carried out using DIG-labeled antisense RNA probes according to standard procedures as described. The number of embryos with decreased expression of genes was analyzed. Stained embryos were examined with BX61 and SZX12 microscopes (Olympus), and images were captured with a DP70 digital camera (Olympus).

RNA isolation and Q-PCR

Total RNA was extracted from embryos using TRIzol RNA isolation reagent (Invitrogen, Carlsbad, USA). First-strand cDNA was reverse transcribed using oligo-dT primers and MMLV reverse transcriptase (Promega, Madison, USA). The cDNA was analyzed immediately or stored at −20°C until use.

Quantitative real-time polymerase chain reaction (Q-PCR) was performed using the 7300 Real-Time PCR System (Applied Biosystems, Carlsbad, USA). The sequence-specific primers were designed using Primer Express 2.0 software (Applied Biosystems) and listed in Table 1. The SYBR Green method was used to quantify cDNA. *β-actin* was used as the internal control. The value of expression level of each gene was divided by the amount of *β-actin* in individual cDNA samples. The specificity of each reaction was controlled by melting curve analysis. Results were analyzed using the 7300 Real Time PCR System software (Applied Biosystems).

Statistical analysis

Data were presented as the mean±standard deviation (SD). Comparisons between groups were analyzed by ANOVA (*t*-test with Bonferroni correction). $P<0.05$ was considered statistically significant.

Table 1. Sequence of the primers used in this study

Genes	Primer sequence
tbx1	F: 5′-GAGACTGTGATCCCGAGGAC-3′
	R: 5′-TCATGATTTGTAGCGAGCCT-3′
hand2	F: 5′-GCCAAAGAAGAAAGGCGAAA-3′
	R: 5′-TGTCATTGCTGCTCCCTGAA-3′
ngn1	F: 5′-GTGACCAGAACCCCGTTAGA-3′
	R: 5′-TGAGGGTTTCTTCGGGTCAA-3′
huc	F: 5′-TCGTCAACTACCTGCCTCAG-3′
	R: 5′-GACTCATGGTTTTGGGCAGG-3′
flk-1	F: 5′-CAATGGCAGGATTCACTTTGAG-3′
	R: 5′-TTCATAAGGAGCGGATCAATCGTACTCACC-3′
gata-1	F: 5′-GAATTCCAGGAGATAAGCAAG-3′
	R: 5′-CTTGCTTATCTCCTGGAATTC-3′
c-myb	F: 5′-CAGCACTTCCTCCGAAGAG-3′
	R: 5′-CTCTTCGAGGAGGAAGTGCTG-3′
titin	F: 5′-CCTGTCAAGTCCCCTGTGAT-3′
	R: 5′-CCTCTTTGACACCTGCAACC-3′
β-actin	F: 5′-GTCCACCTTCCAGCAGATGT-3′
	R: 5′-GAGTCAATGCGCCATACAGA-3′

Results

Ethanol exposure induced the pharyngeal arch arteries and vessels defects, impaired the cardiac function and reduced the expressions of *tbx1* and *flk-1*

In controls, the pharyngeal arch arteries (Fig. 1A,a) and intersomitic vessels (Fig. 1E) could be clearly observed at 60 hpf by microangiography. Compared with controls, ethanol-treated embryos showed the dysplastic pharyngeal arch arteries (15.67±2.22; Fig. 1B,b) and vessels were unclear and vague (13.67±1.11; Fig. 1F). Using the transgenic zebrafish line *Tg(fli1:neGFP)*^{y7} embryos, which expressed GFP in endothelial and endocardial cells and allowed to better visualize the endothelial nuclei, we observed that ethanol exposure led to vessels containing less endothelial cells (Fig. 1J) than that in the control embryos (Fig. 1I) at 36 hpf.

In the ethanol-treated group, the heart rate and VSF were decreased compared with those in the control group (Fig. 1M,N), which indicated that ethanol caused impaired cardiac function in embryos. Results from the *in situ* antisense RNA hybridization showed that in controls, *tbx1* expressed in the pharyngeal arch arteries at 60 hpf (Fig. 3A) and *flk-1* was highly expressed in somatic vessels at 24 hpf (Fig. 3B). The expressions of *tbx1* (10.00±1.33; Fig. 3H) and *flk-1* (11.33±1.11; Fig. 3I) were decreased in the ethanol-treated group, which is consistent with the result of Q-PCR (Fig.7).

Ethanol exposure caused craniofacial cartilage and pharyngeal arch defects and decreased the expression of *tbx1* and *hand2* in pharyngeal arches

Ethanol-treated embryos developed craniofacial abnormalities that were apparent at 96 hpf. Alcian blue staining of cartilaginous structures showed shortened Meckel's cartilage, dysplastic ceratohyal arches and pharyngeal arches in ethanol-treated group (13.33±1.11) (Fig. 2B,F).

Tbx1 and *hand2* are expressed at the pharyngeal arches (Fig. 3C,D). The results of *in situ* hybridization showed that in the ethanol-treated group, the expressions of *tbx1* (13.00 ± 1.11) and *hand2* (13.33 ± 1.11) at pharyngeal arches were decreased (Fig. 3J,K). The result of Q-PCR also showed that the relative expression levels of *tbx1* at 36 hpf and *hand2* at 48 hpf in the ethanol-treated group were markedly reduced compared with those in the control group (Fig. 7).

