

Original Article

# Dihydroartemisinin promotes angiogenesis during the early embryonic development of zebrafish

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**Aim:** To investigate the embryotoxicity of dihydroartemisinin (DHA), the main active metabolite of artemisinin, in zebrafish, and explore the corresponding mechanisms.

**Methods:** The embryos of wild type and TG (*flk1:GFP*) transgenic zebrafish were exposed to DHA. Developmental phenotypes of the embryos were observed. Development of blood vessels was directly observed in living embryos of TG (*flk1:GFP*) transgenic zebrafish under fluorescence microscope. The expression of angiogenesis marker genes *vegfa*, *flk1*, and *flt1* in the embryos was detected using real-time PCR and RNA *in situ* hybridization assays.

**Results:** Exposure to DHA (1–10 mg/L) dose-dependently caused abnormal zebrafish embryonic phenotypes in the early developmental stage. Furthermore, exposure to DHA (10 mg/L) resulted in more pronounced embryonic angiogenesis in TG (*flk1:GFP*) zebrafish line. Exposure to DHA (10 mg/L) significantly increased the mRNA expression of *vegfa*, *flk1*, and *flt1* in the embryos. Knockdown of the *flk1* protein partially blocked the effects of DHA on embryogenesis.

**Conclusion:** DHA causes abnormal embryonic phenotypes and promotes angiogenesis in zebrafish early embryonic development, demonstrating the potential embryotoxicity of DHA.

**Keywords:** artemisinin; dihydroartemisinin; zebrafish; *flk1*; VEGF; angiogenesis; embryotoxicity

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## Introduction

Artemisinin, a sesquiterpene lactone derived from the sweet wormwood plant *Artemisia annua*, is a new antimalarial compounds discovered by Chinese scientists. Artemisinin and its bioactive derivatives are used to effectively treat different forms of malarial parasites<sup>[1,2]</sup>. As the main active metabolite of artemisinin, dihydroartemisinin (DHA) has more effective anti-malarial effects than artemisinin. In addition, artemisinin and its derivatives exhibit potent anti-cancer effects in a variety of human cancer cell model systems<sup>[3–6]</sup> in which the apoptotic response, cell cycle arrest and nuclear receptor response are involved. The anti-cancer activity of artesunate, a semi-synthetic derivative of the sesquiterpene artemisinin, has been verified in mice bearing human xenograft tumors and patients with uveal melanoma and non-small cell lung cancer<sup>[7–9]</sup>.

However, treatment with artemisinin analogs has been

reported to be associated with different types of toxicity, such as neurotoxicity, embryotoxicity and genotoxicity, in cellular and animal models<sup>[10,11]</sup>. Among the side effects arising from the use of artemisinin-related compounds, the danger of embryotoxicity appears to be more serious. The typical embryotoxic effects of artemisinin that have been observed in mice, rats, rabbits and monkeys include embryonic death, developmental retardation, skeletal defects and cardiovascular malfunction<sup>[12–16]</sup>. In view of these side effects, artemisinin and its derivatives are not recommended for use in the first trimester of pregnancy by the World Health Organization (WHO). Thus, there is an urgent need for artemisinin safety control for patients, particularly pregnant women.

One mechanism of embryotoxicity appears to be related to vasculogenesis and angiogenesis. Artemisinin was shown to inhibit angiogenesis and decrease hypoxia-induced factor 1 $\alpha$  (HIF1 $\alpha$ ) and vascular endothelial growth factor (VEGF) expression in mouse embryonic stem cell-derived embryoid bodies<sup>[12]</sup>. DHA caused a reduction of primitive red blood cells in *Xenopus* larvae and rats<sup>[13,17]</sup>. Moreover, artemether, another artemisinin derivative, could induce cardiovascular

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malformation and embryonic erythroblast defects in rats, rabbits and monkeys<sup>[14-16]</sup>. However, the detailed mechanisms of artemisinin action require further study. In addition, although several animal models have been used to study the embryotoxicity of artemisinin, fewer studies have reported using zebrafish, particularly for the effect of artemisinin on vasculogenesis and angiogenesis, although zebrafish is a useful model system for the study of vasculogenesis and angiogenesis in embryonic development<sup>[18]</sup>.

