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Impact of molecular hydrogen treatments on the innate immune activity and survival of zebrafish (*Danio rerio*) challenged with *Aeromonas hydrophila*

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ABSTRACT

Recently, molecular hydrogen has been reported to have a suppressive effect on inflammation in human and rodent models. The aim of this study was to evaluate the preventive effects of hydrogen-rich water (HRW) on zebrafish challenged by *A. hydrophila*. We have found an increased survival rate of bacteria-challenged zebrafish subjected to the HRW immersion treatment. Furthermore, we have revealed that HRW was able to block multiplication of *A. hydrophila* in zebrafish. In addition, treatment of zebrafish infected by *A. hydrophila* with effective concentrations of HRW strongly affected the expression of genes mediating pro-inflammatory and anti-inflammatory cytokines. There were down-regulation of selected pro-inflammatory immune response genes (IL-1β, IL-6, and NF-κB), and up-regulation of the anti-inflammatory cytokine gene (IL-10) in the spleen, kidney, and liver. This study is the first one to investigate the effects of HRW on fish infected with bacteria, and might shed new light on hydrogen's antimicrobial effects and further application in aquaculture fish species.

1. Introduction

Interest in the effect of molecular hydrogen in various diseases has been spurred since Ohsawa and colleagues reported the astonishing therapeutic effects of molecular hydrogen on a rat model of cerebral infarction in Nature Medicine [1]. Their paper ignited a number of original articles demonstrating the effect of molecular hydrogen including the anti-oxidative stress, anti-inflammatory, and anti-apoptotic effects [2–5]. Drinking water containing a therapeutic dose of hydrogen (hydrogen-rich water) represents an alternative mode of delivery of molecular hydrogen. It has potential therapeutic value in the prevention of anti-inflammatory diseases. Up till now, at least 63 disease categories including essentially all organs including the brain, spinal cord, eye, ear, lung, heart, liver, kidney, pancreas, intestine, blood vessel, muscle, cartilage, metabolism, perinatal disorders, and inflammation/allergy [6]. It has been reported that hydrogen-rich water (HRW) regulate immunity by functioning as immunomodulators, showing effects by modulating activities and expressions of various molecules such as intercellular-adhesion-molecule-1 (ICAM-1) and myeloperoxidase [7]; B-cell lymphoma 2 (Bcl2) and Bcl2-associated X protein (Bax) [8]; matrix metalloproteinases (such as MMP3 and MMP13) [9]; neuronal nitric oxide synthase (nNOS) [10], fibroblast growth factor 21 (FGF21) [11], extracellular signal-regulated kinase (ERK) [12], c-Jun N-terminal kinase (JNK) [13], Nuclear factor κB (NF-κB) [13–15], vascular endothelial growth factor (VEGF) [12]. They not only boost the immune response to prevent infection but also suppress other proinflammatory responses to avoid uncontrolled inflammation, thereby resulting in homeostasis of innate cellular defense within an organism [6]. Recently, it has been found that hydrogen regulates miRNA expressions and modifies expressions of inhibitor of IκB kinase-beta (IKK-β), NF-κB, and programmed cell death protein4 (PDCD4) in lipopolysaccharide (LPS)-activated retinal microglia cells [16,17].

As a widely used model organism to study vertebrate development and hematopoiesis, Zebrafish (*Danio rerio*) has caught increasing growing interest of immunologist in the field of human disease and cancer in recent years [18,19], as well as the field of fish immunology [20]. The zebrafish has a complete immune system (including both innate and adaptive immune system), it has

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advantages over other vertebrate infection models, such as fruit flies, nematodes, mice and rats, because of its evolutionarily closer to humans [21]. It has relatively small size and rapid life cycle, and it is ease of breeding, transparency of early life stages allowing real-time visualization. These make zebrafish a perfect model being used for study of experimental infections for fish pathogens [22–28].

Aeromonas hydrophila has been reported as an important pathogen for lower vertebrates including amphibians, reptiles and fish [29]. These Gram-negative aquatic bacteria can cause fatal hemorrhagic septicaemia in a wide variety of fresh water fish species [30,31], In particular, unusual and sometimes high mortality rates caused by *A. hydrophila* infections have been repeatedly reported from zebrafish facilities [32,33]. Some strains of *A. hydrophila* are also harmful to humans that might cause systemic illness of immune-compromised patients [34,35].

Hence, a study to demonstrate antimicrobe effect and immune modulation of molecular hydrogen in fish will be a very fascinating topic, and the aim of our study was to investigate the preventive effects of HRW on bacterial infection in zebrafish. We have analyzed the anti-inflammatory and pro-inflammatory immune system parameters in response to HRW treatment caused by a pathogenic *A. hydrophila in vivo*.