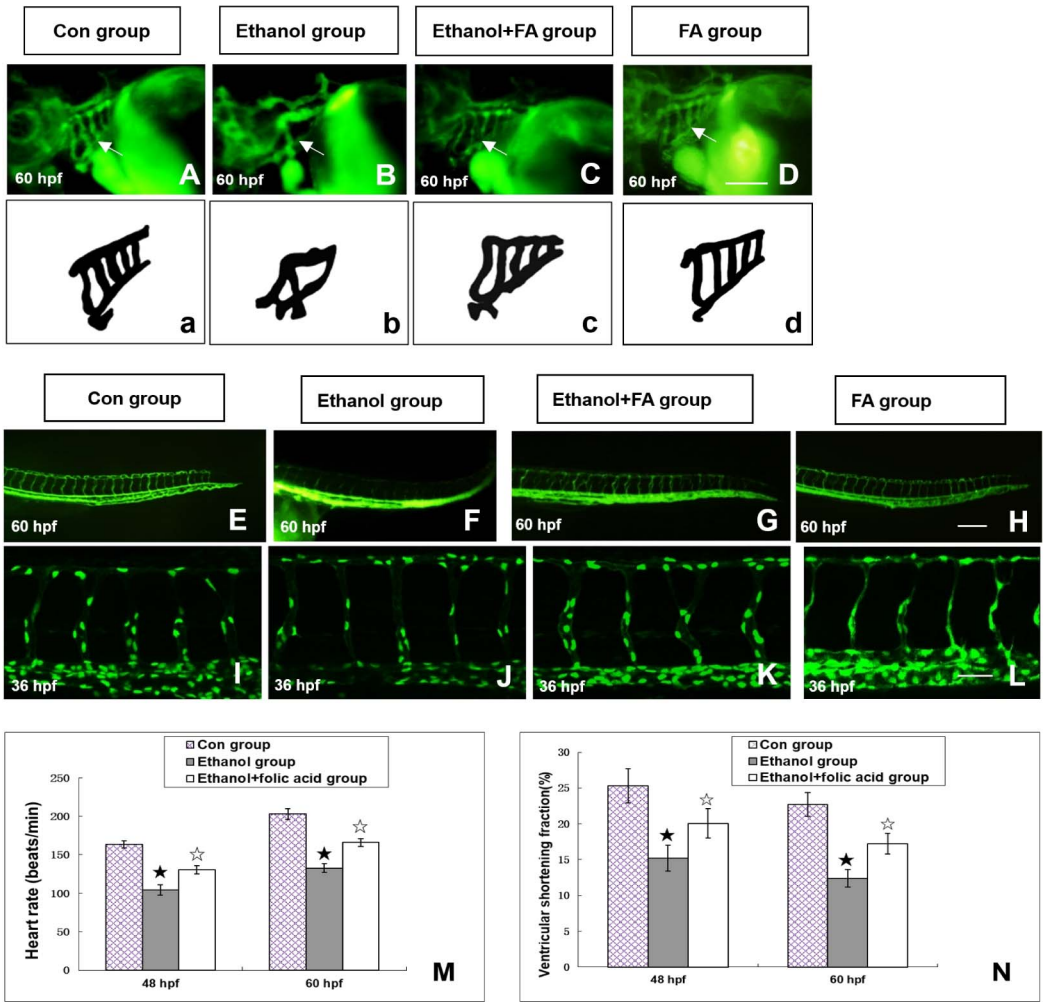

Figure 1. The development of pharyngeal arch arteries and vessels and cardiac function analysis (A–D) Fluorescent images of the pharyngeal arch arteries (white arrows) detected by fluorescein microangiography. (a–d) Conceptual diagram of A–D. (E–H) Fluorescent images of the vessels detected by fluorescein microangiography. (I–L) Fluorescent image of the vessels of live *Tg(fli1:neGFP)y7* embryos. The experiment was repeated three times (n=20). (M,N) Cardiac function was analyzed by heart rate and VSF. The experiment was repeated three times (n=100). Data are shown as the mean±SD. Comparisons between groups were made with ANOVA (t-test with Bonferroni correction). ★P<0.05 compared with control group. ☆P<0.05 compared with ethanol group. Bar: 125 μm (A–D), 125 μm (E–H), 30 μm (I–L).

Ethanol exposure induced defects in the brain and decreased the expression of *huc* and *ngn1*

The paraffin section and H&E staining showed craniocerebral dysplasia in the ethanol-treated embryos (14.00±2.00). The number of brain cells and the craniocerebral volume were decreased in the ethanol-treated group compared with those in the control group (Fig. 2J).

In controls, *huc* was expressed in the brain and neural tube, while *ngn1* was expressed in the brain and spinal chord (Fig. 3E,F). Compared with control group, the expressions of *huc* (14.67±1.78; Fig. 3L) and *ngn1* (14.67±1.56; Fig. 3M) were both decreased in the ethanol-treated group. The results of Q-PCR confirmed that the expression levels of *huc* and *ngn1* in ethanol-treated group were reduced when compared with those in the control group (Fig. 7).

Ethanol exposure perturbed hemoglobin formation

Erythrocytes in the 48 hpf embryos were identified by O-dianisidine staining, in which the presence of hemoglobin in erythrocytes was shown by a brown color after staining. The results of O-dianisidine staining showed decreased hemoglobin synthesis in the ethanol-treated group (15.67±1.11) compared with the controls (Fig. 4A,B). The expressions of *gata-1* and *c-myb* were detected in hematopoietic cells. Compared with the controls, ethanol-treated group showed decreased expressions of *gata-1* (12.00±0.67; Fig. 4D,E) and *c-myb* (14.00±1.33; Fig. 4G,H), as revealed by both *in situ* hybridization and Q-PCR (Fig. 7).

Ethanol exposure produced defective somites

Compared with the controls, the tail of the ethanol-treated embryos was shortened with dysplasia (16.00±0.67). Ethanol exposure resulted in dysplasia of the chevron-shaped somites with an angle of 120° (Fig. 2N,n), while that angle in the controls was 90° (Fig. 2M,m).

In the control group, *titin* was high expressed at somite (Fig. 3G). The expression of *titin* at somite in ethanol-treated embryos was reduced (13.67±0.89) (Fig. 3N). The result of Q-PCR showed that