Thus, we used zebrafish as a model to demonstrate that DHA, the main artemisinin derivative, causes zebrafish embryotoxicity, including pericardial edema and a larger yolk sac, and induces vasculogenesis/angiogenesis and the overexpression of *vegfa*. Our results not only confirm toxicity phenotypes such as malformation, embryonic death and heart defects in zebrafish, but they also innovatively demonstrate the pro-angiogenic effects of DHA on vessel formation.

## Materials and methods

### Zebrafish maintenance

Zebrafish maintenance, breeding and staging were performed by standard methods<sup>[19]</sup>. The TG (*flk1:GFP*) transgenic zebrafish line was a kind gift from Dr Ting-xi LIU, Institute of Health Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences. The zebrafish facility and zebrafish study were approved by the institutional review board (IRB) of the Institute of Nutritional Sciences, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences (Protocol Approval Number: 2011-AN-1).

### Collection and treatment of zebrafish embryos

Embryos were collected and staged following standard methods<sup>[19]</sup>. The stock solutions for the different concentration of each compound were prepared in DMSO. The treatment solutions were diluted 1000 times from the stock solutions with system water. All stock and treatment solutions were freshly made before each use. For exposure, 10 hpf (hours post fertilization) embryos were placed in 6-cm Petri dishes containing 10 mL of treatment solution. Untreated embryos and embryos treated with 0.1% DMSO were used as control groups. Exposures were performed at 28.5°C for 38 h–6 d. The control/treatment solutions were changed daily. Three replicates of 60 embryos were used for each concentration group. The developmental phenotypes of zebrafish embryos were directly observed in the dish using an Olympus SZX9 system (Tokyo, Japan) every 12 h. Such phenotypes, including mortality, spontaneous movement, hatching success, heartbeat, pericardial edema and curved body axis, were selected and examined to determine the embryotoxicity effects of the compounds. Artemisinin and its derivative DHA were kind gifts from Zhejiang Yiwu Golden Fine Chemical Co Ltd.

### Real-time PCR

Total RNA was extracted from 20 zebrafish embryos using TRIzol reagent (Invitrogen, Shanghai, China). RNA was reverse transcribed using the PrimeScript RT reagent kit

(TaKaRa, Shiga, Japan). Real-time quantitative PCR for all genes was performed with the fluorescent dye SYBR Green. Amplification reactions were performed in a total volume of 20 µL with Real-time PCR Master Mix (TOYOBO, Osaka, Japan) and 10 pmol of each primer. PCR was performed for 40 cycles consisting of 95°C for 10 s, 60°C for 30 s, and 72°C for 30 s using the CFX96™ real-time system (Bio-Rad, Hercules, CA, USA). The gene-specific primers are shown in Table 1. The results were normalized to β-actin, which served as a loading control.

**Table 1.** Primers for real-time PCR.

Genes	Sense (5′–3′)	Antisense (5′–3′)
β-Actin	ATGCCCTCGTGCTGTTTTTC	GCCTCATCTCCCACATAGGA
<i>vegf</i>	TCTCCTCCATCTGTCTGC	CAGGAGCATTTCACAGGTG
<i>flt1</i>	AGGCTTTTGCAGGACAGAAA	TGTGATGGTGAGGTTCTGGA
<i>flk1</i>	GATGGAGATACACACCTTCAG	TGCGTACCGATGACACATTTTC

### RNA *in situ* hybridization

For whole-mount RNA *in situ* hybridization (WISH), 48 hpf embryos were collected and fixed in 4% paraformaldehyde overnight at 4°C. cDNA sequences of specific genes were PCR-amplified and cloned into the pGEM-T Easy vector (Promega, Madison, WI, USA) as templates to generate an antisense riboprobe for *in situ* hybridization (Roche, Shanghai, China). WISH was performed using the standard methods<sup>[20]</sup>, and the primers are shown in Table 2.

