

Effects of methotrexate on the developments of heart and vessel in zebrafish

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Methotrexate (MTX), an antagonist of folic acid, can inhibit dihydrofolate reductase (DHFR) which is of great importance in the synthesis of tetrahydrofolic acid and embryonic development. In this study, we found that after being exposed to 1.5 mM MTX at 6–10 hours post-fertilization, zebrafish embryos fail to form normal cardiovascular system. In MTX-treated embryos, the morphological development of ventricle and atrium was disrupted, the cardiac twist was abnormal, the heart rate and ventricular shortening fraction were reduced, and the vascular development was disrupted. We also found that either microinjection with *dhfr-gfp* mRNA or treatment with folic acid calcium salt pentahydrate (CF) could cause improved development in the heart and vessels in MTX-treated embryos, which proved that MTX induced the malformations by inhibiting DHFR. The transcript levels of genes such as *hand2*, *mef2a*, *mef2c*, and *flk-1* were reduced in MTX-treated embryos. Compared with the MTX-treated group, the transcript levels of *hand2*, *mef2a*, *mef2c*, and *flk-1* were increased in the MTX + *dhfr-gfp* mRNA-injected group and in the MTX + CF group. Our results indicated that the disrupted development of the heart and vessels in MTX-treated embryos is related to the reduced transcript levels of *hand2*, *mef2a*, *mef2c*, and *flk-1*.

Keywords methotrexate; folic acid; heart; vessel; gene transcript level; zebrafish

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Introduction

The cardiovascular defects are the major cause of deaths, and the incidence of congenital heart defects (CHDs) is about 1% of live birth [1,2]. Recently, more and more

studies have shown that folic acid dysfunction is related with CHD [3]. Methotrexate (MTX) is the antagonist of folic acid. MTX has multiple therapeutic uses including treatment for cancers, ectopic pregnancy, and autoimmune disorders. More frequent use of MTX may result in an increased number of exposures in pregnant women and their fetuses. Analysis of a large case–control study suggested that during early pregnancy, the use of MTX increased the risk of congenital heart disease in new-borne babies [4]. MTX can inhibit the function of dihydrofolate reductase (DHFR) that is critical for nucleotide synthesis and methylation, both of which play essential roles in embryonic development. DHFR structure is highly conserved in mammals. Zebrafish DHFR is about 60% identical to human DHFR. Similar structural and kinetic properties were revealed between zebrafish and human DHFR [5]. A better understanding of effects of MTX and DHFR on cardiovascular development may provide important clues to the study of the relationship between folic acid deficiency and CHD in human.

Folic acid dysfunction leading to malformations in cardiovascular system has been proved in different animal models, such as chick, mouse, and so on. These animal models showed delayed heart development [6], reduced thickness of cardiac ventricular compact walls, fewer embryonic myocardium [7], and conotruncal heart defects [8]. However, none of the fetuses in these studies can live to birth; few of these investigations referred to the transcript levels of genes and none was carried out with zebrafish. No investigation about the effects of MTX on zebrafish development was found in literatures. Zebrafish offers several distinct advantages for genetic and embryological studies including external fertilization, rapid development, and optical clarity of its embryos [9]. It is an attractive and widely used vertebrate model for studying cardiovascular development.

Zebrafish is particularly suitable for our study because severe defects in the heart do not lead to immediate lethality as in many vertebrate models, and developing zebrafish can survive the first week of life without functional circulation [10].

In this study, we treated zebrafish embryos with MTX and observed the malformations in the heart and vessels of MTX-treated embryos. The transcript levels of genes that are very important for cardiovascular development were detected by real-time polymerase chain reaction (PCR). *dhfr*-increased transcribing experiment and tetrahydrofolic-acid-rescuing experiment were also carried out to investigate how MTX induced the malformations.

Materials and Methods

Materials

Folic acid antagonist, MTX of >99.0% purity (HPLC) was from Sigma (St. Louis, USA) (Product Number M8407). Folinic acid calcium salt pentahydrate (CF) of $\geq 99.0\%$ purity (HPLC) was from Fluka (St. Louis, USA) (Product Number 47612). Wild-type AB line zebrafish was obtained from University of Oregon (Eugene, USA). The breeding facility was bought from Aquatic Habitats Corporation (Apopka, USA). Fish and embryos were maintained, collected, and staged as previously described [11,12].

Drug exposure

MTX was dissolved in 250 mM sodium bicarbonate to prepare the stock solution (200 mM, pH 7.8). To obtain a series of work solutions, stock solution was diluted with egg water (60 mg/l Instant Ocean salts, pH 7.2). Control group was treated with same concentration of sodium bicarbonate solution. CF was dissolved into egg water to a final concentration of 5 mM. Each group contained 50 embryos and after exposure, the embryos were transferred into the egg water.

Microinjection of *dhfr-gfp* mRNA

dhfr-gfp (green fluorescent protein) mRNA was synthesized using the mMACHINE system (Ambion, Austin, USA) from the linearized pT7TS-*dhfr-gfp* plasmids (zebrafish *dhfr*, digested with *EcoRI*, transcribed with T7); *dhfr-gfp* mRNA was diluted to 40 $\mu\text{g/ml}$ in solution (0.1% phenol red, 0.2 M KCl, pH 7.0). Embryos were divided into five groups. Embryos in each group were microinjected with 1, 3, 6, 9, or 12 nl of *dhfr-gfp* mRNA, respectively, into the

blastomeres of one-to-two-cell-stage embryos. After the microinjection, the numbers of embryos with green fluorescence at 12 hours post-fertilization (hpf) were calculated under the fluorescent microscope and the numbers of embryos with malformation were calculated under the optical microscope at 48 hpf in each group.

Morphological observation and functional evaluation of the heart

The abnormal phenotypes of the heart were observed under microscope at 48 hpf and the numbers of abnormal cardiac embryos were calculated in each group. Embryos were removed and transferred to modified Tyrode's solution (136 mM NaCl, 5.4 mM KCl, 0.3 mM NaH_2PO_4 , 1.8 mM CaCl_2 , 1 mM MgCl_2 , 10 mM HEPES, 5 mM glucose, pH 7.3) at 48, 54, 60, and 72 hpf to obtain the heart rate record and ventricular contractility analysis. Heart rates were measured under a dissecting microscope. The diastolic and systolic lengths of ventricle were measured with a TK-C1381 video camera (JVC, Tokyo, Japan). Ventricular shortening fraction (VSF) was calculated with the following formula according to the standard method previously described [13,14]: $\text{VSF} = (\text{ventricular length at diastole} - \text{ventricular length at systole}) / \text{ventricular length at diastole}$.

