

# Resveratrol Treatment Attenuates the Wound-Induced Inflammation in Zebrafish Larvae through the Suppression of Myeloperoxidase Expression

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## ABSTRACT

Resveratrol, a polyphenolic phytoalexin found in many plants, was reported to exhibit anti-inflammatory effects, but its molecular mechanism is not fully understood. This study was aimed to investigate the anti-inflammatory effects of resveratrol *in vivo* using a zebrafish model of wound-induced inflammation. Caudal fin-wounded zebrafish larvae were treated with resveratrol for 8 h. Neutrophil recruitment was visualized in transgenic line “Tg (*mpx*: GFP)” expressing GFP-tagged neutrophil-specific myeloperoxidase (*mpx*). The enzymatic activity of Mpx was evaluated by histochemical staining. Relative mRNA levels of *mpx* and cyclooxygenase-2 (*cox2*) were quantified by qRT-PCR, and the protein expression levels of Mpx and Cox2 were detected by immunostaining. Results showed that wound-induced neutrophil recruitment in zebrafish was not affected by resveratrol, but Mpx enzymatic activity in zebrafish was significantly suppressed by resveratrol in a dose-dependent manner. Moreover, both mRNA and protein expression levels of Mpx and Cox2 were significantly down-regulated by resveratrol. Taken together, our results provide *in vivo* evidence that the anti-inflammatory effects of resveratrol on wound-induced inflammation are significantly mediated through the suppression of Mpx and Cox2 at both transcriptional and protein levels, rather than the down-regulation of neutrophil recruitment. In conclusion, this *in vivo* zebrafish model can be readily applied to screen other potential anti-inflammatory compounds at a whole-organism level.

Key words: cyclooxygenase, inflammation, myeloperoxidase, resveratrol, zebrafish

## INTRODUCTION

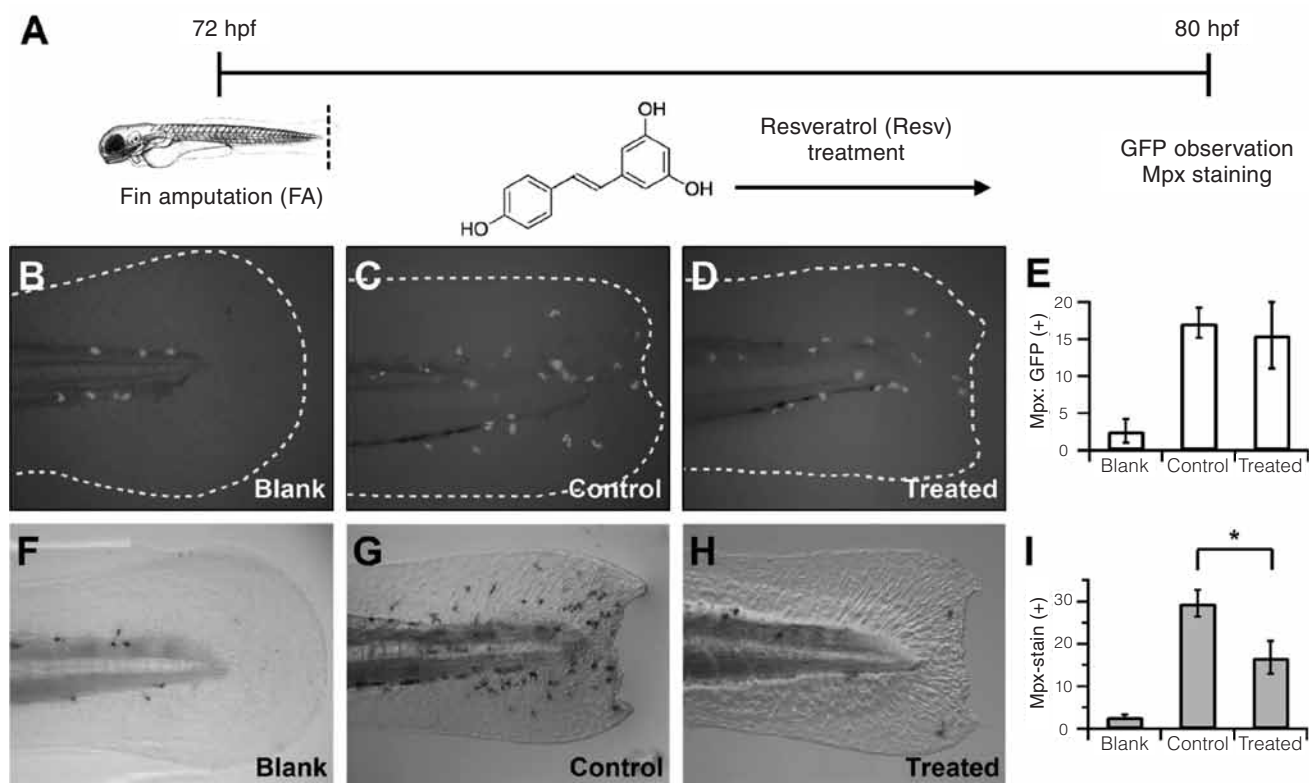
The wound-induced inflammation involves dynamic regulation of pro-inflammatory mediators and enzymes accompanying the recruitment of blood cells to wound sites. Circulating leukocytes are the key cells, and the chemotaxis of neutrophils to sites of inflammation is essential for physiological responses to tissue injury and infection<sup>(1)</sup>. However, dysregulated or excessive inflammation may lead to pathological consequences. To attenuate potential adverse effects of wound-induced inflammation, it is essential to identify or develop effective anti-inflammatory compounds.