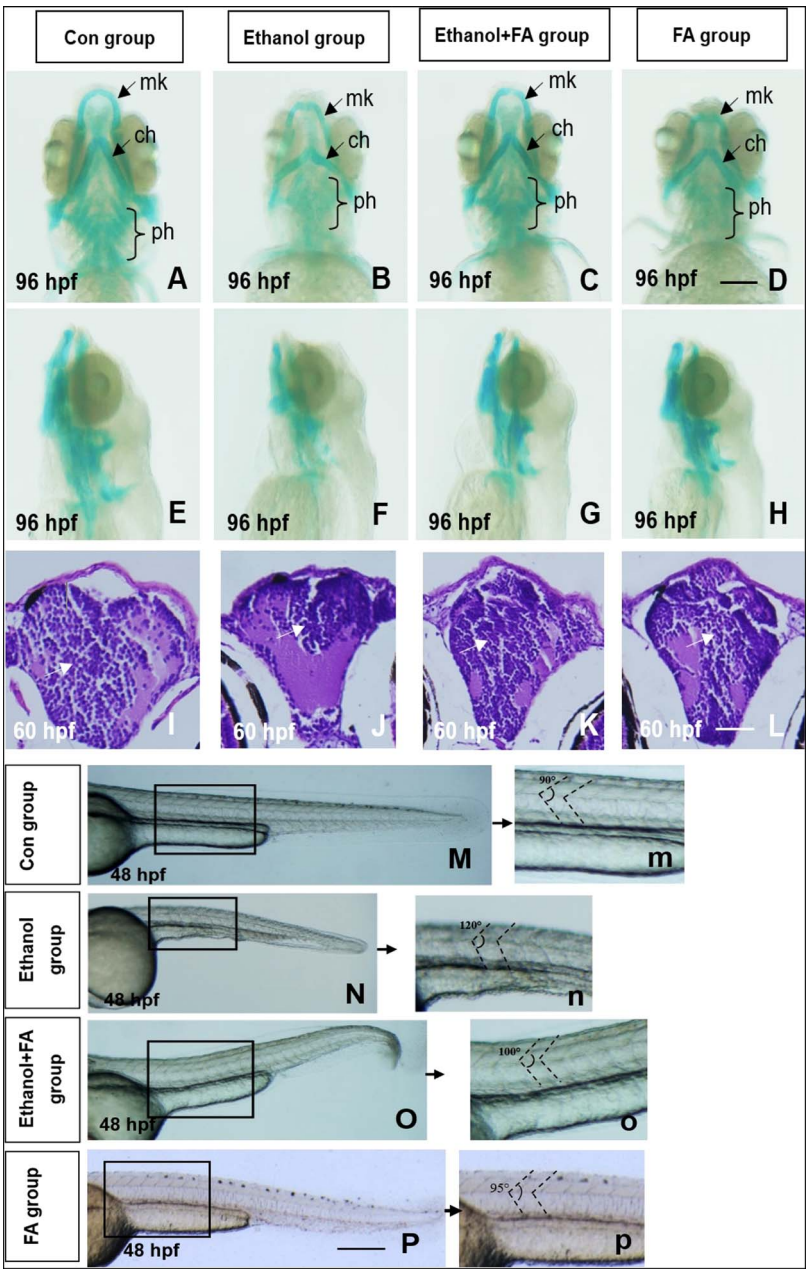

Figure 2. The development of craniofacial cartilages, pharyngeal arches, brain and somites (A–H) Craniofacial cartilages and pharyngeal arches were shown by Alcian blue staining. mk: Meckel's cartilage, ch: ceratohyal arch, ph: pharyngeal arches. (I–L) Craniocerebral were shown by paraffin section and H&E staining. The white arrow indicates the brain cells. (m–p) The enlarged images of black rectangles in M–P. (A–D) Ventral views, heads to the top. (E–H) Left-lateral views with heads to the top. (I–L) Dorsal views, heads to the top. (M–P) Left-lateral views, head to the left. All experiments were repeated three times (n=20). Bar: 125 μm (A–H), 25 μm (I-L), 250 μm (M-P).

the expression level of *titin* at 36 hpf was also decreased in the ethanol-treated group compared with that in the control group (Fig. 7).

Ethanol exposure increased apoptosis

Compared with the controls (Fig. 5A,D), ethanol-treated embryos exhibited the increased apoptosis in the upper part of the body at 10 hpf (13.67±1.53) and in the entire body at 20 hpf (16.33±1.15; Fig. 5B,E).

FA supplementation at 6–12 hpf can best rescue the teratogenic effects of ethanol

The number of dysplasia embryos at 72 hpf and the number of dead individuals at fifth day after fertilization were counted in all groups. Comparing with the ethanol control, five groups with FA supplementation at 0–6 hpf, 6–12 hpf, 12–18 hpf, 18–24 hpf and 24–30 hpf showed obviously decreased number of malformation and death (Fig. 6A,B). These results demonstrated that FA supplementation before 30 hpf can rescue the teratogenic effects of ethanol. Among these five groups, the number of malforma-

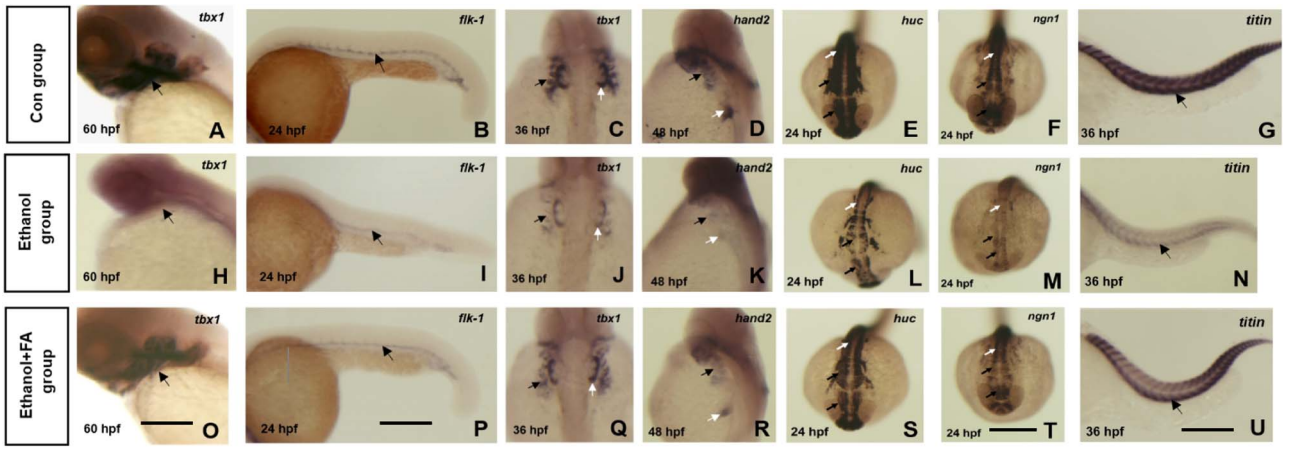

Figure 3. Expressions of *tbx1, flk-1, hand2, huc, ngn1* and *titin* The expression of genes was detected by whole-mount *in situ* hybridization. (A,H,O) The expression of *tbx1* in pharyngeal arch arteries (black arrows). (B,I,P) The expression of *flk-1* in vessels (black arrows). (C,J,Q) The expressions of *tbx1* in pharyngeal arches (black arrows) and ears (white arrows). (D,K,R) The expressions of *hand2* in pharyngeal arches (black arrows) and fins (white arrows). (E,L,S) The expressions of *huc* in brain (black arrows) and neural tube (white arrows). (F,M,T) The expressions of *ngn1* in brain (black arrows) and spinal chord (white arrows). (G,N,U) The expressions of *titin* in somites (black arrows). All experiments were repeated three times (n=20). Bar: 125 μm (A,H,O), 165 μm (B–D,I–K,P–R), 250 μm (E,F,L,M,S,T), 225 μm (G,N,U).