Microangiography

Fluorescein (2 – 5 nl) was micro-injected into the heart of living zebrafish embryos at 60 hpf. About 2 – 3 min later, vascular images were observed on a BX61 fluorescent microscope (Olympus, Tokyo, Japan) and collected with a DP70 digital camera (Olympus).

Whole-mount *in situ* hybridization

Whole-mount *in situ* hybridization was performed with RNA anti-sense probes which were labeled with digoxigenin for the following genes: dihydrofolate reductase gene (*dhfr*; Accession No. NM 131775), ventricular myosin heavy chain (*vmhc*; Accession No. NM 001112733), atrial myosin heavy chain (*amhc*; Accession No. NM 198823), the heart and neural crest derivatives expressed transcript 2 (*hand2*; Accession No. NM 131626), myocyte-specific enhancer factor 2A (*mef2a*; Accession No. NM 131301), myocyte-specific enhancer factor 2C (*mef2c*; Accession No. NM 131312), and kinase insert domain receptor (*flk-1*; Accession No. NM 131472). Embryos older than 24 hpf were treated with 0.003% phenylthiourea (sigma) in egg water to inhibit pigment production. Whole-mount *in situ* hybridization ($n = 20$) was performed according to standard

Table 1 Sequence-specific primers used in this study

<i>hand2</i>	F: 5'-GCCAAAGAAGAAAGGCGAAA-3'	R: 5'-TGTCATTGCTGCTCCCTGAA-3'
<i>mef2a</i>	F: 5'-GAACCGGCAGGTTACCTTTA-3'	R: 5'-GGGCAATCTCACAGTCACAC-3'
<i>mef2c</i>	F: 5'-AATCCGAGGACAAATATCGC-3'	R: 5'-TTAGACTGAGGGATGGCACA-3'
<i>flk-1</i>	F: 5'-CAATGGCAGGATTCACCTTGAG-3'	R: 5'-TTCATAAGGAGCGGATCAATCGTACTCACC-3'
β -actin	F: 5'-GTCCACCTCCAGCAGATGT-3'	R: 5'-GAGTCAATGCGCCATACAGA-3'

procedures [15]. Images were acquired using BX61 and SZX12 microscopes equipped with a DP70 digital camera.

RNA extraction and semi-quantitative real-time PCR analysis

Total RNA was extracted using TRIzol reagent (Invitrogen) according to the manufacturer's protocol. RNAs extracted from micro-dissected hearts of embryos of 48 hpf ($n = 50$) and 18 hpf ($n = 50$) were used to detect transcript levels of *hand2*, *mef2a*, and *mef2c*. RNAs extracted from embryos of 24 hpf ($n = 50$) and 36 hpf ($n = 50$) were used to detect the transcript level of *flk-1*. RNAs were reverse-transcribed using oligo-dT primer. SYBR green method was used to quantify cDNA. The sequence-specific primers were listed in **Table 1**. Real-time quantitative PCR was done using the ABI Prism 7000 Sequence Detection System (Perkin-Elmer Applied Biosystem, Inc.). Amplification conditions were identical for all reactions and consisted of: 95°C for 3 min, then 45 s at 95°C, annealing for 45 s at 56°C, and extension for 45 s at 60°C for 40 cycles. Relative expression levels of each gene were computed with respect to the amount of β -actin. Value of expression level of each gene was divided by β -actin in different individual cDNA samples. Results thus show each value as the mean \pm SD of three separate semi-quantitative PCRs ($n = 3$). Specificity of each reaction was controlled by melting curve analysis, which began at 50°C and increased to 95°C in 1°C increments.

Statistical analysis

All the data were presented as mean \pm SD. Comparisons between groups were made with ANOVA (t -test with Bonferroni correction). A P -value < 0.05 was considered statistically significant. All the data were analyzed with the SPSS 13.0 software (SPSS, USA).

Results

Expression of *dhfr* in zebrafish at 10 hpf

The result of *in situ* hybridization showed that at 10 hpf, *dhfr* expressed in whole organism that includes the head, the mesoderm, and the tail bud (**Fig. 1**).

MTX-induced malformations of the heart and vessels in zebrafish

We found that exposure of embryos before 6 hpf to MTX cause embryo death or severe overall malformations (data not shown). When embryos after 10 hpf were exposed to MTX, less effects of MTX on embryonic development were observed (data not shown). The dose-response curve in 6–10 hpf exposing stage showed that treatment with 1.5 mM MTX induced evident cardiac malformations in about 78% embryos [**Fig. 2(A)**], but less injury in developments of the head, fins, and somites. Therefore, in following experiments, embryos exposed to 1.5 mM MTX at 6 – 10 hpf were defined as MTX-treated group. In the MTX-treated group, embryos developed abnormal cardiac morphology and pericardial edema at 48 hpf. Cardiac morphologic abnormalities included hypogenetic ventricle with dilating atrium, both ventricle and atrial dilating, or both ventricle and atrium hypogenetic as a linear tube

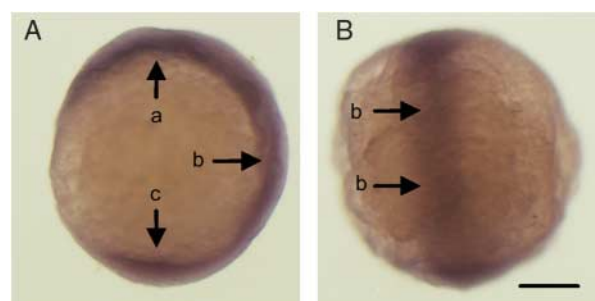


Fig. 1 Expression of *dhfr* in zebrafish at 10 hpf Expression of *dhfr* was detected in whole organism as arrows indicated. (A) Left lateral view, head toward top. (B) Dorsal view, head toward top. a, head; b, mesoderm; c, the tail bud. Bar = 300 μ m.