Resveratrol (*trans*-3,4',5-trihydroxystilbene, Figure 1A) is a natural polyphenolic phytoalexin found in many plants.

Resveratrol has been reported to exhibit anti-inflammatory activity through down-regulation of the inflammatory responses, but the molecular mechanism is not fully understood. In isolated leukocytes of mice or human cell lines, resveratrol has been reported to inhibit pro-inflammatory mediators and enzymes, such as Cox2, TNF-induced activation of nuclear transcription factors NF- $\kappa$ B, and NF- $\kappa$ B-dependent gene expression<sup>(2-5)</sup>. In isolated neutrophils of turbot (*Psetta maxima*), resveratrol is shown to inhibit the activity and transcription of *mpx* gene<sup>(6)</sup>. However, current knowledge regarding the anti-inflammatory effects of resveratrol in vertebrates has been reported *in vitro*. Thus, it is essential to establish an effective animal model to study the *in vivo* anti-inflammatory effects of resveratrol.

Zebrafish is a widely used model organism because of its morphological and physiological similarity to mammals, and the optical transparency of zebrafish embryos allows noninvasive and dynamic imaging the inflammation *in*

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**Figure 1.** Myeloperoxidase (Mpx) expression in zebrafish larvae in response to wounding or resveratrol treatment. (A) Experimental scheme for detecting Mpx expression in zebrafish larvae. (B-D) The distribution of neutrophils in living Tg(*mpx*:GFP) zebrafish larvae using GFP fluorescence as a marker. Dashed line outlines the region of caudal fin. (E) Comparison of Mpx:GFP-expressing cells among groups (B-D). (F-H) The endogenous myeloperoxidase (Mpx) activity in wild-type zebrafish larvae. (I) Comparison of Mpx enzymatic activity among groups (F-H). Abbreviations: FA, fin amputation; Resv, resveratrol; Blank, unwounded group; Control, wounded group without treatment; Treated, wounded group treated with 25 ppm of resveratrol.

*in vivo*. The innate immunity of vertebrates is evolutionarily conserved from zebrafish to mammals. Zebrafish embryos develop several immune cells homologous to mammalian counterparts, and the innate immunity of zebrafish even appears highly evolved with potentially enhanced functionality in comparison to those of mammals<sup>(7,8)</sup>. Neutrophils are the most abundant leukocytes in adult zebrafish, and myeloperoxidase (Mpx) is an abundant peroxidase specifically expressed in zebrafish neutrophils<sup>(7)</sup>. Zebrafish *mpx* gene is expressed in embryos from 18 hpf on, thus neutrophil recruitment in zebrafish can be observed using *mpx* as a neutrophil marker<sup>(9,10)</sup>. In addition to Mpx, cyclooxygenase2 (Cox2) is an inducible inflammatory enzyme catalyzing the rate-limiting step in prostaglandin synthesis in response to inflammation. Zebrafish *cox2* gene is genetically and functionally homologous to its mammalian orthologs<sup>(11,12)</sup>.