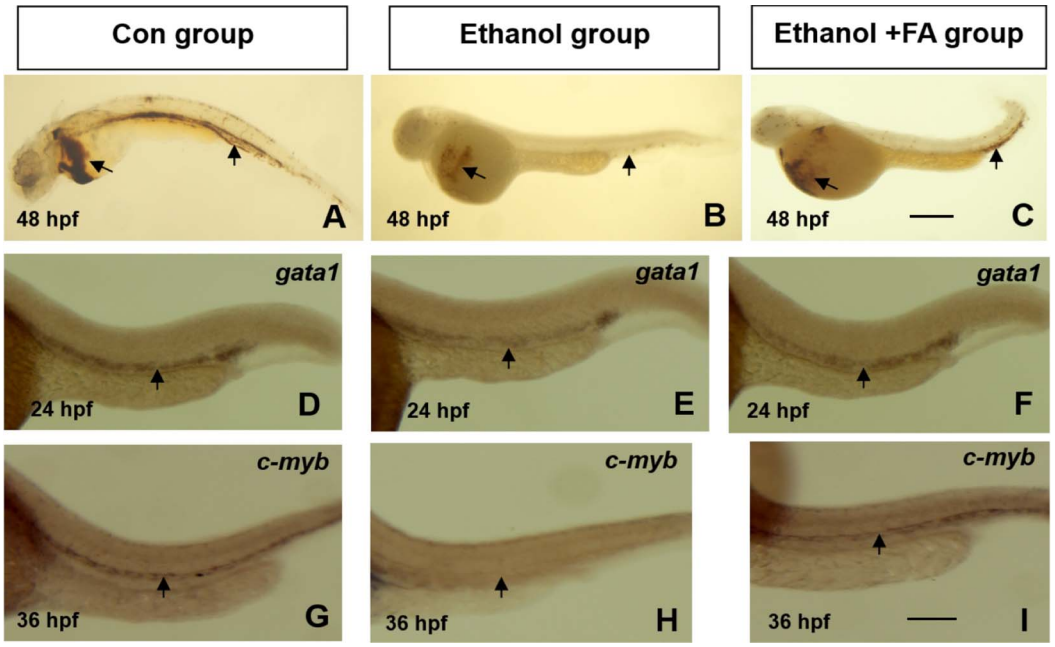

Figure 4. Hematopoiesis and expressions of *gata-1* and *c-myb* (A–C) O-dianisdine staining. The hemoglobin in erythrocytes was shown by brown color after staining (black arrows). (D–F) The expressions of *gata1* in erythroid progenitor cell (black arrows). (G–I) The expressions of *c-myb* in hematopoietic stem cell (black arrows). All experiments were repeated three times (n=20). Bar: 250 μm (A–C), 125 μm (D–F,G–I).

tion and death was reduced most evidently when FA was supplemented at 6–12 hpf (Fig. 6A,B). These results indicated that FA supplementation at 6–12 hpf can best rescue the teratogenic effects of ethanol, then ethanol-treated embryos with FA supplementation at 6–12 hpf was defined as ethanol+FA group. Ethanol-treated embryos developed obvious dysplasia (Fig. 6D), and the malformation of ethanol+FA embryos was improved (Fig. 6E). FA-treated embryos showed no obvious developmental abnormalities (Fig. 6F).

FA supplementation prevented ethanol-induced defects and apoptosis

Compared with that in the ethanol group, the number of embryos with malformations of the pharyngeal arch arteries (8.00±0.67; Fig. 1C,c) and intersomitic vessels (9.67±1.11; Fig. 1G,K) in the FA+ethanol group was reduced, and the heart rate and VSF were increased (Fig. 1M,N), which indicated that the abnormal cardiac function and the malformation of the pharyngeal arch arteries and intersomitic vessels were obviously improved. The expressions of

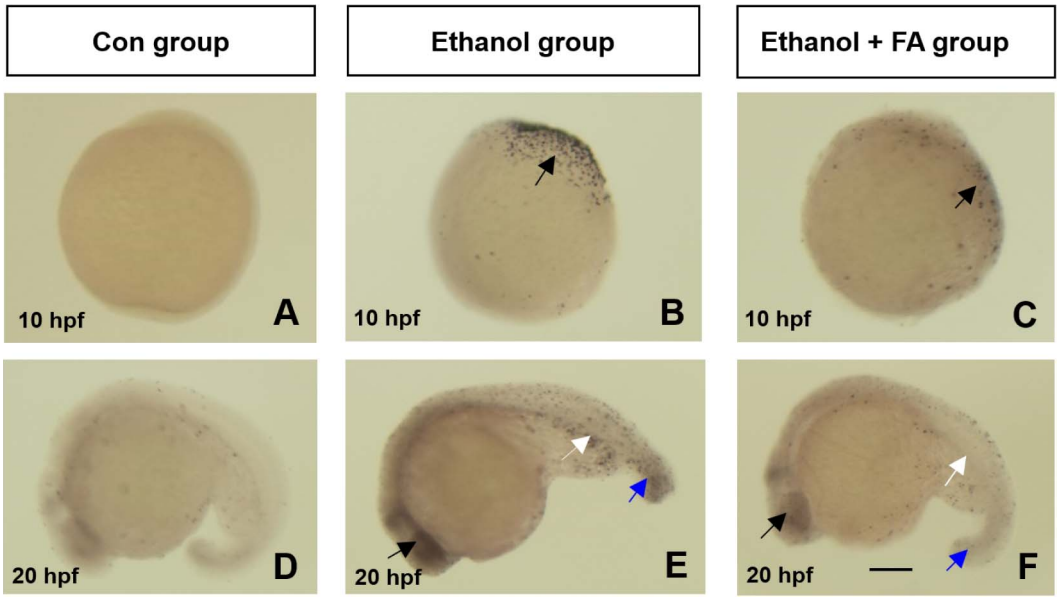

Figure 5. Cell apoptosis detected by whole-mount TUNEL staining (A,D) Control group at 10 hpf and 20 hpf. (B) Ethanol group at 10 hpf, the apoptosis was increased in upper part of the body (black arrow). (E) Ethanol group at 20 hpf, enhanced apoptosis was detected at the head (black arrow), body (white arrow) and tail (blue arrow). (C,F) Ethanol+FA group at 10 hpf and 20 hpf, apoptosis was decreased compared with the ethanol group. All experiments were repeated three times (n=20). Bar: 125 μm.