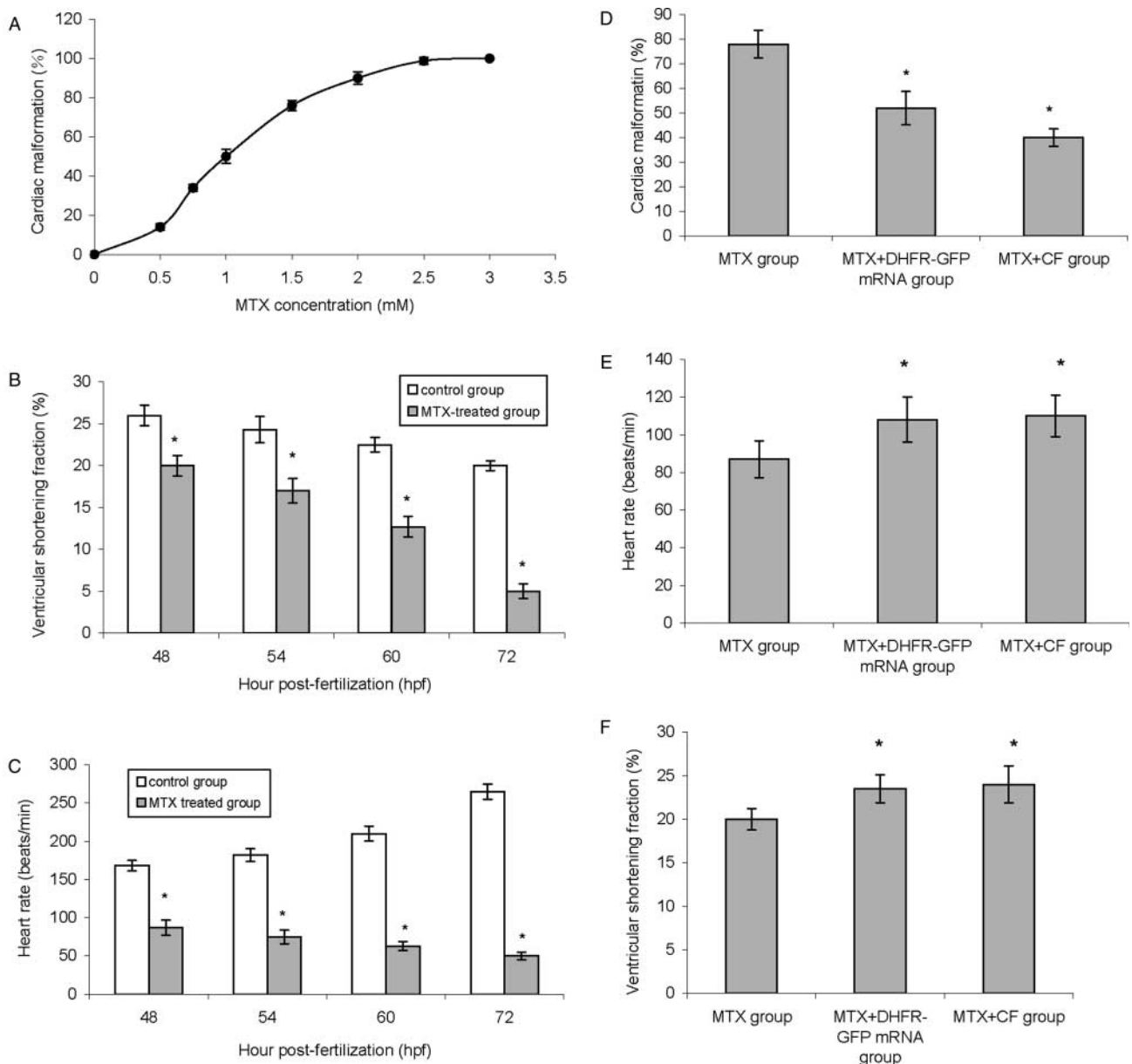


Fig. 2 Effects of MTX on the heart development of zebrafish (A) Dose–response curve of cardiac malformation caused by MTX. (B, C) Effects of MTX on the heart rate and VSF of zebrafish embryos. (D, E, F) At 48 hpf, cardiac abnormal ratio, heart rate, and VSF in MTX-treated group, MTX + CF group, and MTX + *dhfr–gfp* mRNA group. Data are presented as mean \pm SD ($n = 50$). * $P < 0.05$.

[Fig. 3(D)–(F)]. In controls, the heart rate progressively increased with maturity, whereas in the MTX-treated group, the heart rate progressively decreased and was lower than in controls. Compared with the controls, the heart rate and VSF markedly decreased in every time point in the MTX-treated group [Fig. 2(B) and (C)]. Using microangiography, we detected vascular malformation in MTX-treated embryos. In controls, at 60 hpf, intersomitic vessels and cranial vessels can be clearly observed [Fig. 3(G) and (K)]. In MTX-treated embryos,

vessels were unclear and even could not be observed [Fig. 3(H) and (L)].

Microinjecting *dhfr–gfp* mRNA induced increase in transcript level of *dhfr*

Curves in Fig. 4(A) and (B) indicated that when microinjection volume was 6 nl, GFPs were observed in about 94% of embryos and number of malformation was only about 10%. When the microinjection volume was < 6 nl, the number of embryos with GFPs was