In this study, a wounded zebrafish model was used to assess the anti-inflammatory effects of resveratrol on wound-induced inflammation *in vivo*. We observed a transgenic zebrafish line Tg (*mpx*:GFP) expressing green fluorescent protein (GFP) under the control of neutrophil-specific *mpx* promoter<sup>(13)</sup> to visualize the recruitment of neutrophils to wounded sites. We also evaluated the Mpx expression by histochemical staining<sup>(14)</sup>, and examined the transcriptional

and protein levels of two evolutionarily conserved pro-inflammatory enzymes (Mpx and Cox2) upon resveratrol treatment.

## MATERIALS AND METHODS

### I. Zebrafish Larvae, Fin Amputation and Resveratrol Treatment

Zebrafish larvae (wild-type, WT; AB strain) and Tg (*mpx*:GFP)<sup>(13)</sup> were raised at 28.5°C and staged according to standard protocols<sup>(15,16)</sup>. All larvae were grown in 0.003% N-phenylthiourea (PTU, Sigma-Aldrich) to suppress melanization. To trigger wound-induced inflammation, caudal fin of zebrafish larvae at 72 hours post-fertilization (hpf) was half-amputated, around 50% of caudal fin area was removed (Figure 1A). To make wounds as consistently sized as possible, fin amputation (FA) was performed using a cryostat blade under a dissecting microscope.

Resveratrol (C<sub>14</sub>H<sub>12</sub>O<sub>3</sub>, Sigma-Aldrich) was dissolved in dimethylsulfoxide (DMSO) as stock solution (2,500 ppm), and diluted to desired concentrations for treatment. Unwounded zebrafish larvae (FA-) were randomly divided into "blank" group (FA-, RESV-) and "resv" group (FA-,

RESV+), whereas wounded larvae (FA+) for no-treatment “control” group (FA+, RESV-) or resveratrol-“treated” group (FA+, RESV+). Each group was exposed to DMSO (RESV-) or resveratrol (RESV+) in the dark at 28.5°C for 8 hours (Figure 1A). The final concentration of DMSO used in all groups was less than 1%. The possible effects of DMSO (1%) could be neglected because the same amount of vehicle solvent was applied to all groups.

## II. Myeloperoxidase Staining

Myeloperoxidase staining of zebrafish larvae was based on the method of Kaplow<sup>(14)</sup>. Larvae at 72 hpf were fixed with 10% formaldehyde/ethanol at room temperature for 1 min. After washing with double-distilled water (ddH<sub>2</sub>O) for 30 s, myeloperoxidase (Mpx) of larvae was stained with incubation mixture (30% ethanol, 0.3% benzidine dihydrochloride, 0.38% ZnSO<sub>4</sub>•7H<sub>2</sub>O, 1% sodium acetate, 0.02% H<sub>2</sub>O<sub>2</sub>, 0.015N NaOH, pH 6) at room temperature for 5 - 10 min, and stopped by washing with ddH<sub>2</sub>O for 30 s. After staining, Mpx-stain (+) cells were shown in brown.

## III. Quantitative Reverse Transcription-Polymerase Chain Reaction (qRT-PCR)

Total RNAs were extracted from zebrafish and reverse-transcribed by the standard procedure as described previously<sup>(17,18)</sup>. Gene-specific PCR primer sequences were listed in Table 1. Quantitative PCR was performed for 2 min at 50°C, 10 min at 95°C, and 40 cycles of 15 s at 95°C, and 1 min at 60°C, using 2X Power SYBR Green PCR Master Mix (Applied Biosystems) and 200 nM of forward and reverse primers (Table 1). Each assay was performed in triplicates on an Applied Biosystems 7300 Real-Time PCR system, and expression fold-changes were derived by the comparative C<sub>T</sub> method. The β-actin mRNA was used as an internal control for relative quantification.