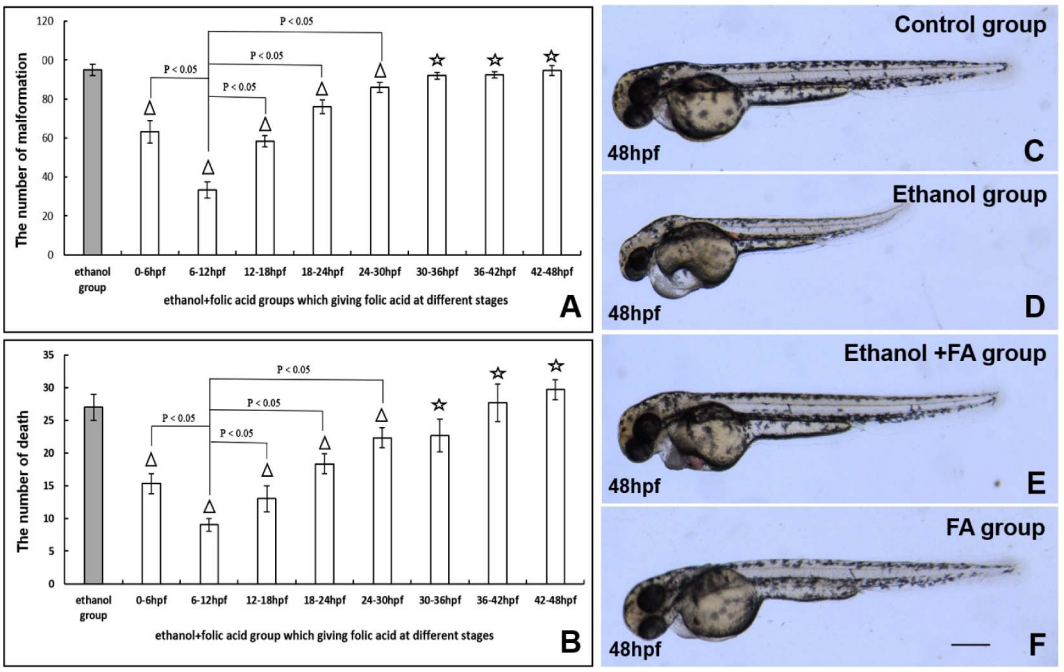

Figure 6. FA supplementation rescued the teratogenic effects of ethanol Ethanol group was treated with 400 mM ethanol at 2–48 hpf. FA rescue groups were given FA (75 μM) to ethanol group at 0–6 hpf, 6–12 hpf, 12–18 hpf, 18–24 hpf, 24–30 hpf, 30–36 hpf, 36–42 hpf and 42–48 hpf. (A) Analysis of the number of dysplasia embryos in the ethanol-treated group and FA rescue groups at 72 hpf. (B) Analysis of the number of dead embryos in the ethanol-treated group and FA rescue groups on Day 5 after fertilization. Data are shown as the mean±SD. (n=100, the experiment was repeated three times). Comparisons between groups were made with ANOVA (t-test with Bonferroni correction). $\Delta P<0.05$ compared between ethanol group and FA rescue groups. ☆$P>0.05$ compared between ethanol group and FA rescue groups. FA rescue group (6–12 hpf) were compared with other FA rescue groups (0–6 hpf, 12–18 hpf, 18–24 hpf, 24–30 hpf), respectively, $P<0.05$. (C) Control group. (D) Ethanol group. (E) Ethanol+FA (6–12 hpf) group. (F) FA group (treated with 75 μM FA at 6–12 hpf). Bar: 250 μm.

tbx1 in the pharyngeal arch arteries at 60 hpf (Fig. 3O) and the expressions of *flk-1* (Fig. 3P) at 24 hpf were increased in the FA+ethanol group compared with that in the ethanol group. The number of embryos with decreased *tbx1* expression (7.00±0.67 vs 10.00±1.33) and *flk-1* expression (6.67±1.11 vs 11.33±1.11) in the FA+ethanol groups was reduced compared to those in the ethanol group.

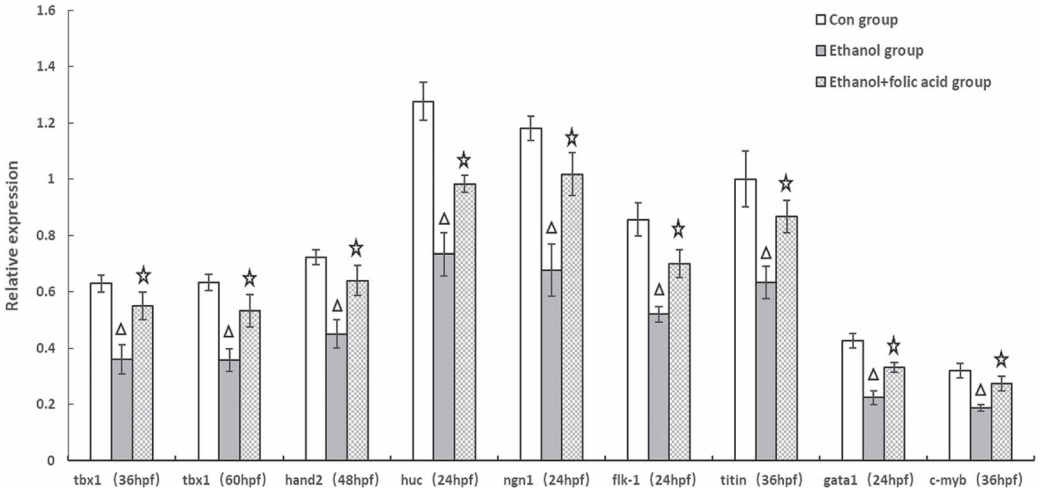

Figure 7. Expressions of genes detected by Q-PCR Data are expressed as the mean±SD of three separate Q-PCR. Comparisons between groups were made with ANOVA (*t*-test with Bonferroni correction). $\Delta P<0.05$ compared with the control group, $\star P<0.05$ compared with the ethanol group.

The dysplastic of craniofacial cartilage and pharyngeal arches in the FA+ethanol group was improved compared with the ethanol-treated group (Fig. 2C,G). In the FA+ethanol group, the number of embryos with dysplastic cartilaginous structures was reduced (8.33±0.44). The expressions of *tbx1* and *hand2* at pharyngeal arches were increased in the FA+ethanol group (Fig. 3Q,R). The number of embryos with decreased *tbx1* expression (7.00±0.67 vs 13.00±1.11) and *hand2* expression (6.67±1.11 vs 13.33±1.11) in the FA+ethanol groups was reduced compared with that in the ethanol group.