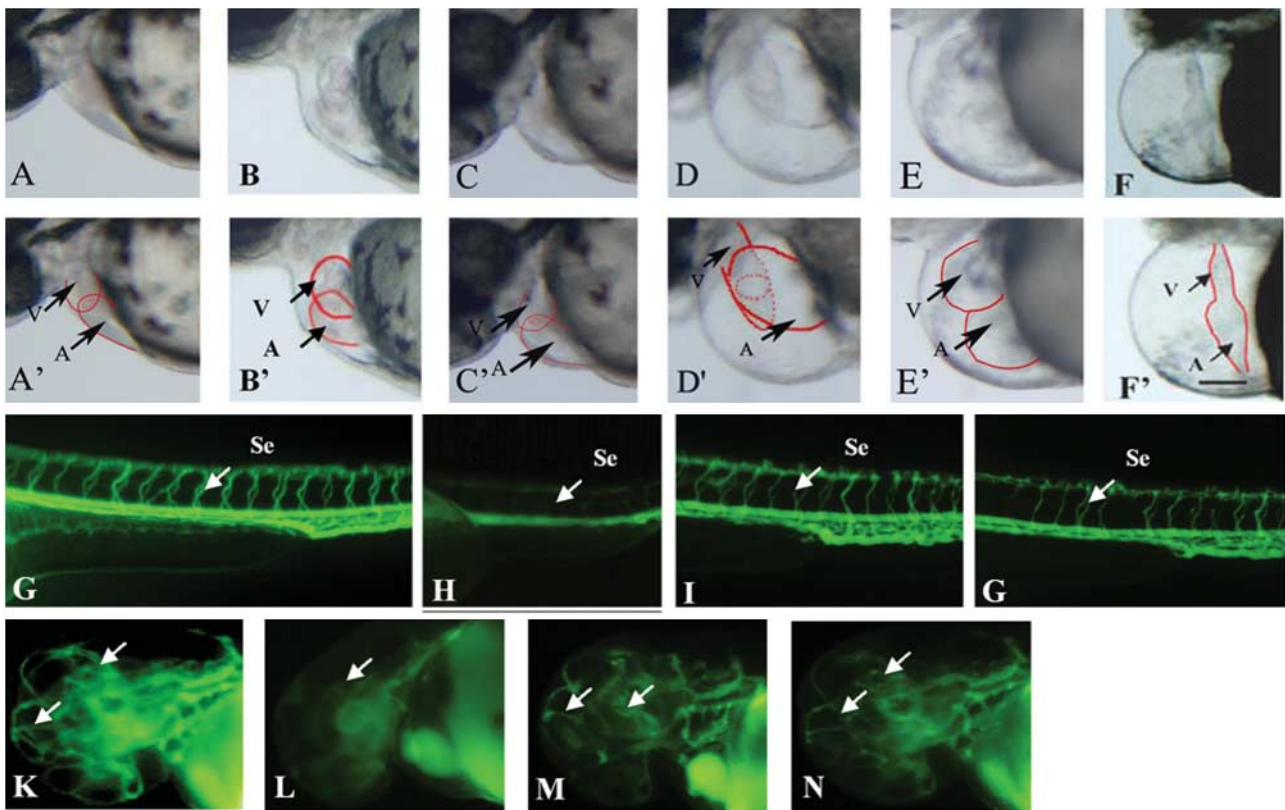


Fig. 3 Morphological observation of heart and vessels (A–F) Morphological observation of hearts. (G–J) Development of somatic vessels which was observed under fluorescent microscope. (K–N) Development of cranial vessels which was detected by fluorescein microangiography. (A, G, and K) Control group, (B, I, and M) MTX + *dhfr-gfp* mRNA-injected group, (C, J and N) MTX + CF-treated group, (D, E, F, H, and L) MTX-treated group. (A'–F') Conceptual diagram of A–F. In K, L, M, and N, arrows indicate cranial vessel. V, ventricle; A, atrium; Se, somatic vessels. Bars = 150 μ m. A–N: left lateral views, heads toward left.

obviously reduced. When the microinjection volume was >6 nl, the number of embryos with malformation was markedly increased. So, we select 6 nl as the microinjecting volume in following experiments. In controls, no green fluorescence was observed. However, green fluorescence was detected in *dhfr-gfp* mRNA-injected groups, which indicated that microinjecting *dhfr-gfp* mRNA results in increased transcript of *dhfr* and GFP and induces increase in transcript level of *dhfr*.

Increasing transcript level of *dhfr* and CF treatment can rescue MTX-induced malformations of the heart and vessels

Compared with group which was only exposed to MTX, the MTX + CF group (treated with 1.5 mM MTX + 5 mM CF at 6–10 hpf) and the MTX + *dhfr-gfp* mRNA group have improved development in the heart. In the MTX + CF group and MTX + *dhfr-gfp* mRNA group, ventricle and atrium appeared morphologically normal [Fig. 3(B) and (C)], cardiac abnormal percentage was lower, heart rate was higher, and VSF was increased

[Fig. 2(D)–(F)]. Microangiographically, we also observed clear images of intersomitic and cranial vessels in both the MTX + CF and MTX + *dhfr-gfp* mRNA groups [Fig. 3(I), (J), (M), and (N)].

Effects of MTX on transcript levels of *vmhc*, *amhc*, *hand2*, *mef2a*, *mef2c*, *flk-1*

Results of *in situ* hybridization with *vmhc* and *amhc* at 48 hpf showed that the transcript levels of *vmhc* and *amhc* in the MTX-treated group appeared normal [Fig. 5(E) and (F)]. The results of *in situ* hybridization with *vmhc* and *amhc* at 20 hpf showed that in the control group, primitive heart tubes on each side of midline fused to form circular heart tube [Fig. 5(C)]. But in MTX-treated embryos, primitive heart tubes remained on both sides of midline without fusing, which demonstrated delayed development in the heart [Fig. 5(G)]. Results of *in situ* hybridization with *vmhc* and *amhc* at 48 hpf showed that compared with controls, cardiac twist was disrupted in MTX-treated embryos [Fig. 5(D) and (H)].