## IV. Whole-Mount Immunostaining

Whole-mount immunostaining was performed as previously described, with minor modifications<sup>(19)</sup>. Expression levels of Mpx and Cox2 were detected with rabbit polyclonal primary antibodies against either Mpx (1 : 20; AnaSpec) or Cox2 (1 : 10; Cayman Chemicals), and visualized with Alexa Fluor 568-conjugated goat-anti-rabbit IgG (1 : 200; Invitrogen) as the secondary antibody.

## V. Image Acquisition and Analysis

Images were acquired using a camera (Leica DFC 280) attached to a stereomicroscope (Leica DM 2500; objective: 20x; filter: for GFP or RFP fluorescence). Acquired images were analyzed by using built-in functions (“Analyze Particles” and “Find Maxima” for automated counting; “Cell Counter” for manual counting) of ImageJ (NIH Image software). Results from automated counting were confirmed in consistency with

**Table 1.** Primers used in this study

Primer	Sequence (5' → 3')
β-actin-F	CAGCAAGCAGGAGTACGATGAGT
β-actin-R	TTGAATCTCATTGCTAGGCCATT
Danio <i>cox2</i> -F	CTGCACTCTTGAGCTGTTCAT
Danio <i>cox2</i> -R	AGAATACACCTGGACGTGAAACAA
Danio <i>mpx</i> -F	GAGTCTCTGCCCTTTACTAGTGT
Danio <i>mpx</i> -R	TCACCCGCAATGAAGCAA

those from manual counting. To quantify the relative intensity of immunostaining images, each image was transformed into grayscale format and analyzed by using Igor Pro software (WaveMetrics) to compute the value of “Intensity<sub>(mean/max)</sub>”, i.e. average intensity normalized to the maximum.

## VI. Statistical Analysis

Quantitative data were expressed as mean ± SEM (standard error of the mean). Statistical significance was determined by two-sample *t*-test. *p*-value of less than 0.05 was considered statistically significant.

# RESULTS

## I. Zebrafish Model of Wound-Induced Inflammation

To investigate wound-induced inflammation *in vivo*, we used transgenic zebrafish Tg (*mpx*: GFP) expressing GFP-tagged neutrophils which has been used to visualize the resolution of inflammation<sup>(1,10,20,21)</sup>. Because the inflammatory response to wounding depends on the wound size, we half-amputated caudal fin of zebrafish (Figure 1A) to obtain relatively consistent wound sizes and inflammatory responses in comparison with those by tip amputation<sup>(1,20)</sup> and complete transection of caudal fin<sup>(10,21)</sup>.

Green fluorescent neutrophils (hereafter cited as Mpx: GFP(+) cells) were observed during wound-induced inflammation (Figure 1B-E). In unwounded zebrafish, Mpx: GFP(+) cells were observed within the blood circulation (Figure 1B, Blank). In contrast, circulating Mpx: GFP(+) cells tended to migrate toward the wound site upon fin amputation in wounded zebrafish (Figure 1C, Control). A significant increase in Mpx: GFP(+) cells was observed in the wounded group (Figure 1E, Control, Mpx: GFP(+): 17 ± 2, n = 17) compared with the unwounded group (Figure 1E, Blank, Mpx: GFP(+): 2.6 ± 1.6, n = 5), demonstrating a wound-induced recruitment of Mpx: GFP-expressing neutrophils similar to previous studies<sup>(1,10,20,21)</sup>.