This is the first study to investigate the effects of HRW with fish infected with bacteria, and to show that the appropriate hydrogen concentrations have an effect on fish survival when challenged with *A. hydrophila*. Furthermore, the anti-inflammatory gene was up-regulated in the kidney, spleen and live, the pro-inflammatory genes were generally down regulated. The present work has shown *in vivo* study of HRW treatment on the innate immune system of zebrafish, while further studies are needed to gain better insight of the mechanism of immunological signalling pathway activation.

2. Materials and methods

2.1. Zebrafish husbandry and sample collection

For most experiment, zebrafish wild type AB adults (4-6 month-old) were used and held in 200 L tanks with freshwater at 28 °C. Fish were fed twice a day and acclimatized for 2 weeks. Prior to the experiments, zebrafish were fasted for 1 day. All zebrafish were handled in compliance with the local animal welfare regulations and maintained according to standard protocols which were designed to minimize pain and discomfort to the animals. For challenge experiment, zebrafish were anaesthetized with MS-222 (tricaine methanesulpho-nate, Argent Chemical Laboratories, USA). Euthanasia of zebrafish was achieved by anaesthetic overdose. The samples were collected and immediately frozen in liquid nitrogen and stored at −70 °C before use.

2.2. Bacteria culture conditions and production of HRW

The Gram-negative bacterium *A. hydrophila* was preserved by our laboratory [36]. When needed, the *A. hydrophila* strain was streaked onto a Luria Bertani (LB) plate and incubated at 28 °C for 24 h, then single colonies were picked and inoculated into 100 μl liquid LB medium, and incubated at 28 °C for 12 h with continuous shaking at 200 rpm. After centrifugation at 8000 rpm for 5min, the harvested bacterial pellet was re-suspended twice in 20 mM sterilized phosphate-buffered saline (PBS) (pH 7.4) to yield a concentration of 5×10^9 CFU/ml, and stored at 4 °C until used.

HRW was produced in an acrylic resin tube by using apparatus (SHC-300, Haowei Co. Jinan, China) producing molecular hydrogen gas in a polyethylene terephthalate (PET) bottle for 2 h so as to dissolve molecular hydrogen into pure water. HRW was prepared just before the experiment in this study, and the hydrogen concentration of the HRW used was measured to be 6 ppm [15]. 1% and 4% HRW were produced by diluting 10 ml and 40 ml HRW in 1 L purified water.

2.3. Zebrafish survival assay

To assess the protective role of HRW *in vivo*, 120 adult zebrafish were randomly divided into 6 groups (20 fish per group) and individually cultured in a 10 L tank for the *A. hydrophila* challenge. Then adult zebrafish were individually injected intraperitoneally with 10 μl of live *A. hydrophila* suspension (3.3×10^7 CFU/ml). Three groups injected with 10 μl of sterilized PBS (control) and three groups injected with A. hydrophila. After injection, challenged fish were cultured in water with 0, 1% or 4% HRW. The survival rates of zebrafish were recorded every 6 h after the bacterial challenge, and the cumulative mortality was calculated, the relative percent survival (RPS) was calculated by the formula:

RPS = [1−(mortality of experimental group/mortality of control group)] × 100, as described by Amend [37].

2.4. HRW bactericidal activity in vivo

To validate the protective effect of HRW against *A. hydrophila in vivo*, adult zebrafish were individually injected intraperitoneally with 10 μl of *A. hydrophila* (3.5×10^7 CFU/ml). After injection, 20 adult zebrafish were immersed in water containing 1% HRW, the other 20 adult zebrafish were immersed in water as a control. A total of five fish were sampled at 6 h, 12 h, 24 h, and 48 h after injection. The zebrafish was anesthetized in 0.2% tricaine and then euthanized via incubation in ice water for 15 min. Each zebrafish was washed with sterile water for three times and cut into pieces via a sterile surgical scissors. Then 10 ml of PBS was added and mixed with a homogenizer for 30mim at 28 °C. One microliter from each solution was collected for a serial dilution, 100 μl of each diluted solution were streaked onto a trypticase soya agar (TSA) plate and incubated at 28 °C for 24 h. *A. hydrophila* were calculated by count the colonies.

2.5. Bacterial challenges of fish

Experimental infections were performed to investigate the immune-related gene expression under HRW treatment. For intraperitoneal infections, tripulicated groups of 20 adult zebrafish were injected with 10 μl of *A. hydrophila* (3.3×10^7 CFU/ml), and then immersed in water containing 1% HRW. Spleen, kidney, and liver were sampled at 0, 6, 12 and 24 h post infection from a total of five zebrafish of each group. All samples were stored at −80 °C until used.