**Table 2.** Primers for *in situ* hybridization.

Genes	Sense (5′–3′)	Antisense (5′–3′)
<i>vegf</i>	TGCCACATACCCAAAGAAG	GCAAGGCTCACAGTGTTTTTC
<i>flt1</i>	GCCGTTGATGGTCATTGTTG	TCTCCGTTCCGCACATAGTCT
<i>flk1</i>	GAGATGGAGGAGGAAGTGGTG	GATAGCCGCTGGTCTGATTG

### Knockdown of *flk1* and *flt1* expression with antisense morpholino oligonucleotides

Antisense morpholino oligonucleotides (MO) for *flk1* and *flt1* were obtained from Gene Tools, LLC (Philomath, OR, USA). The sequences used are as follows: 5′-CCG AAT GAT ACT CCG TAT GTC AC-3′ for *flk1*, 5′-CAG CAG TTC ACT CAC ATC TCC GTT C-3′ for *flt1*<sup>[21]</sup>, and 5′-CCT CTT ACC TCA GTT ACA ATT TAT A-3′ for the control. For microinjection, a 1-nl volume was injected into 1- to 2-cell stage embryos.

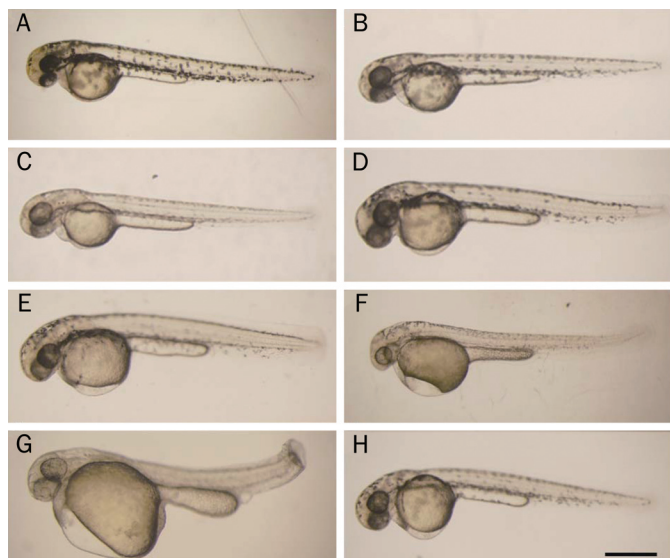
### Statistical analysis

Experimental data were expressed as the mean±SD unless indicated, and the significance of differences was analyzed using Student's *t*-test, as appropriate.

## Results

### DHA induces morphological abnormalities during the early embryonic developmental stages of zebrafish

To investigate the overall effects of DHA and artemisinin on zebrafish early embryonic development, embryos were exposed to different concentrations of drugs beginning at 10 hpf. Morphologically, the treated embryos began to exhibit obvious abnormalities at the 48 hpf stage from 1 mg/L drug treatment, including pericardial edema, a larger yolk sac, shorter yolk sac extension, a curving trunk, a shorter trunk axis and less melanin pigmentation, compared with untreated and DMSO-treated embryos (Figure 1A-1H). Moreover, the drug caused the dilation of pericardial edema and other abnormal phenotypes in a dose-dependent manner (Figure 1C-1G and data not shown). In addition, we found that DHA more severely induced morphological abnormalities in embryos than artemisinin (Figure 1F and 1H). Therefore, DHA was selected for subsequent experiments.