## II. Effects of Resveratrol on Wound-Induced Neutrophil Recruitment and Mpx Enzymatic Activity

Upon treatment with 25 ppm of resveratrol for 8 h, the



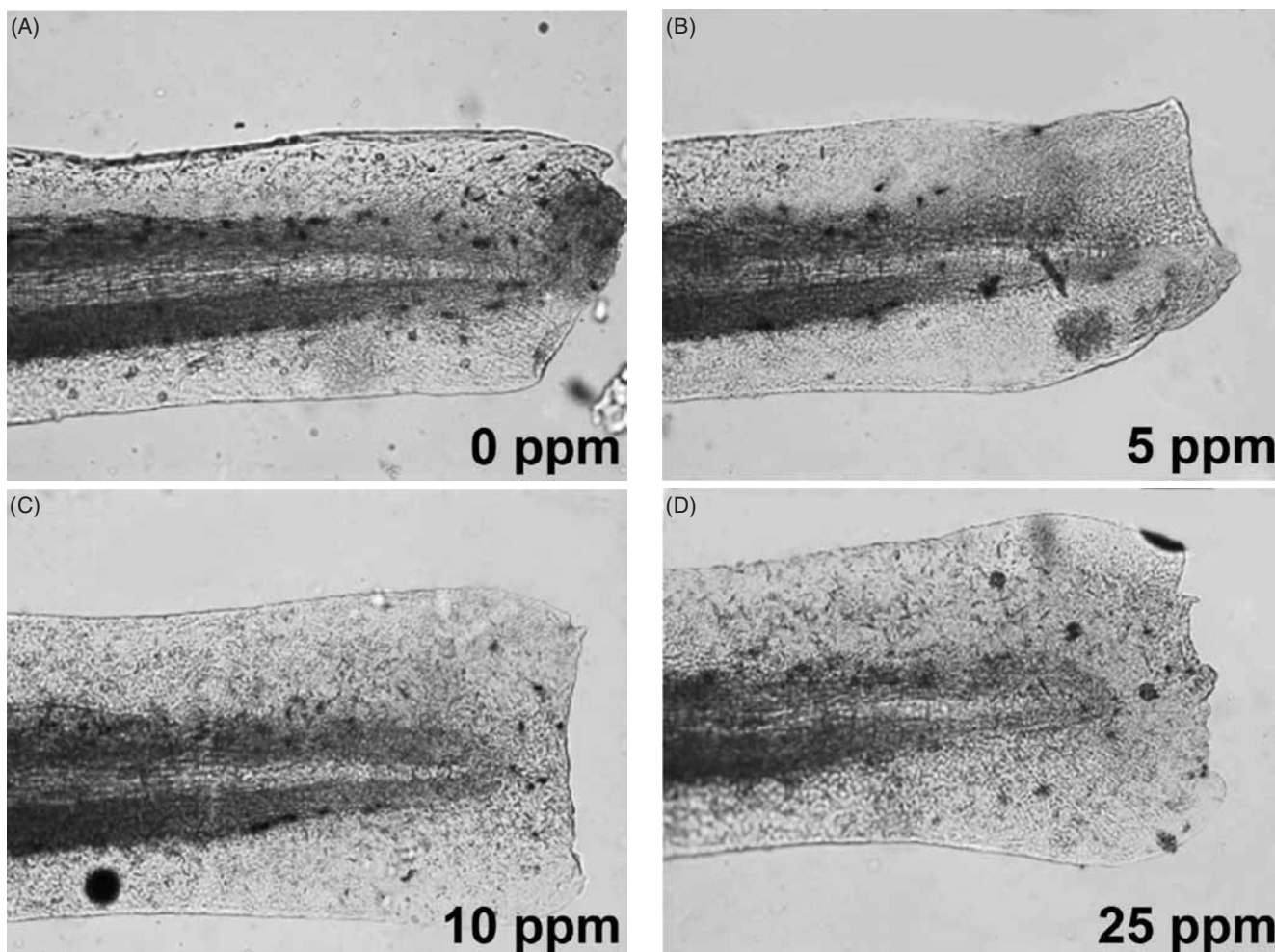
wound-induced neutrophil recruitment was slightly attenuated in the resveratrol-treated group (Figure 1D, Treated). However, the difference between resveratrol-treated group (Figure 1E, Treated, Mpx:GFP(+):  $16 \pm 4.5$ ,  $n = 8$ ) and no-treatment control (Figure 1E, Control) was not statistically significant (Figure 1E, Treated versus Control,  $p > 0.05$ ). These *in vivo* results preliminarily demonstrated that wound-induced neutrophil recruitment in living zebrafish was not affected by resveratrol treatment.