2.6. Total RNA extraction and cDNA production

The total RNAs were extracted from grinded samples by using Trizol (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions. Complete DNA removal was achieved by treating the supernatant from the RNeasy processed samples with RNase-Free DNase Set (Qiagen, Valencia, CA). Gel electrophoresis was used to confirm that isolated RNA was intact, and the concentration and purity of the RNA were quantified by using NanoDrop® ND-1000 (NanoDrop Technologies, Dela-ware, USA). 1 μg of total RNA was amplified in cDNA by using Superscript III Reverse Transcriptase (Invitrogen) following the manufacturer's instructions, and the cDNAs were stored at −20 °C for real-time PCR.

2.7. Gene expression profile by real-time PCR

Specific primers for β-actin, IL1β, IL6, IL-10, and NF-κB were designed by using the Primer 3 software based on their available sequences in the Gene Bank database (Table 1). Each pair of primer (10 μM) was mixed with 18 μl of EXPRESS SYBR GreenER qPCR Supermix (Invitrogen). Two μl of template cDNA was used. Real-Time PCR tests were performed in duplicate with a Stratagene detection system (Stratagene, La Jolla, CA, USA) using optical grade 96-well plates. The cycling profile was as follows: 94 °C for 2 min, followed by 40 cycles of 94 °C for 15 s and 55–60 °C for 15 s and 72 °C for 30 s. To determine the specificity of amplification, analysis of the product melting curve was performed after the last cycle of the amplification. After the PCR program, data were analyzed using Bio-Rad iQ5 2.1 Standard Edition Optical System Software (Bio-Rad, Hercules, California, USA). The baseline was set automatically by the software to maintain consistency. The 2-△△CT method was used to analyze the expression level of each cytokine gene was normalized to β-actin as previously described in our lab [36]. All data were given in terms of relative mRNA expressed as mean ± S.E ($N = 3$).

2.8. Statistical analysis

Each experiment was performed in triplicate. Data from bacterial challenge on zebrafish responses and antibacterial assay were analyzed by using the SPSS 22.0 software. Differences with $p < 0.05$ were considered statistically significant and those with $p < 0.01$ were considered highly significant.

3. Results

3.1. The immersion of HRW treatment enhances the bacteria challenged zebrafish survival rate

To examine if HRW has any antimicrobial activity, different concentrations of HRW were applied to adult *D. rerio*, after intraperitoneally injection with live *A. hydrophila*. Fig. 1 showed that the zebrafish survival rate at 48 h post-infection was 100% for the group injected with PBS. However, the zebrafish survival rates at 48 h post-infection were dramatically reduced, high mortality of the fish treated with HRW occurred at 18–48 h after the bacterial challenge. A gradual mortality started among fish injected with bacteria without HRW treatment at 18 h with 10% and reached highest at 48 h with 40%. The RPS of HRW treatment at the dose of 1% were 100%, 90%, 85%, 85%, 85%, 80% at 18 h, 24 h, 30 h, 36 h, 42 h, and 48 h, individually, which was in contrast to the RPS of PBS group without HRW treatment was 60%. Compared with the group injected with bacteria alone without HRW treatment at 48 h post-infection, the survival rate increased significantly from 60% to 80%. The zebrafish injected with bacteria with HRW treatment at the dose of 4% showed similar results with milder protection with 65%. Taken together, our results indicated that HRW treatment could protect zebrafish from pathogenic *A. hydrophila* infection *in vivo*, especially 1% HRW treatment was capable of significantly enhancing the survival rate of zebrafish.

Table 1
Primer sequences of zebrafish immune-related genes for real time PCR.

Gene	Primer sequence (5′–3′)	Size(bp)	Accession number
β-actin	ATGGATGAGGAAATCGCTGC ATGCCAACCATCACTCCCTG	139	NM131031.1
IL-1β	TGGACTTCGCAGCACAAAATG GTTCACTTCACGCTCTTGGATG	149	NM212844.2
IL-6	AGACCGCTGCCTGTCTAAAA TTTGATGTCGTTCACCAGGA	135	NM001261449.1
NF-κB	TTTACTGCCAGGTGAAGGTGC TGACATAGCCAGACTTCTCAAACTC	124	NM001003414.1
IL-10	TGGAGACCATTCTGCCAACA GCATTTCACCATATCCCGCT	112	NM001020785.2