The development of craniocerebral (the number of dysplasia was 6.67±1.11 vs 14.00±2.00; Fig. 2K) and somites (the number of dysplasia was 8.67±1.11 vs 16±0.67; Fig. 2O,o) was recovered in the FA+ethanol group. The angle of chevron-shaped somites in the FA+ethanol group was 100° (Fig. 2o), while that angle in the ethanol group was 120° (Fig. 2n). The expressions of *huc*, *ngn1* and *titin* were increased in the FA+ethanol group (Fig. 3S,T,U). In the FA+ethanol groups, the number of embryos with decreased *huc, ngn1* and *titin* expressions (7.00±1.33 vs 14.67±1.78, 8.00±0.60 vs 14.67±1.56, 7.00±1.33 vs 13.67±0.89) was reduced, compared with those in the ethanol group.

The hemoglobin synthesis detected by O-dianisidine staining (the number of decreased hemoglobin synthesis was 7.00±0.67 vs 15.67±1.11; Fig. 4C) and the expressions of *gata-1* and *c-myb* detected by *in situ* hybridization in the FA+ethanol group were all increased (Fig. 4F,I). In the FA+ethanol groups, the number of embryos with decreased *gata-1* and *c-myb* expressions (6.30±1.10 vs 12.00±0.67 and 9.00±1.78 vs 14.00±1.33, respectively) was reduced compared with those in the ethanol group.

Compared with those in the ethanol group, the overall expression levels of *tbx1* (both at 60 hpf and 36 hpf), *flk-1*, *hand2*, *ngn1*, *huc*, *titin*, *gata-1* and *c-myb* were all enhanced in the FA+ethanol group, as measured by Q-PCR (Fig.7).

Apoptosis in the FA+ethanol group were reduced both at 10 hpf (the number of apoptosis embryos was 5.33±0.58 vs 13.67±1.53) and at 20 hpf (8.33±1.53 vs 16.33±1.15) compared with those in their corresponding ethanol groups (Fig. 5C,F).

In order to determine whether the administration of FA (75 μM) at 6–12 hpf can cause embryonic abnormalities or not, we observed the development of FA-treated embryos. Compared with the controls, the overall development (Fig. 6F), the pharyngeal arch arteries, vessels (Fig. 1D,d,H,L), craniofacial cartilage (Fig. 2D,H), brain and somites (Fig. 2L,P,p) of the FA-treated embryos showed no obvious developmental abnormalities.

Discussion

Embryonic development is sensitive to environmental teratogens, such as ethanol, which produce severe defects in the embryo [21,22]. Ethanol is able to cross the placental barrier, allowing direct access to the developing embryo. In humans, FASD is associated with maternal alcohol consumption. Numerous potential mechanisms have been proposed to explain the effects of ethanol on the developing embryo. Ethanol exposure changes the gene expression and cell adhesion, increases cellular apoptosis and disrupts cellular metabolism [23,24]. Ethanol exposure also changes the DNA methylation pattern and causes epigenetic changes [25,26]. Investigations in ethanol teratogenicity in animal models can help to clarify the mechanism and to provide clues for effective prevention strategies of FASD.

In this study, we explored the teratogenic effects of ethanol on zebrafish multiple organs, such as pharyngeal arch arteries, vessel, craniofacial cartilage, pharyngeal arches, brain, somite and hematopoietic system. Previous study by Sarmah and Marrs [14] showed that ethanol perturbed multiple steps of cardiogenesis, and we further investigated whether ethanol can cause impaired cardiac function. We also explored whether FA supplementation can effectively prevent these ethanol-induced defects.

Our results demonstrated that ethanol, which is the environmental teratogens, produced severe defects in multiple organs, which exhibited similar malformations with human FASD, such as the craniofacial abnormalities, central nervous system damage, vascular dysplasia and short tails with abnormal somite. We also found that ethanol caused cardiac dysfunction and disrupted the formation of pharyngeal arches and hemoglobin. The expression levels of genes that play important roles in the development of above organs in several vertebrate species were further detected.

Tbx1 is a member of the T-box family of transcriptional regulators and *tbx1* is the major gene underlying del22q11.2 or the DiGeorge syndrome in humans. It is required for the formation of out-flow tract and pharyngeal arches [27–29]. *Hand2* is a basic helix-

loop-helix transcription factor, and it is expressed in the neural crest derivative tissues, lateral plate mesoderm, pharyngeal arches and heart [30,31]. *Ngn1* (neurogenin1) is also the basic helix-loop-helix transcription factor and is expressed in specific regions within the developing brain and spinal cord. *Ngn1* directs neuronal differentiation of progenitor cells during development [32–34]. *Huc* is the important neuronal marker, which is necessary for nervous system development. Huc belongs to the family of RNA-binding proteins implicated in neuronal differentiation and maintenance [35,36]. *Flk-1* is the receptor of VEGF and one of the earliest markers for angioblasts, which is indispensable for the migration of angioblasts from ventral mesoderm to midline [37]. *Flk-1* is crucial for further formation of the vascular system [38]. *Gata-1* is essential for erythropoiesis and erythroid development. The dysfunction of *gata-1* is related to aplastic anemia [39,40]. *C-myb* plays critical roles in the differentiation of hematopoietic progenitor cells [41,42]. *Titin* acts as a scaffold for signaling proteins in muscle and is responsible for establishing and maintaining the structure and elasticity of sarcomeres in striated muscle [43,44]. In this study, we found that the expression levels of *tbx1*, *flk-1*, *hand2*, *ngn1*, *huc*, *titin*, *gata-1* and *c-myb* were reduced in ethanol-treated group.

Ethanol exposure may result in DNA instability and reduced cell proliferation. We detected apoptosis by using TUNEL assay, which is a method for detecting DNA breakage by labeling the terminal end of nucleic acids. In this study, excessive apoptosis were detected in ethanol-treated embryos, which indicated that ethanol could promote apoptosis.

Our results showed that the expression levels of *tbx1*, *flk-1*, *hand2*, *ngn1*, *huc*, *titin*, *gata-1* and *c-myb* were reduced and the apoptosis was increased in ethanol-treated group, and the disrupted development in the ethanol-treated embryos was related to the reduced transcript levels of these genes and increased apoptosis.

The effects of ethanol on development may be influenced by comorbid environmental and nutritional factors. FA is an essential vitamin that participates in nucleic acid synthesis and repair. FA is also required for the production of methyl groups, which are subsequently used to methylate DNA during epigenetic events. FA also has antioxidant properties [45,46]. Exogenous FA may prevent abnormal epigenetic processes and may prevent disruptions generated by ethanol exposure. In the present study, we found that the diverse teratogenic effects of ethanol on zebrafish embryos could be extensive rescued by FA supplementation. Moreover, after FA supplementation to the ethanol-treated group, the reduced expression of genes in the ethanol-treated embryos was improved and the increased apoptosis in the ethanol-treated group was reduced. It is possible that the mechanism of FA rescuing teratogenic effects of ethanol may be related to the increased expressions of these genes and reduced apoptosis.