To further evaluate the effect of resveratrol treatment on Mpx enzymatic activity in neutrophils, endogenous Mpx enzymatic activity in wild-type zebrafish was observed by histochemical staining with peroxidase substrate benzidine (Figure 1F-I). Positively-stained cells (hereafter cited as Mpx(+) cells) revealed the Mpx activities *in vivo* and *in situ*. Based on the Mpx(+) cells observed in the tailfin region, wounded zebrafish (Figure 1G, Control, Mpx(+):  $30 \pm 3$ ,  $n = 32$ ) revealed a significant increase in Mpx enzymatic activity (Figure 1I,  $p < 0.05$ ) versus the corresponding blank group (Figure 1F, Blank, Mpx(+):  $2.8 \pm 0.47$ ,  $n = 24$ ). Upon resveratrol treatment, a significant decrease (Figure 1I,  $p < 0.05$ ) of the Mpx(+) cells was observed in resveratrol-treated

group (Figure 1H, Treated, Mpx(+):  $17 \pm 3.8$ ;  $n = 25$ ) versus no-treatment control (Figure 1G, Control). Taken together, resveratrol significantly attenuated the Mpx enzymatic activity (Figure 1I), rather than the wound-induced neutrophil recruitment *in vivo* (Figure 1E).

### III. Dose-Dependent Attenuation of Mpx Enzymatic Activity by Resveratrol

The dose-dependency of the Mpx enzymatic activity on resveratrol treatment was further examined by exposing wounded zebrafish larvae at 72 hpf to different doses of resveratrol (0, 5, 10, and 25 ppm, respectively) for 8 h (Figure 2). In the absence of resveratrol treatment (0 ppm), Mpx(+) cells were widely distributed around wounded sites (Figure 2A). Upon treatment with 25 ppm of resveratrol, Mpx(+) cells were significantly reduced (Figure 2D). With increasing resveratrol doses (5 ppm, 10 ppm, and 25 ppm), there was a tendency towards reduction in Mpx(+) cells in the caudal fin region of wounded zebrafish larvae (Figure 2B-D), suggesting that resveratrol attenuated Mpx enzymatic activity in a dose-dependent manner.



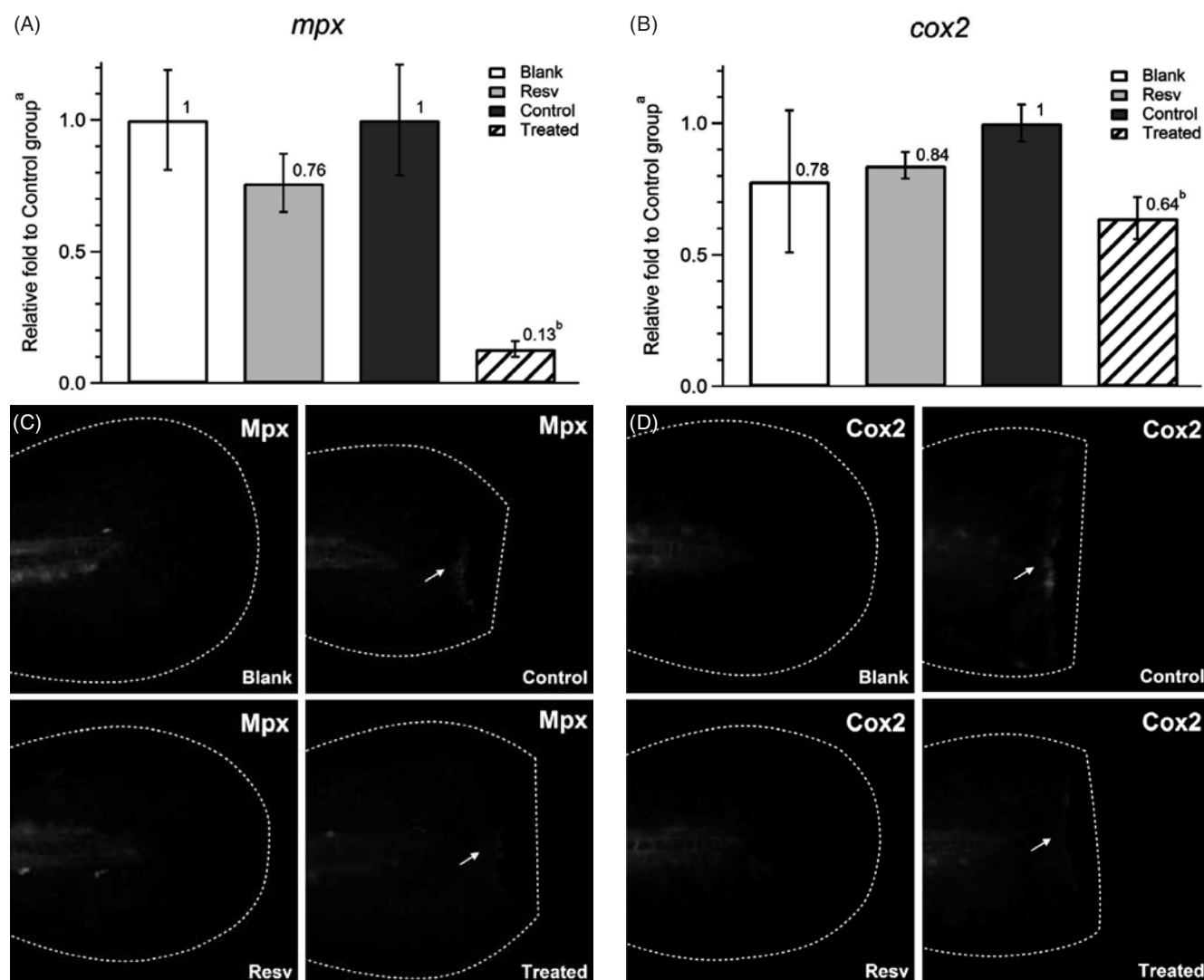
**Figure 2.** Dose-dependent suppression of the Mpx-positive cells in wounded zebrafish larvae upon resveratrol treatment. (A) No-treatment control (0 ppm) and (B-D) resveratrol-treated groups (B, 5 ppm; C, 10 ppm; and D, 25 ppm).