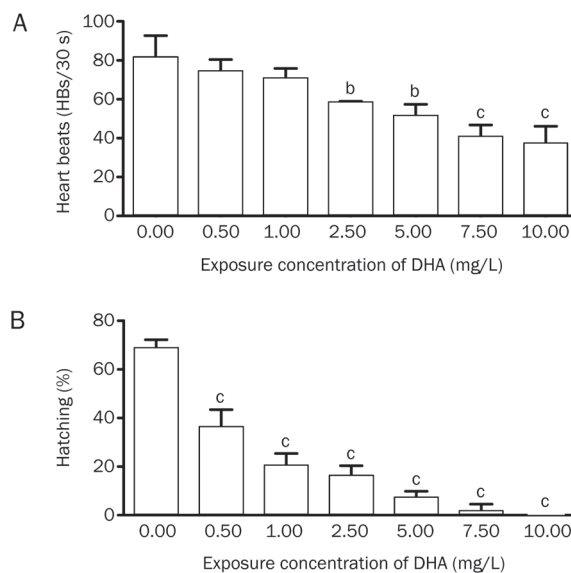


**Figure 1.** Morphological changes induced by dihydroartemisinin (DHA) and artemisinin during zebrafish early embryonic development. Embryos treated with different concentrations of compounds were imaged at 48 hpf. (A) No treatment; (B) 0.1% DMSO; (C) 1.0 mg/L DHA; (D) 2.5 mg/L DHA; (E) 5.0 mg/L DHA; (F) 10 mg/L DHA; (G) 15 mg/L DHA; (H) 10 mg/L artemisinin. The scale bar represents 500  $\mu$ m for all panels.

### DHA exposure slows down heartbeats and causes hatching failure

After treatment with DHA for 50 h at different concentrations, all embryos were alive, even in the highest concentration group (20 mg/L). However, the treated embryos began to die at 72 hpf from 5 mg/L treatment (when the heart in the embryo stopped beating, the embryo was considered to be dead). During this process, the heartbeat rates of embryos began to decrease in the DHA-exposed embryos, beginning at 60 hpf, compared with untreated and DMSO-treated embryos.

Thus, we examined the heartbeat rates at various DHA concentrations at 60 hpf, and the results showed that DHA exposure decreased heartbeat rates in a dose-dependent manner (Figure 2A).

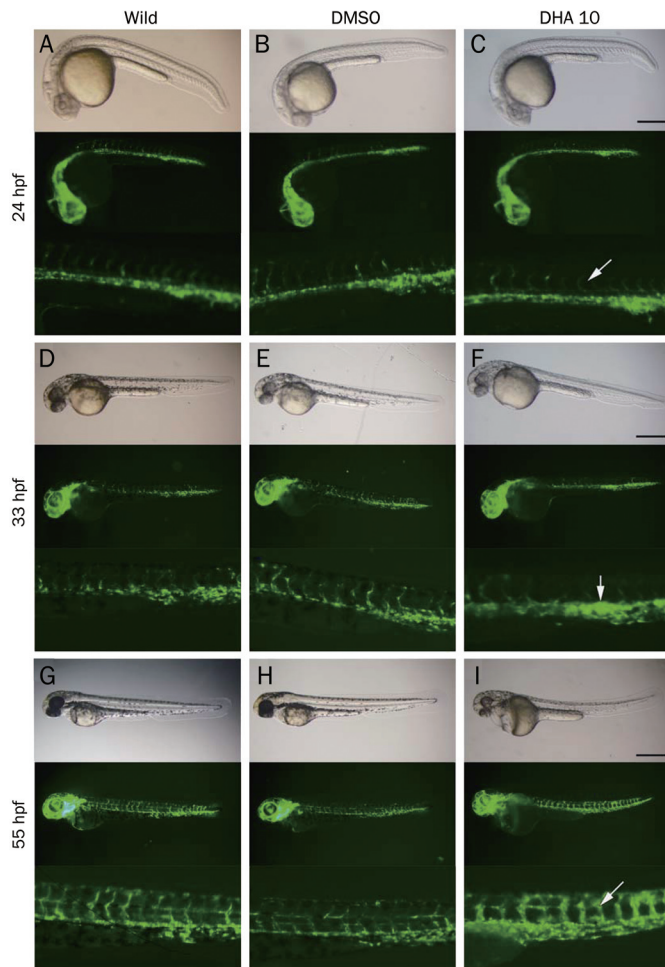


**Figure 2.** The heartbeats and hatching success of embryos exhibit a dose-dependent decrease. (A) Embryos were treated, and the heartbeats were examined at 60 hpf. Three replicates of 60 embryos were used for each concentration group. (B) A total of 60 embryos were used per group. Shown are results representative from three independent experiments. <sup>b</sup> $P < 0.05$ , <sup>c</sup> $P < 0.01$  compared with the control embryos. DHA, dihydroartemisinin.