3.2. HRW blocks bacterial multiplication in zebrafish in vivo

To examine if HRW has any antimicrobial activity, *D. rerio* were injected with live *A. hydrophila*, immediately followed by immersion of 1% HRW, and then the bacterial numbers in the fishes were measured at 6 h, 12 h, 24 h and 48 h. HRW treatment at dose of 1% was applied to adult *D. rerio*, based on the previous experimental result of zebrafish survival rate. As shown in Fig. 2, *A. hydrophila* was detected in zebrafish with or without HRW treatment, the number of *A. hydrophila* detected represented only a small fraction (ca 2.5×10^4 CFUs) of the bacterial number initially injected. By 12 h, the CFUs were similar with those at 6 h, indicating that *A. hydrophila* did not multiplied in zebrafish until 12 h after the bacterial injection, which reached 2.05×10^5 CFUs and 4.72×10^5 CFUs at 24 h and 48 h. By contrast, the CFUs in zebrafish with 1% HRW treatment were significantly reduced at 24 h and 48 h than those of the corresponding fishes without HRW treatment from 2.05×10^5 CFUs to 1.49×10^5 CFUs and from 4.72×10^5 CFUs to 2.03×10^5 CFUs (Fig. 2). These demonstrated that HRW caused a marked decrease in the numbers of *A. hydrophila,* suggesting that HRW was able to block multiplication of *A. hydrophila* in zebrafish.

3.3. Effects of HRW on the expression of immune-related genes in spleen

To test if HRW functions as an immunomodulatory, the mRNA expression levels of the immune-related genes in different tissues in the zebrafish without or with the HRW immersion treatment were investigated using real-time PCR. The pro-inflammatory cytokine genes IL-1β, IL-6, and NF-κB, as well as the anti-flammatory cytokine gene IL-10.

In spleen, IL-1β expression levels in bacteria-injected zebrafish showed an immediate response, with a 5-fold increase over control levels at 6 h p.i. and reached 6-fold at 12 h p.i. After HRW treatment, it had a decreased trend throughout the different time points at 6 h, 12 h, and 24 h p.i. IL-6 expression levels had a 5-fold induction at

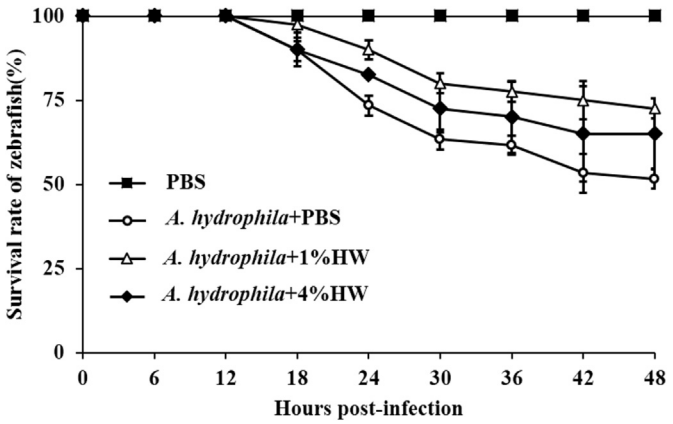

Fig. 1. Survival rate of zebrafish after injection with *A. hydrophila*, followed by immersion in 1% and 4% HRW. The mortality was recorded every 6 h and continued up to 48 h after the bacterial challenge. PBS was injected into zebrafish as control.

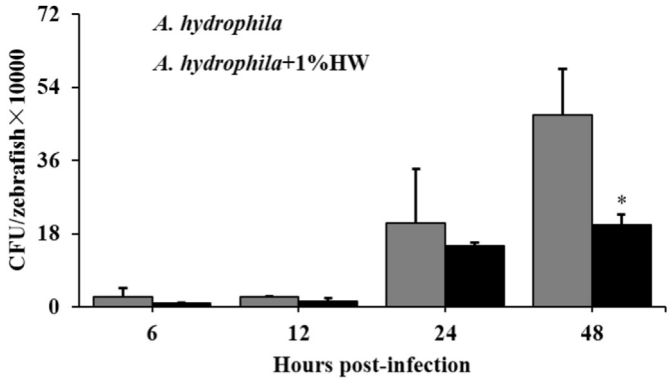

Fig. 2. The *in vivo* suppressive effect of HRW on *A. hydrophila* growth in zebrafish. The live bacterial numbers after *A. hydrophila* injection in zebrafish with/without HRW immersion were calculated. Values on graph represent mean ± standard error (SE). Significant differences were indicated with an asterisk at $p < 0.05$.

6 h p.i. In bacteria-injected fish, began to reach peak at 12 h p.i., and returned back at 24 h p.i. After HRW treatment, they dropped at 6 h, 12 h, and 24 h p.i., respectively. NF-κB expression levels peaked at 24 h p.i. With about a 3.5-fold increase over the control levels, at the levels decreased at 6 h, 12 h, and 24 h p.i. The IL-10 expression levels in spleen of zebrafish with HRW treatment showed an approximately 12-fold increase at 24 h p.i. The expression levels at 6 h and 12 h p.i. Had a 2.5-fold and 3.5-fold increase. (see. Fig. 3).