#### IV. Molecular Mechanism of Anti-Inflammatory Effects of Resveratrol

To further investigate the molecular mechanism of anti-inflammatory effects of resveratrol, we examined the effects of resveratrol on Mpx and Cox2 at transcriptional and protein levels (Figure 3). The relative mRNA expression levels of *mpx* and *cox2* in zebrafish larvae at 80-hpf were quantified by qRT-PCR assays (Figure 3, A-B). Figure 3A shows the transcriptional levels of *mpx* relative to control group. Blank group ( $1 \pm 0.19$ ) and Resv group ( $0.76 \pm 0.11$ ) showed comparable levels to the control group ( $1 \pm 0.21$ ), whereas resveratrol-treated group showed significant decrease ( $0.13 \pm 0.03$ ,  $p < 0.05$ ) compared with no-treatment control group. These results showed that resveratrol attenuated the mRNA expression of *mpx*, correlated well with above-mentioned results

revealed by transgenic line (Figure 1B-E, Mpx:GFP(+) cells). Similarly, Figure 3B presents the transcriptional levels of *cox2* relative to control group. Blank group ( $0.78 \pm 0.27$ ) and Resv group ( $0.84 \pm 0.05$ ) showed comparable levels to the control group ( $1 \pm 0.07$ ), whereas resveratrol-treated group showed significant decrease ( $0.64 \pm 0.08$ ,  $p < 0.05$ ) compared with no-treatment control group. These results revealed that resveratrol suppressed the transcriptional levels of *mpx* and *cox2* in wound-induced inflammation.

Moreover, the effects of resveratrol on Mpx and Cox2 at protein level were also examined. The protein expression levels of Mpx and Cox2 in zebrafish larvae at 80-hpf (8 h post-treatment) were detected by whole-mount immunostaining using antibodies against Mpx or Cox2 (Figure 3, C-D). Figure 3C shows the relative protein expression levels of Mpx in the caudal-fin region. In unwounded groups, Mpx