Zebrafish embryo hatching in the untreated and DMSO-treated groups normally began at 48 hpf and ended at 72 hpf. However, the hatching ability of DHA-treated embryos was significantly impaired. At 60 hpf, the hatching rate began to decrease with increasing DHA concentrations ranging from 0.5 to 10 mg/L (Figure 2B). High concentrations ( $\geq 5$  mg/L) of DHA significantly inhibited embryo hatching with only sporadic hatching.

### DHA accelerates the vasculogenesis and angiogenesis processes

It has been reported that pericardial edema may result from a change in vascular permeability<sup>[18]</sup>. To investigate the effect of DHA on vasculature development, the TG (*flk1:GFP*) zebrafish model was used. In this model, the development of GFP-labeled blood vessels could be directly observed in live embryos under a fluorescence microscope. At 24 hpf, the characteristic “S” pattern of the intersegmental blood vessel (ISV) was present along the dorsal-ventral axis (Figure 3A-3C). Compared with untreated and DMSO-treated embryos, the curvature of the “S” pattern increased with the DHA exposure (Figure 3C), suggesting that DHA could promote the extension of the ISV. At 33 hpf, the dorsal aorta (DA) and pericardial vein (PCV) were more emanative and showed stronger GFP fluorescence intensity under DHA exposure (Figure



**Figure 3.** Embryos from the TG (*flk1:GFP*) zebrafish line display angiogenesis abnormalities with dihydroartemisinin (DHA) exposure. Three groups of embryos were used to compare changes including the untreated, DMSO-treated and DHA-treated (10 mg/L DHA) groups, and different developmental stages were selected to show changes, including 24 hpf (A, B, C), 33 hpf (D, E, F), and 55 hpf (G, H, I). The white arrows in panels C, F, and I indicate abnormalities in embryos compared with untreated and DMSO-treated embryos. The embryos shown in all panels are lateral views. The scale bar represents 500  $\mu$ m for the first and second row in each panel and 1500  $\mu$ m for the third row in each panel.

3D–3F), demonstrating that DHA could promote trunk inter-somitic vessel formation. At 52 hpf, the effect of DHA on ISV became more profound (Figure 3G–3I). In addition, we found that abnormal vasculogenesis together with other abnormal phenotypes, such as pericardial edema and a curving trunk, occurred in a dose-dependent manner (data not shown).

#### DHA exerts the embryotoxicity effect through the VEGF signaling pathway

It is well known that the VEGF signaling pathway plays important roles in vasculogenesis and angiogenesis<sup>[22, 23]</sup>. We determined the expression of VEGF family genes in zebrafish embryos treated with DHA at 48 hpf. By performing whole-mount *in situ* hybridization, we found that the mRNA levels

of *vegfa*, a key factor among VEGF family genes in zebrafish, and its two receptor genes, *flk1* and *flt1*, were upregulated compared with the control groups in a dose-dependent manner (Figure 4A–4C). The expression of *vegfa* was strongly enhanced in the adjacent somite areas along the vasculature (Figure 4Ac, 4Ad, 4Ag, 4Ah), while the expression of *flt1* and *flk1* was highly increased in the entire vasculature of the embryos compared with the control embryos (Figure 4Bc, 4Bd, 4Bg, 4Bh, 4Cc, 4Cd, 4Cg, 4Ch). The real-time PCR results consistently showed that the mRNA levels of *vegfa*, *flk1*, and *flt1* were significantly upregulated in DHA-treated embryos at 48 hpf (Figure 4D). Therefore, we speculated that the VEGF pathway might play an important role in the abnormal embryonic angiogenesis induced by DHA.