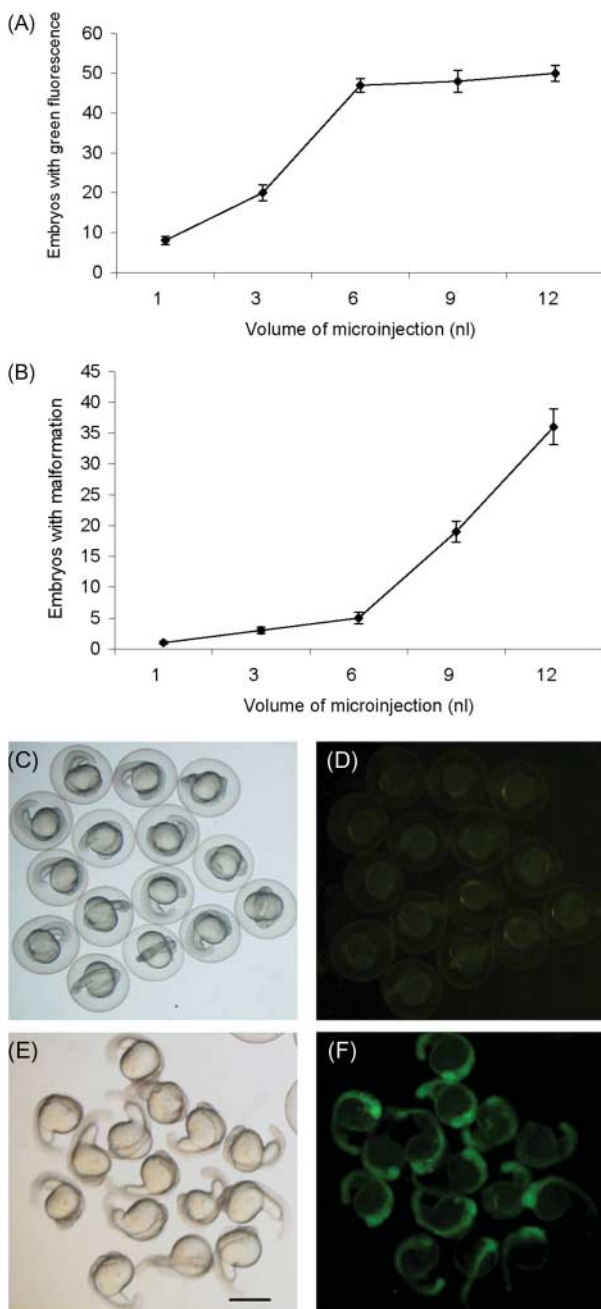


Fig. 4 Microinjecting *dhfr-gfp* mRNA induced an increase in transcript level of *dhfr* (A) Number of embryos with green fluorescence with different *dhfr-gfp* mRNA-injected volumes. (B) Number of abnormal embryos after being injected with different quantities of *dhfr-gfp* mRNA. (C, D) Control group at 18 hpf, observed under microscope. (E, F) MTX + *dhfr-gfp* mRNA-injected group at 18 hpf (volume of microinjection was 6 nl) under fluorescent microscope. Bar = 600 μ m.

Results of *in situ* hybridization showed that in controls at 18 hpf, *hand2* expressed in mesoderm [Fig. 6(A)] and *mef2a* and *mef2c* highly expressed in somites [Fig. 6(E) and (I)]. At 48 hpf, *hand2*

expression in the heart was visible and more intensive in atrioventricular canal [Fig. 7(A)]. The expression of *mef2a* and *mef2c* in the heart of controls were also observed at 48 hpf [Fig. 7(E) and (I)]. Compared with controls, the transcript level of *hand2* in MTX-treated embryos were reduced in mesoderm or in the heart [Figs. 6(B) and 7(B)], and the transcript levels of *mef2a* and *mef2c* were decreased in somites or in the heart [see (F) and (J) in Figs. 6 and 7]. In controls, at 24 hpf, *flk-1* expressed at forming vasculature in trunk axial and crania [Fig. 8(A) and (E)], and at 36 hpf, *flk-1* expression in trunk axial extended to intersomitic region [Fig. 8(I)]. In MTX-treated embryos, the transcript level of *flk-1* at 24 hpf was reduced [Fig. 8(B) and (F)] and at 36 hpf, *flk-1* expression was absent in intersomitic region [Fig. 8(J)].

Real-time PCR analysis also showed that in the MTX-treated group, the transcript levels of *hand2*, *mef2a*, and *mef2c* in whole embryos at 18 hpf and in the heart at 48 hpf were reduced [Fig. 9(A) and (B)]. The transcript levels of *flk-1* were decreased in MTX-treated embryos at both 24 and 36 hpf [Fig. 9(C)].

Increased transcript level of *dhfr* and CF treatment results in increase in transcript levels of *hand2*, *mef2a*, *mef2c*, and *flk-1* in MTX-treated embryos

Compared with the MTX-treated group, in the MTX + CF and MTX + *dhfr-gfp* mRNA groups increased transcript levels of *hand2* in mesoderm at 18 hpf and in the heart at 48 hpf [see (C) and (D) in Figs. 6 and 7] were found. Transcript levels of *mef2a* and *mef2c* in somites at 18 hpf and in the heart at 48 hpf were higher in both MTX + CF and MTX + *dhfr-gfp* mRNA groups than in the MTX-treated group [see (G), (H), (K), and (L) in Figs. 6 and 7]. Transcript levels of *flk-1* in MTX + CF and MTX + *dhfr-gfp* mRNA groups were found to be increased in trunk axial vessels and cranial vessels at 24 hpf and the transcripts were visible in intersomitic vessels at 36 hpf [Fig. 8(C), (D), (G), (H), (K), and (L)]. Real-time PCR analysis also showed that transcript levels of *hand2*, *mef2a*, *mef2c*, and *flk-1* in both MTX + CF embryos and MTX + *dhfr-gfp* mRNA embryos were increased compared with those in the MTX-treated group [Fig. 9(C)].

Discussion

MTX can tightly bind to DHFR and powerfully inhibit this enzyme, leading to a marked reduction in the synthesis of tetrahydrofolic acid and decreased production

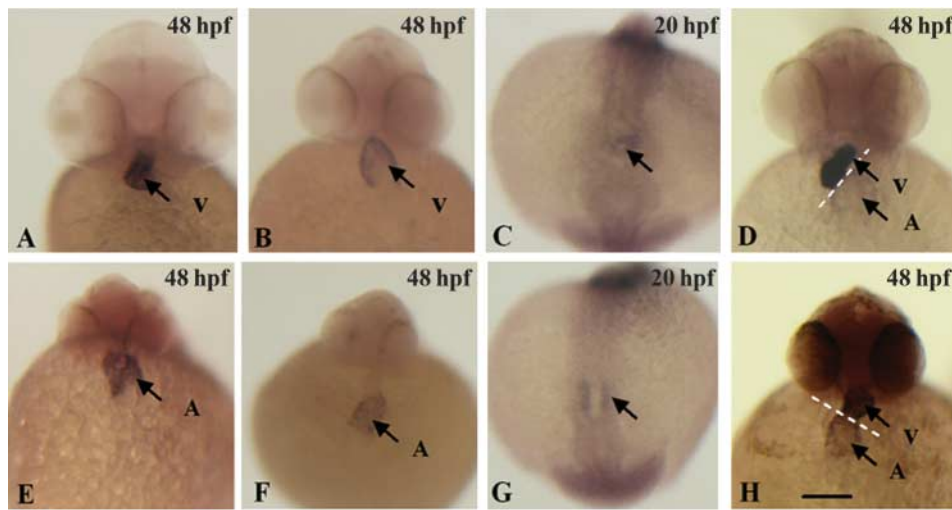


Fig. 5 Expression pattern of *vmhc* and *amhc* detected by *in situ* hybridization (A–D) Control group and (E–H) MTX-treated group. (A, E) Expression of *vmhc* at 48 hpf, (B, F) expression of *amhc* at 48 hpf, (C, G) expression of *amhc* + *vmhc* at 20 hpf, (D, H) expression of *amhc* + *vmhc* at 48 hpf. A, B, D, E, F, and H: ventral views, heads to the top. C and G: dorsal views, tails to the top. Arrows in C and G indicate primitive heart tubes. V, ventricular; A, atrium. Bar in A, B, C, E, F, G: 300 μ m. Bar in D and H: 150 μ m.