Although the specific molecular mechanisms remain to be defined, our investigation confirmed that FA protects the embryos from ethanol-induced defects. We also explored at which stage FA supplementation can most effectively rescue the teratogenic effects of ethanol. The results showed that FA supplementation at 6–12 hpf can best prevent ethanol's teratogenicity. In zebrafish, 6–12 hpf is at the gastrula period (5.25–10 hpf), which is the early embryonic and crucial developmental period. In the gastrula period, the onset of gastrulation occurs at 50%-epiboly (5.25 hpf). Moreover, the gastrula period ends (10 hpf) when epiboly is complete and the tail bud is formed. In the gastrula period, genes are strongly expressed and the morphogenetic cell movements of involution, convergence and extension occur, producing the primary germ layers and the embryonic axis. In this period, the embryo is very sensitive to the environmental factors. Moreover, this period is at the early embryonic development. Our study indicated that embryos with ethanol exposure should take FA supplementation at early stage of embryonic development to best rescue the teratogenic effect of ethanol.

Our study also demonstrated that ethanol exposure induced multiple defects in zebrafish embryos and these defects are similar to FASD in human. We found that the gene expression levels were reduced and the apoptosis was increased in ethanol-treated group. Our findings of ethanol-induced malformations in zebrafish may be an important step to understand the underlying molecular causes for FASD. Our future investigations will focus on the molecular mechanism underlying ethanol-induced abnormalities to better understand ethanol-induced deficits with FASD.

Our study also showed that multiple defects caused by ethanol exposure in zebrafish could be rescued by FA supplementation. We suggest that FA should be given at early stage of embryonic development to best attenuate the teratogenic effect of ethanol to fetus. This study may provide clues to the design of preventive measures for FASD.

The diverse effects of ethanol on zebrafish embryo development and the extensive rescue by FA supplementation suggest that a global development regulatory mechanism affects various developmental events. Our further research will help us to elucidate the teratogenic mechanism of ethanol and to find suitable therapeutic strategies for FASD, which is a frequent and devastating disorder in humans.

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References

1. G1 R, Cimmino L, Grasso GM. Alcohol consumption, pregnancy and fetal alcohol syndrome: implications in public health and preventive strategies. *Ann Ig* 2006, 18: 391–406.
2. Abel EL, Hannigan JH. Maternal risk factors in fetal alcohol syndrome: provocative and permissive influences. *Neurotoxicol Teratol* 1995, 17: 445–462.
3. May PA1, Gossage JP, Marais AS, Hendricks LS, Snell CL, Tabachnick BG, Stellavato C, *et al.* Maternal risk factors for fetal alcohol syndrome and partial fetal alcohol syndrome in South Africa: a third study. *Alcohol Clin Exp Res* 2008, 32: 738–753.
4. May PA, Gossage JP, Kalberg WO, Robinson LK, Buckley D, Manning M, Hoyme HE. Prevalence and epidemiologic characteristics of FASD from various research methods with an emphasis on recent in-school studies. *Dev Disabil Res Rev* 2009, 15: 176–192.
5. Bailey LB, Berry RJ. Folic acid supplementation and the occurrence of congenital heart defects, orofacial clefts, multiple births, and miscarriage. *Am J Clin Nutr* 2005, 81: 1213S–1217S.
6. Ionescu-Ittu R, Marelli AJ, Mackie AS, Pilote L. Prevalence of severe congenital heart disease after folic acid fortification of grain products: time trend analysis in Quebec, Canada. *BMJ* 2009, 338: b1673.