3.4. Effects of HRW on the expression of immune-related genes in kidney

In kidney, the zebrafish with the HRW immersion treatment exhibited significantly immediate 6-fold increased mRNA expression levels of IL-10 at 6 h p.i. The expression levels at 12 h and 24 h p.i. Had a 3.5-fold and 1-fold increase. The zebrafish with the HRW immersion treatment exhibited significantly decreased mRNA expression levels of IL-1β, IL-6, and NF-κB in kidney compared with that observed in the control group. After HRW treatment, mRNA expression levels of IL-1β dropped 5-fold, 1.5-fold, and 4-fold; IL-6 dropped from 3-fold, 8-fold, and 3.5-fold; NF-κB dropped 1-fold, 2-fold, and 1.5-fold at 6 h, 12 h, and 24 h p.i., respectively (Fig. 4).

3.5. Effects of HRW on the expression of immune-related genes in liver

HRW was able to significantly suppress the expression of IL-1β, IL-6, and NF-κB in liver at 6 h, 12 h, and 24 h after injection of *A. hydrophila*: NF-κB expression levels remarkably declined at 24 h p. i. Over the control levels. IL-6 expression levels showed strongest decrease of 25-fold at 12 h p.i. The IL-1 expression levels showed 3-fold decrease at 24 h p.i. Meanwhile, anti-inflammatory cytokine was measured in fish injected with *A. hydrophila* intraperitoneally provided HRW after bacterial injection, and IL-10 increased 5-fold at very early stage at 6 h p.i. (Fig. 5).

In summary, both mRNA expression levels pro-inflammatory and anti-flammatory cytokine regulation genes after HRW

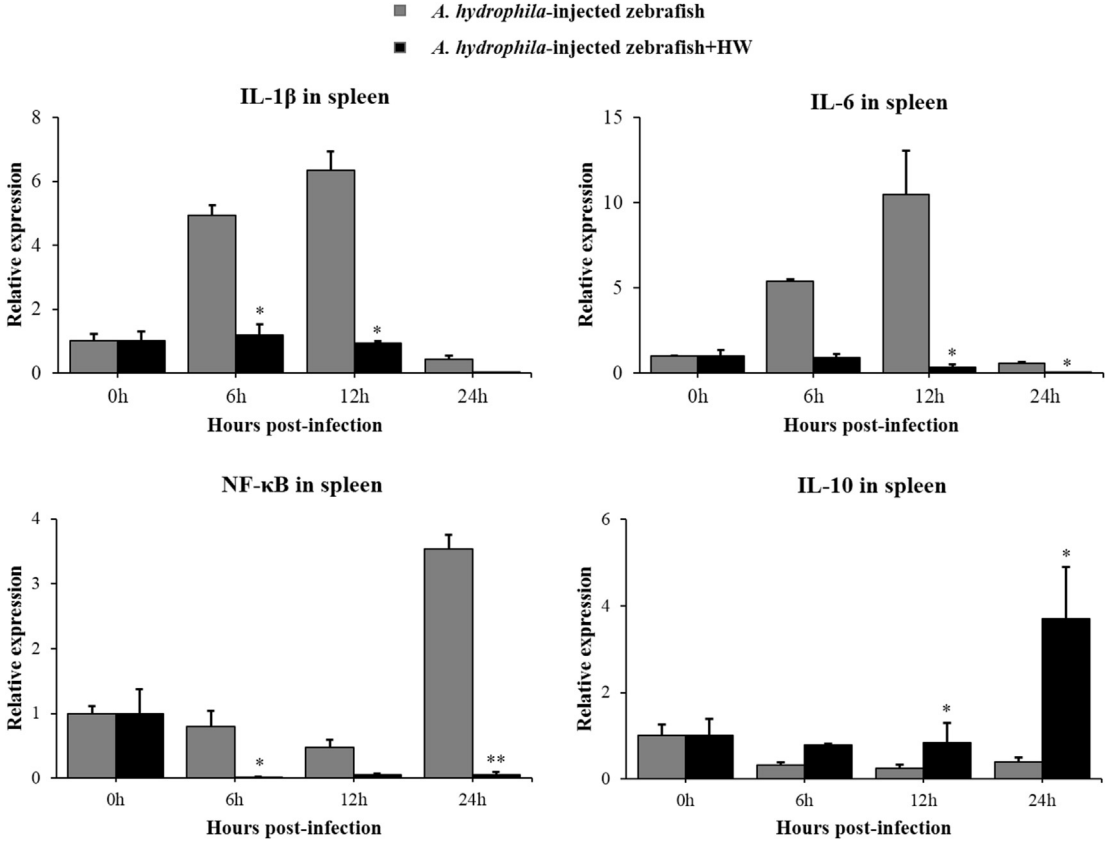

Fig. 3. Expression profiles of the pro-inflamatory and anti-inflammatory cytokine gene expression in spleen after *A. hydrophila* injection in spleen with/without HRW immersion. Relative expressions of the immune-related genes of IL-1β, IL-6, NF-κB and IL-10 were calculated. Values on graph represent mean ± standard error (SE). Significant differences were indicated with an asterisk at $p < 0.05$ and two asterisks at $p < 0.01$.