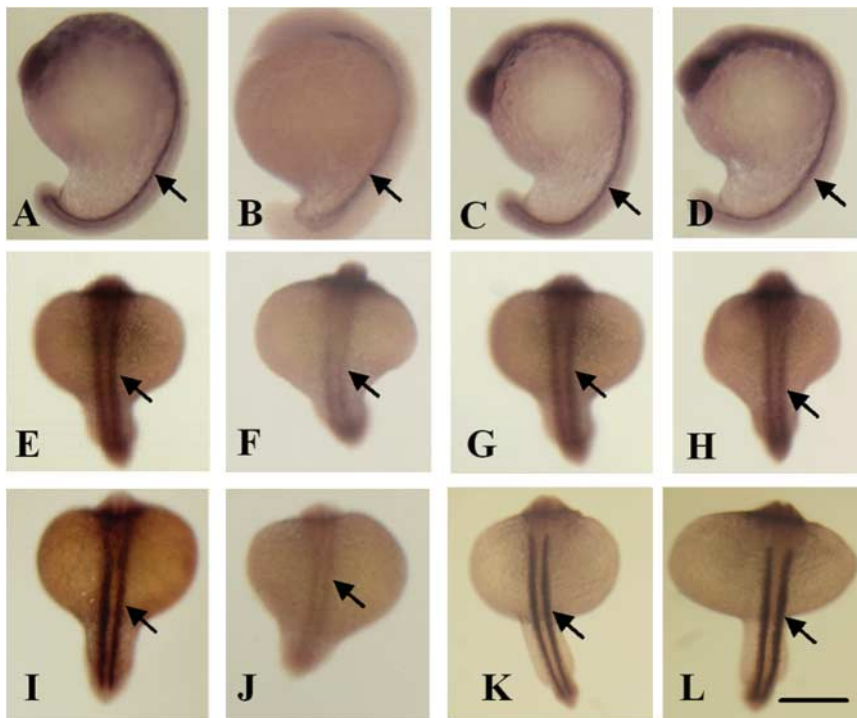


Fig. 6 Effects of MTX on expressions of *hand2*, *mef2a*, and *mef2c* detected by *in situ* hybridization at 18 hpf (A–D) Expressions of *hand2*, (E–H) expressions of *mef2a*, (I–L) expression of *mef2c*. (A, E, and I) Control group, (B, F, and J) MTX-treated group, (C, G, and K) MTX + *dhfr-gfp* mRNA-injected group. (D, H, and L) MTX + CF-treated group. A–D: left lateral views, heads toward top. E–L: dorsal views, heads toward top. A–D: arrows indicate the mesoderm. E–L: arrows indicate the somites. Scale bars: 300 μ m.

of N^5,N^{10} -methyl-tetrahydrofolic acid. As a result, important biological functions such as nucleotide synthesis and methylation reactions are compromised. By this way, biological functions of folic acid are blocked.

In zebrafish, from 1–4 somites to long-pec stage, *dhfr* is expressed in whole organism (neural plate, tail bud, central nervous system, intermediate cell mass of mesoderm, optic tectum, retina, pectoral fin musculature, the

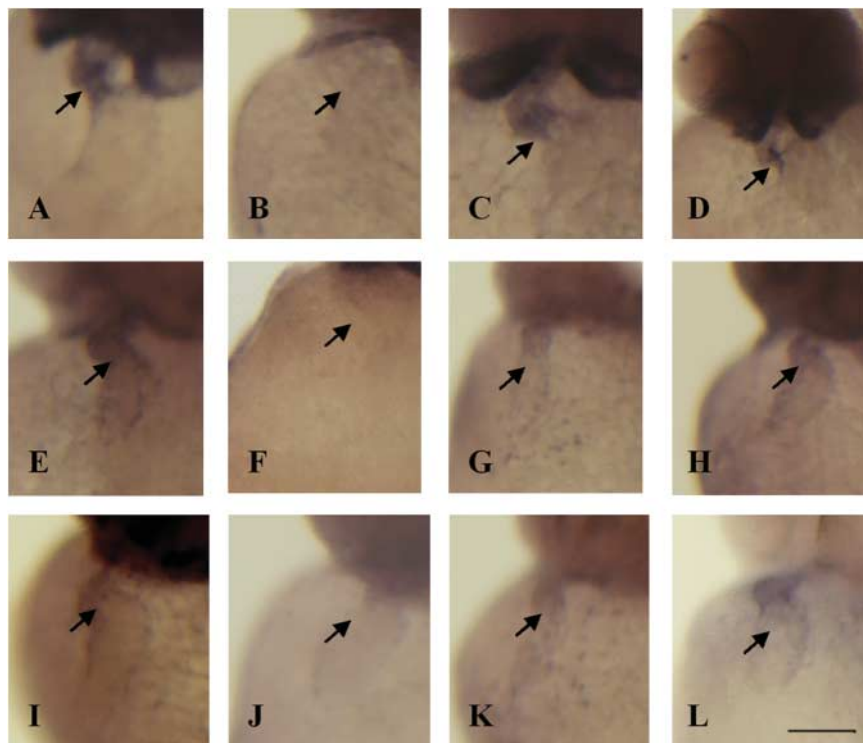


Fig. 7 Effects of MTX on expressions of *hand2*, *mef2a*, and *mef2c* of zebrafish heart detected by *in situ* hybridization at 48 hpf (A–D) Expressions of *hand2*, (E–H) expressions of *mef2a*, (I–L) expression of *mef2c*. (A, E, and I) Control group, (B, F, and J) MTX-treated group, (C, G, and K) MTX + *dhfr-gfp* mRNA-injected group. (D, H, and L) MTX + CF-treated group. A–L: ventral views, heads toward top. Arrows indicate the hearts. Scale bars: 150 μ m.