**Figure 3.** Effects of resveratrol on myeloperoxidase (Mpx) and cyclooxygenase-2 (Cox2) in 80-hpf zebrafish larvae at transcriptional and protein levels. (A-B) Relative mRNA expression levels of *mpx* (A) and *cox2* (B) using the comparative  $C_T$  method ( $C_T$ : cycles of qPCR; <sup>a</sup>: Relative folds to control group  $= 2^{-\Delta\Delta C_T}$ ; <sup>b</sup>: The resveratrol-treated group is significantly different from control group). (C-D) Whole-mount immunostaining of zebrafish larvae with antibodies against Mpx (C) and Cox2 (D). Dashed lines outline the region of caudal fin, and arrows indicate the wounded sites. Abbreviations: Blank, unwounded group; Resv, unwounded group treated with resveratrol (25 ppm); Control, wounded group; Treated, wounded group treated with resveratrol (25 ppm).

expression was detected in blood circulation, but did not appear in the caudal-fin region (Figure 3C, Blank and Resv). After fin amputation, Mpx expression in wounded groups was observed around the wounded sites (indicated by arrows in Figure 3C, Control and Treated). Upon resveratrol treatment, Mpx expression in resveratrol-treated group (Figure 3C, Treated) appeared to decrease compared with no-treatment control group (Figure 3C, Control). These immunostaining results revealed that resveratrol attenuated the protein expression of Mpx in wound-induced inflammation, not only consistent with above-mentioned transcriptional levels of *mpx* (Figure 3A) but correlated well with above-mentioned Mpx enzymatic activity revealed by histochemical staining (Figure 1F-I, Mpx(+) cells).

On the other hand, Figure 3D presents the relative protein expression levels of Cox2 in the caudal-fin region. In unwounded groups, Cox2 expression did not appear in the caudal-fin region (Figure 3D, Blank and Resv). After fin amputation, Cox2 expression in wounded groups was induced around the wounded sites (indicated by arrows in Figure 3D, Control and Treated). Upon resveratrol treatment, Cox2 expression in resveratrol-treated group (Figure 3D, Treated) appeared to decrease compared with no-treatment control group (Figure 3D, Control). These immunostaining results indicated that resveratrol attenuated the protein expression of Cox2 in wound-induced inflammation, correlated well with above-mentioned transcriptional levels of *cox2* (Figure 3B). Taken together, our results suggested that the anti-inflammatory effects of resveratrol on wound-induced inflammation were mediated through the suppression of Mpx and Cox2 expression at both transcriptional and protein levels. In addition, the effects of resveratrol on the suppression of Mpx were more significant than those on the suppression of Cox2.

## DISCUSSION

This study demonstrated that the anti-inflammatory effects of resveratrol on wound-induced inflammation are mediated through the suppression of Mpx and Cox2 expression at both transcriptional and protein levels. To our knowledge this is the first *in vivo* evidence that resveratrol attenuates the wound-induced inflammation through Mpx and Cox2 at a whole-organism level. Our *in vivo* evidence is also consistent with previous *in vitro* one from cell-based or enzyme-based assays in other vertebrate models of inflammation. For example, resveratrol was reported to decrease Mpx enzymatic activity and COX-2 expression level in isolated leukocytes of rats<sup>(22)</sup>. Resveratrol has been demonstrated to inhibit transcription and enzymatic activity of myeloperoxidase in isolated neutrophils of human and other species<sup>(6,23,24)</sup>. Resveratrol was also revealed to inhibit Cox2 transcription and activity in phorbol ester-treated human mammary epithelial cells<sup>(3)</sup>. Resveratrol has been reported to affect several signal transduction pathways (e.g. TNF- $\alpha$ , MAPKs, NF- $\kappa$ B, and so on)<sup>(25,26)</sup>. Whether these signaling mechanisms are directly or indirectly involved in the

anti-inflammatory effects of resveratrol on Mpx and Cox2 needs further investigation.

Based on partial fin amputation used in this study to yield relatively consistent wound sizes and inflammatory responses, this zebrafish model of wound-induced inflammation could be used for better understanding of the resolution of wound-induced inflammation and wound-healing process. Moreover, this zebrafish model of wound-induced inflammation can also be readily applied to *in vivo* screening of the therapeutic potential of anti-inflammatory compounds, which are worth further tests in higher vertebrates. Furthermore, an automated screening platform of zebrafish larvae in high throughput was reported to process a single zebrafish larva in less than 30 seconds<sup>(27)</sup>, demonstrating that zebrafish is emerging as a powerful platform for genetic and chemical screening at a whole-organism level.

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