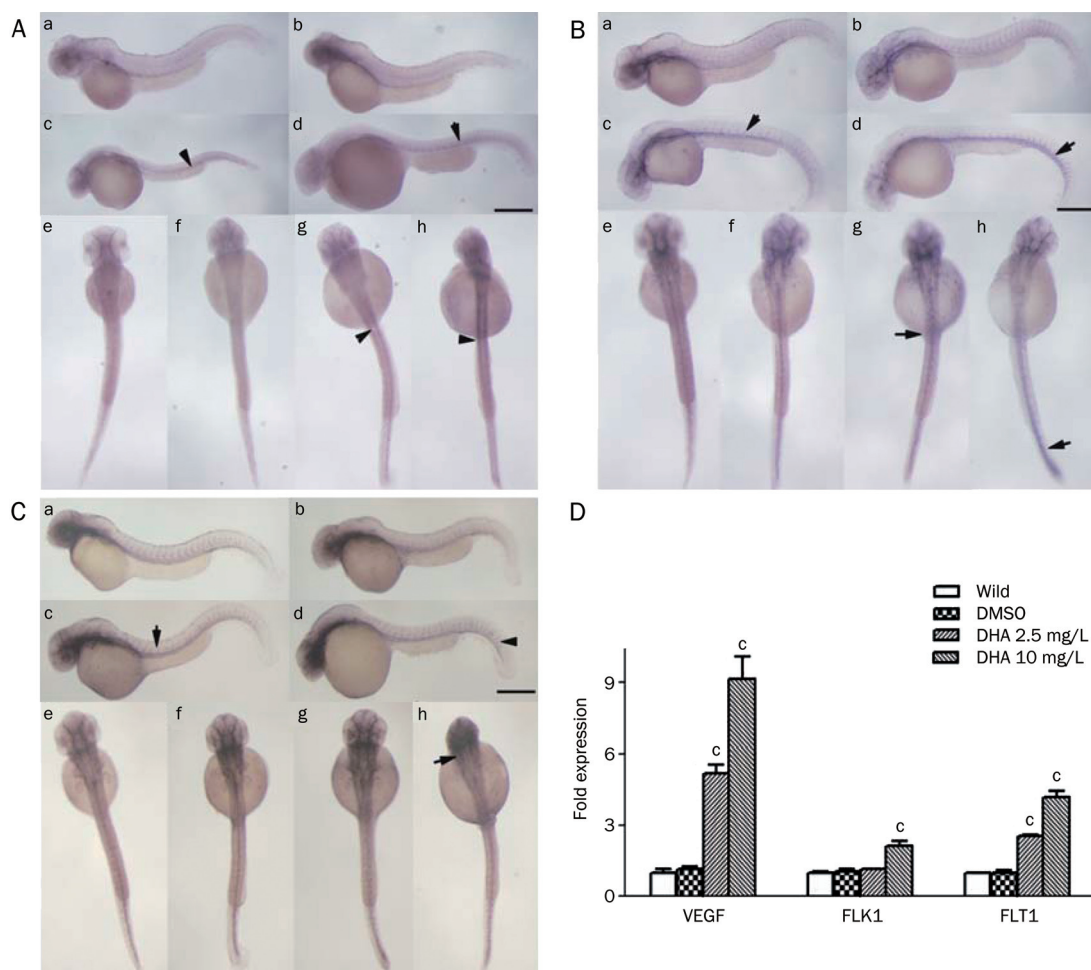
#### The knockdown of *flk1* blocks DHA-induced pericardial edema during zebrafish embryogenesis

To further confirm the role of *flt1* and *flk1* in the abnormal vascular development and pericardial edema of the DHA-treated embryos, we knocked down the expression of *flt1* and *flk1* separately by injecting *flk1*-MO or *flt1*-MO into embryos at the 1- to 2-cell stage. Untreated embryos and embryos injected with std-ctrl-MO were used as controls. The MOs were tagged with green fluorescein at the 3'-end, and a fluorescence signal indicated the successful injection and distribution of the respective MO in the injected-embryos. At 48 hpf, we found that ectopic phenotypes *eg*, dilated pericardial edema, were not present in *flk1*-MO-injected embryos compared with the controls (Figure 5A, 5D, 5G, 5C, 5F, and 5I). However, abnormal edema still occurred in *flt1*-MO-injected embryos (Figure 5A, 5D, 5G, 5B, 5E, and 5H). These results indicate that *flk1* might play an important role in DHA-induced pericardial edema.

#### Discussion

In this study, we investigated the embryotoxicity of DHA exposure during zebrafish early embryonic development and found that DHA exposure could cause abnormal phenotypes including pericardial edema, a larger yolk sac, heart rate inhibition and hatching failure. Using the TG (*flk1:GFP*) zebrafish line, we detected a pro-angiogenic property of DHA, and *flk1* might play a key role in this process. In summary, we propose a potential mechanism in which DHA may cause embryotoxicity (Figure 6). DHA induces VEGF-*flk1* overexpression, promotes angiogenesis and results in abnormal phenotypes in early development, including pericardial edema, which might be related to cardiovascular malfunctions such as hyperpermeability.