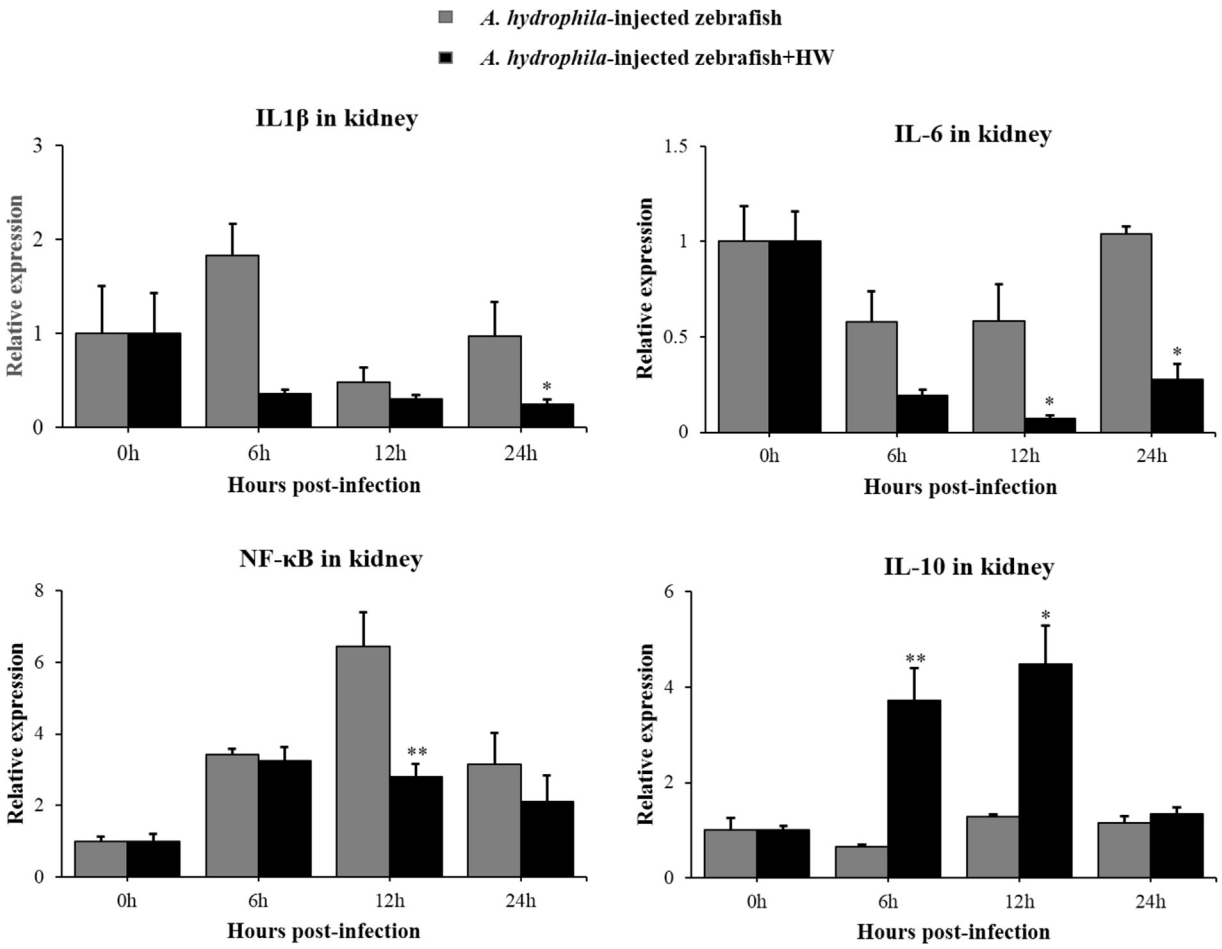

Fig. 4. Relative expressions of the immune-related genes of IL-1β, IL-6, NF-κB and IL-10 in kidney were measured. Values on graph represent mean ± standard error (SE). Significant differences were indicated with an asterisk at $p < 0.05$ and two asterisks at $p < 0.01$.

immersion treatment were determined by real time PCR in kidneys of adult zebrafish inoculated with live *A. hydrophila* bacteria. Consistently, HRW remarkably inhibited the expression of pro-inflammatory and induced anti-inflammatory cytokine genes in three tested tissues at 6 h, 12 h, and 24 h after the bacterial injection. These data suggest that HRW could up-regulate the expression of the anti-flammatory cytokine genes IL-10, and down-regulate the expression of the pro-inflammatory cytokine genes IL-1β, IL-6, and NF-κB, which indicating that HRW was an immunomodulator in zebrafish.