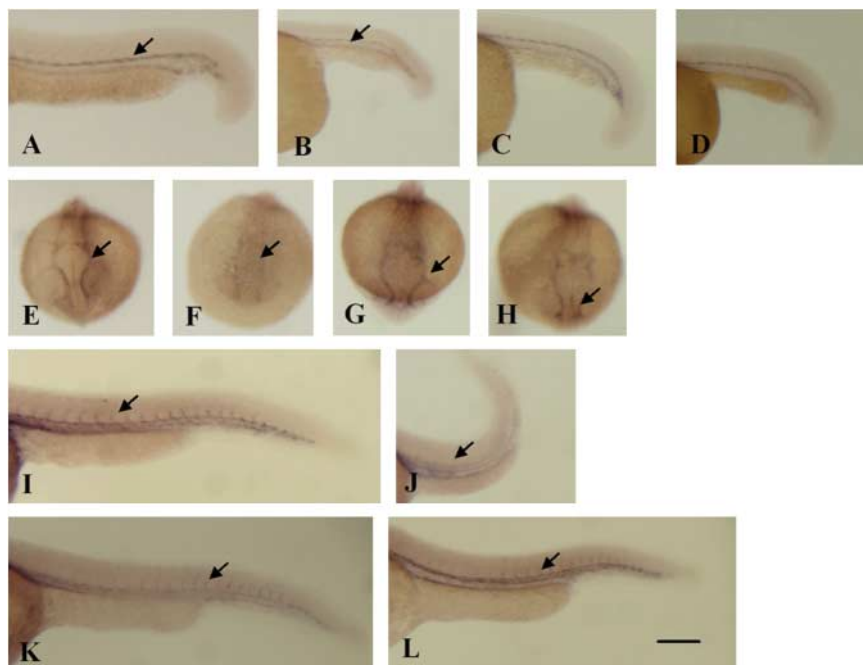


Fig. 8 Effects of MTX on expression of *flk-1* detected by *in situ* hybridization (A–D) Expression of *flk-1* in somatic vessels at 24 hpf, (E–H) expression of *flk-1* in cranial vessels at 24 hpf, (I–L) expression of *flk-1* in somatic vessels at 36 hpf. (A, E, and I) control group, (B, F, and J) MTX treated group, (C, G, and K) MTX + *dhfr-gfp* mRNA injected group. (D, H and L) MTX + CF-treated group. (A–D, I–L) Left lateral view, head toward left. (E–H) Views from the top. Arrows in (A–D) indicated the vessels in trunk axes. Arrows in (E–H) indicated the vessels in crania. Bars = 150 μ m.

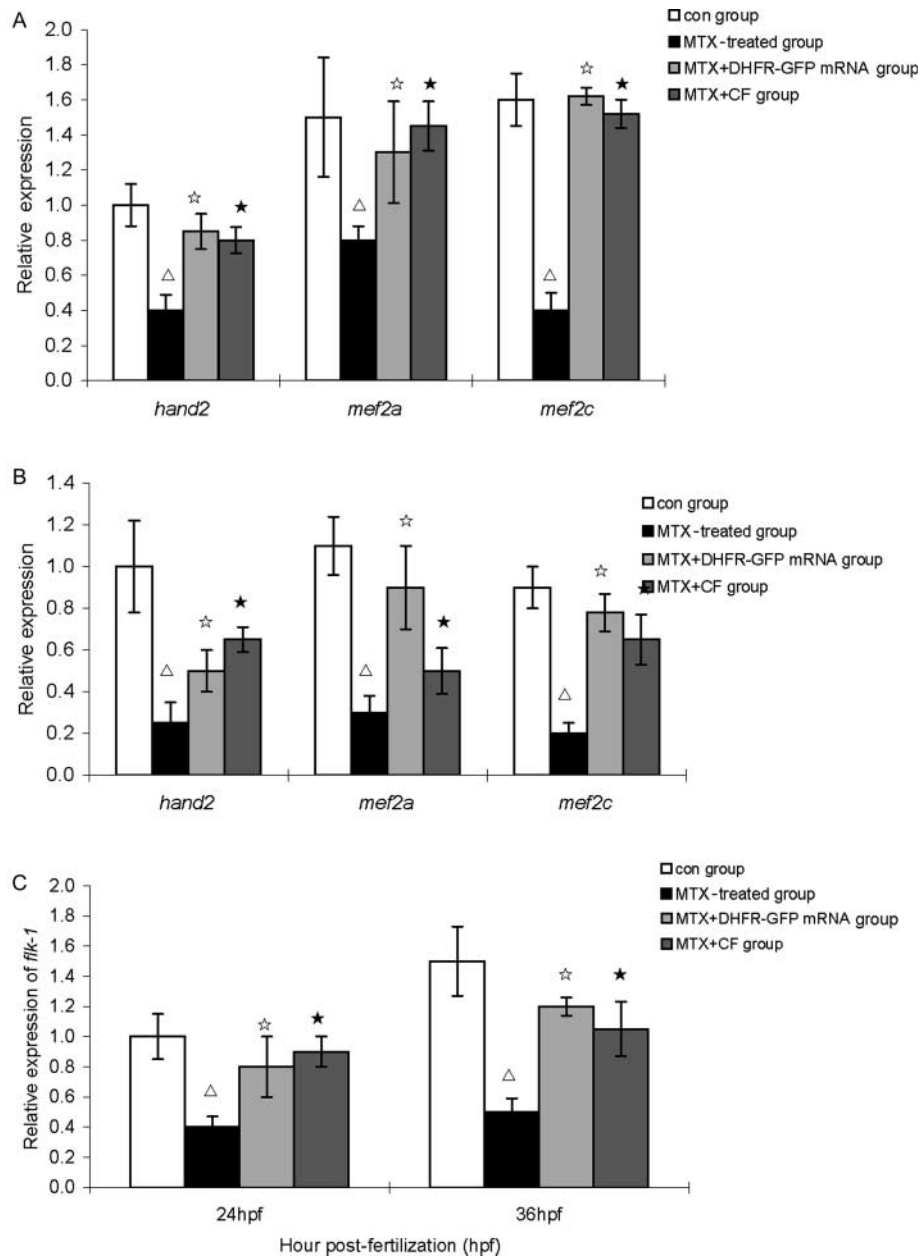


Fig. 9 Expressions of genes detected by real-time PCR (A) Expressions of *hand2*, *mef2a*, and *mef2c* at 18 hpf. (B) Expressions of *hand2*, *mef2a*, and *mef2c* in zebrafish heart at 48 hpf. (C) Expressions of *flk-1* at 24 and 36 hpf. Data are expressed as the mean \pm SD of three separate quantitative PCR ($n = 3$). $\Delta P < 0.05$ compared with control group, $\star P < 0.05$ compared with MTX-treated group, $\star P < 0.05$ compared with MTX-treated group.

heart, muscle, liver, spleen, spinal cord, and pharyngeal arch) (<http://zfin.org>, ZFIN ID: ZDB-FIG-050630-6067, 128, 6110, 2433, 7081) [5]. In this paper, we also observed that at 6–10 hpf, *dhfr* is expressed in whole organism including the head, mesoderm, and tail bud.