Previous animal studies on rats, rabbits and monkeys have shown that artemisinin and its derivatives cause angiogenesis defects, embryonic death, developmental retardation and cardiovascular malfunction<sup>[12–16]</sup>. We consistently found that DHA induces anomalies in zebrafish, including embryonic death, developmental retardation and cardiovascular malfunction. The difference is that we found that DHA promoted angiogenesis in zebrafish early development. This inconsistency may be attributed not only to different species but also

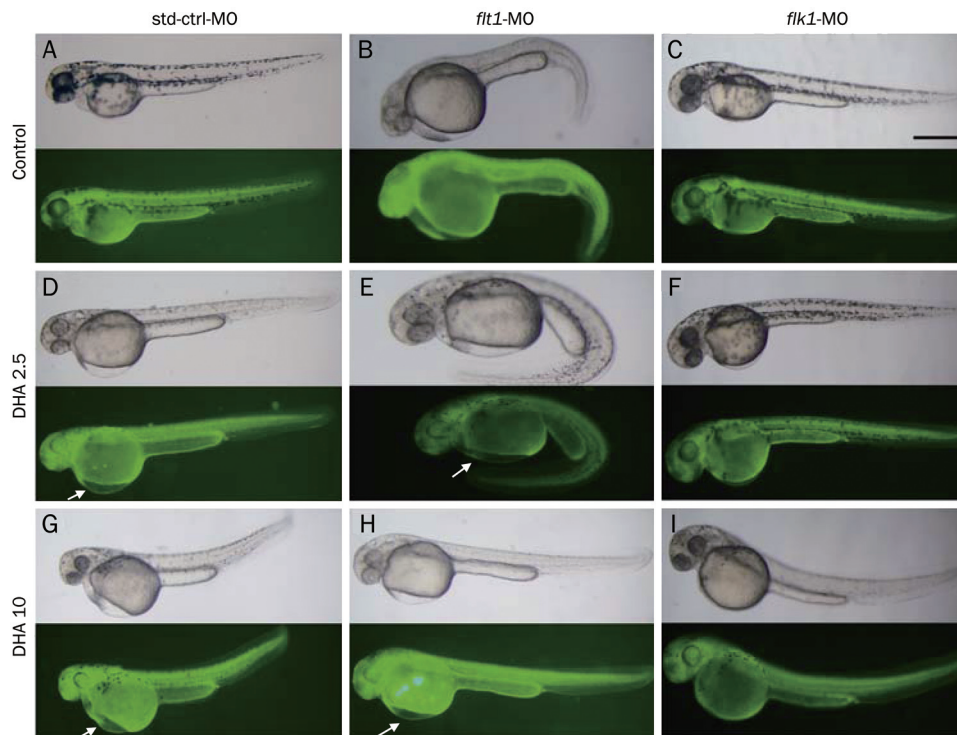


**Figure 4.** Dihydroartemisinin (DHA) enhances the expression of angiogenesis marker genes in zebrafish embryonic development. (A–C) The WISH results for *vegfa* (A), *flk1* (B), and *flt1* (C) expression induced by DHA during early embryogenesis at 48 hpf. The embryos in panels A (a, e), B (a, e), and C (a, e) were untreated, the embryos in panels A (b, f), B (b, f), and C (b, f) were DMSO-treated, the embryos in panels A (c, g), B (c, g), and C (c, g) were treated with 2.5 mg/L DHA, and the embryos in panels A (d, h), B (d, h), and C (d, h) were treated with 10 mg/L DHA. Panels A (a–d), B (a–d), C (a–d) are lateral views and the others are dorsal views. The scale bar represents 650  $\mu$ m for panels A (a–d), B (a–d), C (a–d), and 150  $\mu$ m for panels A (e–h), B (e–h), C (e–h). (D) The mRNA expression level of *vegfa*, *flk1*, and *flt1* at 48 hpf was examined by real-time PCR. Data shown are the mean  $\pm$  SEM for at least three independent experiments.  $^{\circ}P < 0.01$  compared with the control embryos.