4. Discussion

Recently, molecular hydrogen has been reported to have a suppressive effect on inflammation in human and rodent models. Apart from astonishing therapeutic effects of molecular hydrogen on a rat model [1], which ignited interest in the effect of molecular hydrogen in various diseases and has been cited hundreds of times, the number of original articles demonstrating the effect of molecular hydrogen adds up to more than 300. Most of the articles applied mice and rats model, the rest are clinical trials. Hydrogen water and hydrogen gas are mostly reported, hydrogen water is in ad libitum treatments of experiments, and hydrogen gas is given less than 4% by inhalation. It has been found that HRW generally showed a more prominent effect than hydrogen gas [38,39]. Clinical studies showed statistically significant effects in patients, though not as conspicuous as those effects found in rodent models, which might due to a technical difficulty in preparing a high concentration of hydrogen water every day. Most of works are in rodents and human, while only a few in rabbits, pigs, and cultured cells or cultured tissues, while no reports in fish yet [39].

Being prompted by the previous observations in human and rodent model, we have designed our current research work by firstly using zebrafish model to study HRW effects on *A. hydrophila*-infection, as zebrafish has been applied as powerful model to study the inflammation and the innate immune response to infectious diseases [40]. The aim of this study was to evaluate the effects of HRW on the inflammation in zebrafish, and it is very interesting for us to reveal the significant *in vivo* repressive effect on bacterial growth and the increased survival rate of bacteria-challenged zebrafish subjected to the HRW immersion treatment, which motivated us to further investigate the effect of HRW on bacteria-challenged zebrafish immunity. Although there are several preferred approaches to ingest or consume molecular hydrogen, including inhalation of hydrogen gas, drinking HRW and injecting hydrogen-dissolved saline. Compared with inhalation of hydrogen gas or injection of hydrogen-dissolved saline which is relatively impractical for use, we chose to examine HRW immersion in our present study, and this is particularly promising way to ingest molecular hydrogen, as it is easily administered and safe.

Based on the antimicrobial effects, we have designed further experiments by means of HRW immersion treatment, samples were collected on 6 h, 12 h, and 24 h p.i., and the levels of IL-1β, IL-6, NF-κB, and IL-10 were evaluated using real-time PCR. We revealed