In zebrafish, 6–10 hpf is in gastrula period (5.25–10 hpf), which is a crucial stage for cardiac development. Therefore, extrinsic factors can strongly affect the

cardiac development in this stage. In our experiments, MTX affected both the morphological and functional developments of the heart in zebrafish. We found that exposing zebrafish embryos to MTX at 6–10 hpf can induce obvious cardiac malformations. We also found that in the MTX-treated group, the heart rate and VSF decreased, indicating cardiac ventricular contractility was impaired. Using microangiography, we detected that

MTX disrupted the development of vessels. We further detected the transcript levels of some genes which plays important roles in cardiovascular development.

Zebrafish *vmhc* labels ventricular myocardium and *amhc* labels myocardium of atrium [15]. *vmhc* and *amhc* are important for cardiac morphogenesis [16]. *hand2* is a basic helix–loop–helix transcription factor. Functional analysis has shown that *hand2* is involved in the development of the heart and vasculature [17]. *hand2* can promote ventricular cardiomyocyte expansion [18]. Dysfunction of *hand2* was associated with ventricular hypoplasia and cardiac growth delay [19,20]. In zebrafish, *hand2* plays pivotal roles in cardiac morphogenesis and cardiac-specific transcription [21]. *mef2a* and *mef2c* belong to MEF2 family. Dysfunction of MEF2 causes chamber dilation and mechanical dysfunction during the course of cardiac development [22]. *mef2c* have fundamental role in ventricular myocyte development [23]. Inactivation of *mef2c* causes cardiac developmental arrest [24]. In zebrafish, *mef2a* controls cardiac ventricular contractility [13]. *flk-1*, the receptor of VEGF, is one of the earliest markers for angioblasts and indispensable for migration of angioblasts from ventral mesoderm to midline [25]. *flk-1* is crucial for further formation of vascular system, including dorsal artery, axial vein, cranial vessels, and intersomitic vessels [26].

In our study, abnormal cardiac developments in MTX-treated embryos were observed, including dilation or dysplasia of ventricle and atrium, delayed development of the heart, abnormal cardiac twist, and impaired contractility of ventricle. Abnormal formations of cranial vessels and intersomitic vessels in MTX-treated embryos were also found. Due to the central roles of *vmhc*, *amhc*, *hand2*, *mef2a*, *mef2c*, and *flk-1* in cardiac morphogenesis, cardiac developmental process, ventricular contractility, and formation of vessels, the transcript levels of these genes that play vital roles in cardiovascular formation were detected.

In situ hybridization analysis showed that transcript levels of *vmhc* or *amhc* appeared normal in MTX-treated embryos at 48 hpf, suggesting that myocyte differentiations of ventricle and atrium were not affected by MTX. But in MTX-treated embryos, expressions of *vmhc* + *amhc* at 20 hpf demonstrated developmental retardation in the heart and expressions of *vmhc* + *amhc* at 48 hpf showed abnormal cardiac twist. In MTX-treated embryos, reduced transcript levels of *hand2*, *mef2c*, and *mef2a* were detected at 18 hpf, which indicated MTX affected the transcript levels of these genes in the early developmental stage. At 48 hpf,

development of the heart in zebrafish is nearly completed. Cardiac looping and differentiation of ventricle and atrium are finished. Decreased transcript levels of *hand2*, *mef2c*, and *mef2a* in the heart of MTX-treated embryos were detected. Decreased transcript levels of *hand2*, *mef2c*, and *mef2a* may contribute to cardiac abnormalities. At 24 hpf, expressions of *flk-1* were observed at midline in both the control and MTX-treated groups, which demonstrated that MTX did not disrupt the migration of angioblasts to midline. At 36 hpf, *flk-1* expression was absent in intersomitic vessels, which suggested that angioblasts could not constitute the intersomitic vessels in trunk. Malformation of vessels induced by MTX may be related to the reduced transcript level of *flk-1*.

To prove that MTX-induced malformations of the heart and vessels have relationship with decreased transcript levels of *hand2*, *mef2c*, *mef2a*, and *flk-1*, the *dhfr*-increased transcribing experiment and tetrahydrofolic-acid-rescuing experiment were designed. As well known, MTX can inhibit functions of DHFR and results in down-production of tetrahydrofolic acid. If increased transcript levels of *dhfr* and giving tetrahydrofolic acid can rescue cardiovascular malformations and increase transcript levels of *hand2*, *mef2c*, *mef2a*, and *flk-1* in MTX embryos, the conclusion that reduced transcript levels of *hand2*, *mef2c*, *mef2a*, and *flk-1* were related with MTX-induced malformations in the heart and vessels was confirmed. In the *dhfr*-increased transcribing experiment, the *dhfr-gfp* mRNA was synthesized and microinjected into fertilized egg. *dhfr-gfp* mRNA can express both *dhfr* and *gfp* *in vivo*. So if the expression of *gfp* was detected, the expression of *dhfr* is proved. After being microinjected with *dhfr-gfp* mRNA, *gfp* expression was observed in embryos, which demonstrated microinjecting *dhfr-gfp* mRNA resulted in increased transcript level of *dhfr*. In tetrahydrofolic-acid-rescuing experiment, exogenous tetrahydrofolic acid was given by exposing zebrafish embryos to CF solution. CF can be converted to tetrahydrofolic acid *in vivo* and it is more stable than tetrahydrofolic acid when dissolved in water. We found that in the MTX + *dhfr* mRNA and MTX + CF-treated groups, cardiac abnormal ratio was decreased, malformations in the heart were rescued, and the heart rate and VSF were increased. The transcript levels of *hand2*, *mef2a*, *mef2c*, and *flk-1* were increased both in the MTX + *dhfr* mRNA and MTX + CF-treated groups. Then we confirmed that malformations of the heart and vessels caused by MTX were associated with reduced transcript levels of *hand2*, *mef2c*, *mef2a*, and *flk-1*.

Our present findings were expected to provide important clues to investigations about adverse effects of folic acid dysfunction on cardiovascular development in human fetus. However, it is well known that many genes that are very important for cardiovascular development have combinatorial interactions, and the expressions of genes can be regulated by several factors. So, the mechanisms by which folic acid dysfunction induces reduced expression levels of these genes that were detected in our study need further investigation.

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