7. Meijer WM, de Walle HE, Kerstjens-Frederikse WS, de Jong-van den Berg LT. Folic acid sensitive birth defects in association with intrauterine exposure to folic acid antagonists. *Reprod Toxicol* 2005, 20: 203–207.
8. Sun S, Gui Y, Wang Y, Qian L, Liu X, Jiang Q, Song H. Effects of methotrexate on the development of heart and vessel in zebrafish. *Acta Biochim Biophys Sin* 2009, 41: 86–96.
9. Sun S, Gui Y, Jiang Q, Song H. Dihydrofolate reductase is required for the development of the heart and the outflow tract in zebrafish. *Acta Biochim Biophys Sin* 2011, 43: 957–969.
10. Sun SN, Gui YH, Wang YX, Qian LX, Jiang Q, Liu D, Song HY. Effect of dihydrofolate reductase gene knock-down on the expression of heart and neural crest derivatives expressed transcript 2 in zebrafish cardiac development. *Chin Med J (Engl)* 2007, 120: 1166–1171.
11. Ballard MS, Sun M, Ko J. Vitamin A, folate, and choline as a possible preventive intervention to fetal alcohol syndrome. *Med Hypotheses* 2012, 78: 489–493.
12. Wilson RD, Committee G, Wilson RD, Audibert F, Brock JA, Carroll J, Cartier L, *et al*. Pre-conception folic acid and mMultivitamin supplementation for the primary and secondary prevention of neural tube defects and other folic acid-sensitive congenital anomalies. *J Obstet Gynaecol Can* 2015, 37: 534–552.
13. Hutson JR, Stade B, Lehotay DC, Collier CP, Kapur BM. Folic acid transport to the human fetus is decreased in pregnancies with chronic alcohol exposure. *PLoS One* 2012, 7: e38057.
14. Sarmah S, Marrs JA. Complex cardiac defects after ethanol exposure during discrete cardiogenic events in zebrafish: prevention with folic acid. *Dev Dyn* 2013, 242: 1184–1201.
15. Jiang Q, Lagos-Quintana M, Liu D, Shi Y, Helker C, Herzog W, le Noble F. miR-30a acts as a positive regulator of tip cell differentiation and vessel branching by targeting dll4. *Hypertension* 2013, 62: 592–598.
16. Westerfield M. *The Zebrafish Book: A Guide for the Laboratory Use of Zebrafish (Danio rerio)*. Eugene, OR: University of Oregon Press, 1993, 3.1–3.26
17. Kimmel CB, Ballard WW, Kimmel SR, Ullmann B, Schilling TF. Stages of embryonic development of the zebrafish. *Dev Dyn* 1995, 203: 253–310.
18. Jiang Q, Liu D, Gong Y, Wang Y, Sun S, Gui Y, Song H. Yap is required for the development of brain, eyes, and neural crest in zebrafish. *Biochem Biophys Res Commun* 2009, 384: 114–119.
19. Hu J, Sun S, Jiang Q, Sun S, Wang W, Gui Y, Song H. Yes-associated protein (yap) is required for early embryonic development in zebrafish (danio rerio). *Int J Biol Sci* 2013, 9: 267–278.
20. Thisse C, Thisse B. High-resolution insitu hybridization to whole-mount zebrafish embryos. *Nat Protoc* 2008, 3: 59–69.
21. Gilbert-Barness E. Teratogenic causes of malformations. *Ann Clin Lab Sci* 2010, 40: 99e114.
22. Lipinski RJ, Hammond P, O'Leary-Moore SK, Ament JJ, Pecevich SJ, Jiang Y, *et al*. Ethanol-induced face-brain dysmorphology patterns are correlative and exposure-stage dependent. *PLoS One* 2012, 7: e43067.
23. Yelin R, Kot H, Yelin D, Fainsod A. Early molecular effects of ethanol during vertebrate embryogenesis. *Differentiation* 2007, 75: 393–403.
24. Muralidharan P, Sarmah S, Zhou FC, Marrs JA. Fetal alcohol spectrumdisorder (FASD) associated neural defects: complex mechanisms and potential therapeutic targets. *Brain Sci* 2013, 3: 964–991.
25. Mandal C, Halder D, Jung KH, Chai YG. Gestational alcohol exposure altered DNA methylation status in the developing fetus. *Int J Mol Sci* 2017, 18: E1386.
26. Kaminen-Ahola N, Ahola A, Maga M, Mallitt KA, Fahey P, Cox TC, Whitelaw E, *et al*. Maternal ethanol consumption alters the epigenotype and the phenotype of offspring in a mouse model. *PLoS Genet* 2010, 6: e1000811.
27. Xu H, Morishima M, Wylie JN, Schwartz RJ, Bruneau BG, Lindsay EA, Baldini A. Tbx1 has a dual role in the morphogenesis of the cardiac outflow tract. *Development* 2004, 31: 3217–3227.
28. Kelly RG, Papaioannou VE. Visualization of outflow tract development in the absence of Tbx1 using an FgF10 enhancer trap transgene. *Dev Dyn* 2007, 236: 821–828.
29. Morishima M, Yanagisawa H, Yanagisawa M, Baldini A. Ece1 and Tbx1 define distinct pathways to aortic arch morphogenesis. *Dev Dyn* 2003, 228: 95–104.
30. Zhao Y, Samal E, Srivastava D. Serum response factor regulates a muscle-specific microRNA that targets Hand2 during cardiogenesis. *Nature* 2005, 436: 214–220.
31. Laurent F, Girdziusaite A, Gamart J, Barozzi I, Osterwalder M, Akiyama JA, Lincoln J, *et al*. HAND2 target gene regulatory networks control atrioventricular canal and cardiac valve development. *Cell Rep* 2017, 19: 1602–1613.
32. McIntosh R, Norris J, Clarke JD, Alexandre P. Spatial distribution and characterization of non-apical progenitors in the zebrafish embryo central nervous system. *Open Biol* 2017, 7: 160312.
33. Nakada Y, Parab P, Simmons A, Omer-Abdalla A, Johnson JE. Separable enhancer sequences regulate the expression of the neural bHLH transcription factor neurogenin 1. *Dev Biol* 2004, 271: 479–487.
34. Wang L, Zhang ZG, Zhang RL, Jiao ZX, Wang Y, Pourabdollah-Nejad DS, LeTourneau Y, *et al*. Neurogenin 1 mediates erythropoietin enhanced differentiation of adult neural progenitor cells. *J Cereb Blood Flow Metab* 2006, 26: 556–564.
35. Hinman MN, Lou H. Diverse molecular functions of Hu proteins. *Cell Mol Life Sci* 2008, 65: 3168–3181.
36. Diks SH, Bink RJ, van de Water S, Joore J, van Rooijen C, Verbeek FJ, den Hertog J, *et al*. The novel gene asb11: a regulator of the size of the neural progenitor compartment. *J Cell Biol* 2006, 174: 581–592.
37. Liang D, Chang JR, Chin AJ. The role of vascular endothelial growth factor (VEGF) in vasculogenesis, angiogenesis, and hematopoiesis in zebrafish development. *Mech Dev* 2001, 108: 29–43.
38. Zhong TP, Childs S, Leu JP. Gridlock signalling pathway fashions the first embryonic artery. *Nature* 2001, 414: 216–220.
39. Dore LC, Amigo JD, Dos Santos CO, Zhang Z, Gai X, Tobias JW, Yu D, *et al*. A GATA-1- regulated microRNA locus essential for erythropoiesis. *Proc Natl Acad Sci U S A* 2008, 105: 3333–3338.
40. Shimizu R, Trainor CD, Nishikawa K, Kobayashi M, Ohneda K, Yamamoto M. GATA-1 self-association controls erythroid development *in vivo*. *J Biol Chem* 2007, 282: 15862–15871.
41. Greig KT, Carotta S, Nutt SL. Critical roles for c-Myb in hematopoietic progenitor cells. *Semin Immunol* 2008, 20: 247–256.
42. Lidonnici MR, Corradini F, Waldron T, Bender TP, Calabretta B. Requirement of c-Myb for p210(BCR/ABL)-dependent transformation of hematopoietic progenitors and leukemogenesis. *Blood* 2008, 111: 4771–4779.
43. Camara-Pereira ES, Campos LM, Vannier-Santos MA, Mermelstein CS, Costa ML. Distribution of cytoskeletal and adhesion proteins in adult zebrafish skeletal muscle. *Histol Histopathol* 2009, 24: 187–196.
44. Steffen LS, Guyon JR, Vogel ED, Howell MH, Zhou Y, Weber GJ, Zon LI, *et al*. The zebrafish runzel muscular dystrophy is linked to the titin gene. *Dev Biol* 2007, 309: 180–192.
45. Ibrahim W, Tousson E, El-Masry T, Arafa N, Akela M. The effect of folic acid as an antioxidant on the hypothalamic monoamines in experimentally induced hypothyroid rat. *Toxicol Ind Health* 2012, 28: 253–261.
46. Joshi R, Adhikari S, Patro BS, Chattopadhyay S, Mukherjee T. Free radical scavenging behavior of folic acid: evidence for possible antioxidant activity. *Free Radic Biol Med* 2001, 30: 1390–1399.