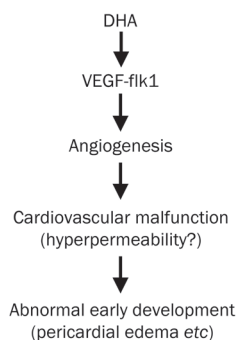
to different experimental settings. In these studies, artemisinin was delivered *po* in the gestational period for approximately 10 d (rats and rabbits) or more than 30 d (monkeys). In the current study, we directly added the drug into the system water and cultured the zebrafish embryos continuously for up to approximately 6 d, which represents a distinct culture system and drug delivery route. In addition, *in vitro* studies show that the activity of DHA varies with different cell types, suggesting that the physiological environment might also confer this variability. Because the culture condition and drug delivery route are distinct between zebrafish and other lab animals, we cannot compare the concentrations of DHA among them. However, in patients, the plasma level of artemisinin derivatives varies from 0.51 to 200  $\mu$ g/mL, depending on the drug delivery route, and these levels are close to the concentrations that we used<sup>[10]</sup>.

Previous studies have shown that the overexpression of VEGF can induce pericardial edema<sup>[18, 24]</sup>, and our real-time PCR and *in situ* hybridization results also supported the notion that VEGF and its receptors play important roles in this process. In zebrafish, *flk1* has an essential role in vasculogenesis, angiogenesis and hematopoiesis, which can mediate vascular dilation, endothelial cell proliferation and epidermal hyperplasia by binding VEGF<sup>[25]</sup>. In our studies, the knockdown of *flk1* with antisense MOs blocked the pericardial edema phenotype, which confirmed that DHA exerted its effects through the VEGF-*flk1* pathway.

In addition to inducing pericardial edema, VEGF plays important roles in angiogenesis. Studies with *in vitro* cell models, such as endothelial cells, and *ex vivo* models, such as the microvessel-like formation assay, indicate that DHA, along with other derivatives, can downregulate VEGF expression



**Figure 5.** Antisense knockdown of *flk1* in zebrafish abolishes dihydroartemisinin (DHA)-induced pericardial edema. (A–C) Embryos were treated with DMSO. (D–F) Embryos were treated with 2.5 mg/L DHA. (G–I) Embryos were treated with 10 mg/L DHA. NC-MO injected embryos are shown in panels A, D, and G. *flt1*-MO injected embryos are shown in panels B, E, and H. *flk1*-MO injected embryos are shown in panels C, F, and I. The scale bar represents 500  $\mu$ m for all panels.



**Figure 6.** The proposed mechanism by which dihydroartemisinin (DHA) exposure causes abnormal early embryonic development in zebrafish.

and inhibit angiogenesis<sup>[24, 26–28]</sup>. Moreover, the cytotoxicity of DHA might play a role in its anti-angiogenic function in *ex vivo* models inhibiting the proliferation of vessels in the medium by killing single cells. However, we found that DHA increased *vegfa* expression and promoted angiogenesis. This inconsistency may be attributed to a difference between the *in vitro* and *in vivo* contexts. Considering that our studies were performed in live animals, the pro-angiogenic effect observed in zebrafish embryos may have more biological relevance.

This study demonstrated the potential toxicity of DHA on the early embryonic development of zebrafish and its cor-

responding mechanisms. Interestingly, we found that DHA exerted a distinct effect on angiogenesis in zebrafish in which the VEGF-*flk1* pathway might be involved. Further studies to uncover the mechanism of the embryotoxicity of DHA are warranted to guide the clinical use of artemisinin and its derivatives with less toxicity and more significantly anti-malarial and anti-tumor effects.

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### Author contribution

Hui WANG and Rui-ai CHU designed the research and revised the manuscript; Qian BA, Juan DUAN, and Jia-qiang

TIAN performed the research and wrote the manuscript; Ziliang WANG analyzed the data; Tao CHEN, Xiao-guang LI, Pei-zhan CHEN, Song-jie WU, Li XIANG, and Jing-quan LI performed the experiments.

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