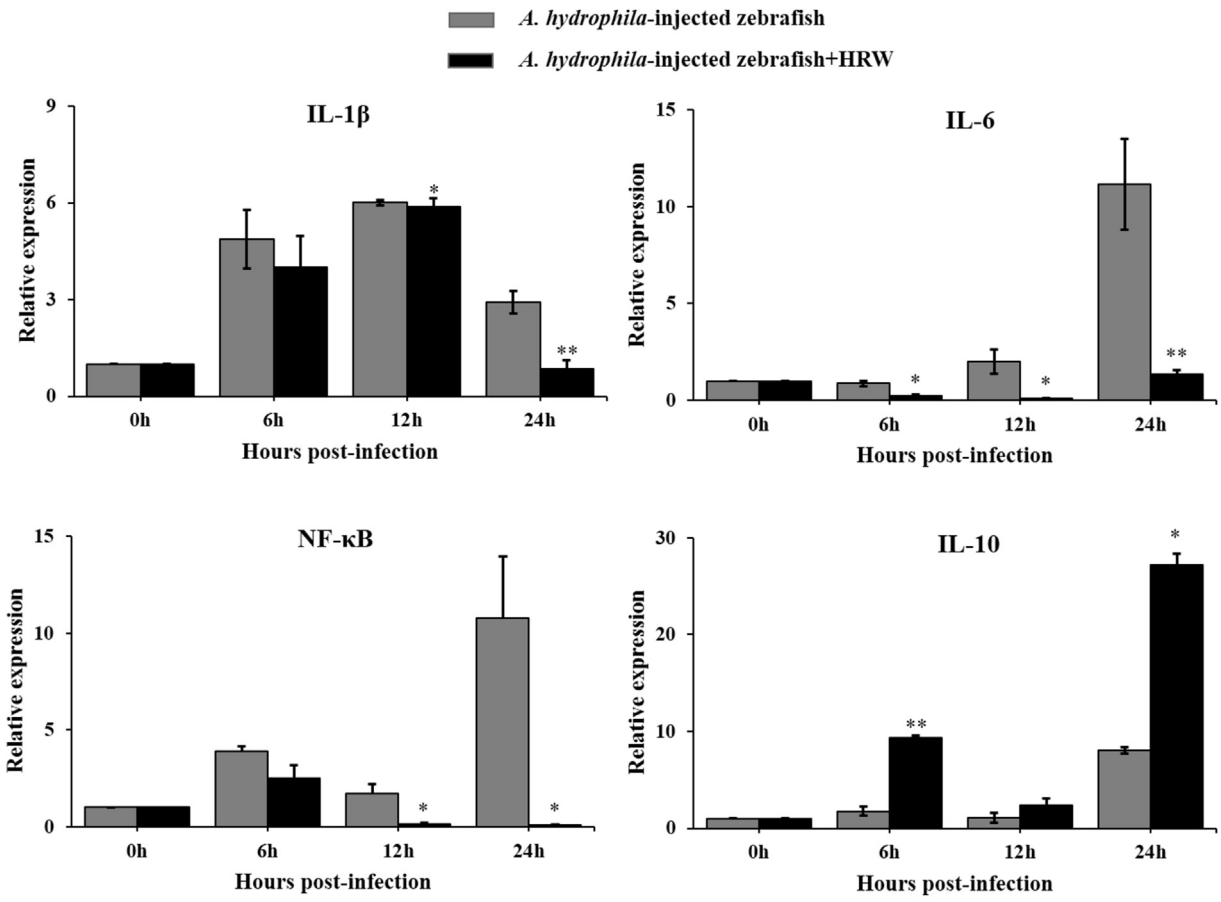

Fig. 5. Expression profiles of the immune-related genes of IL-1β, IL-6, NF-κB and IL-10 in liver were measured. Values on graph represent mean ± standard error (SE). Significant differences were indicated with an asterisk at $p < 0.05$ and two asterisks at $p < 0.01$.

HRW significantly suppressed the expressions of IL-1β, IL-6, and NF-κB in the spleen, head kidney, and liver of A. hydrophila-infected zebrafish, whereas remarkably enhanced the expressions of IL-10 in the same tissues. In the current study, in all tissues measured the pro-inflammatory genes were generally down regulated and the anti-inflammatory gene was up regulated. HRW have been shown to regulate immunity by functioning as immunomodulators, including the ability to alter host gene expression, and we have demonstrated the effects of HRW on the responses of cytokine gene expression in A. hydrophila-infected zebrafish, and these cytokines exhibit opposite effects: IL-1β, IL-6, and NF-κB are pro-inflammatory and IL-10 is anti-inflammatory. Our findings were in accordance with earlier reports relating molecular hydrogen water to influence post-transcriptional cytokine production. Expression analysis of rat liver showed that hydrogen has an effect on expression levels of oxidoreduction-related genes in rats [41]. Hydrogen could down-regulate pro-inflammatory cytokines including tumor necrosis-factor-alpha (TNFα), IL-1β, IL-6, IL-12, in many disease models [13,41–44]. Therefore, our results showed in the suppression of gene expression levels for the cytokines IL-1β, IL-6, NF-κB after bacterial injection with HRW immersion treatments. Cytokines secreted from macrophages and monocytes regulate the host response to infection, the HRW-induced cytokine expression in zebrafish suggesting that HRW enhanced immunity against bacterial infection.

Toll-like receptors (TLRs) are cellular receptors mediating macrophage and monocyte activation, and TLR4 is the major receptor for reorganizing LPS, which is a major component of the Gram-negative bacteria outer membrane. The TLR4 signaling pathway is an established major pathway that activates NF-κB, which induces the expression of pro-inflammatory genes. We found that HRW immersion treatment decreased NF-κB expression in the A. hydrophila-infected zebrafish, which suggesting that HRW may suppress A. hydrophila-induced NF-κB activation. IL-10 is a potent immunosuppressive cytokine for maintaining balance in the immune system through inhibiting pro-inflammatory cytokine production. In a mouse model, the IL-10 deficiency generated aggravating inflammation disease in a mouse model, and the induced IL-10 expression and suppressed inflammatory cytokine expression suggest that HRW has an anti-inflammation function that protects bacteria-infected zebrafish [45]. The HRW immersion treatment after live bacterial infection in this study could increase the expression of IL-10, as a way to evade host defense. In addition, in the spleen, the ratio between IL10 and NF-κB increased with HRW was added (Fig. 2), illustrating the strong anti-inflammatory effect of HRW, and this might explain the possible signal transduction pathway influenced by selective transcription factors. The different expression of the pro-inflammatory and anti-inflammatory cytokines might indicate that HRW has an immunological effect by mediating the expression of these genes.

Aeromonas hydrophila is Gram-negative bacteria which causes aeromonad septicaemia, which is a major fresh water disease affecting aquaculture worldwide. The current work is the first combined *in vivo* study of HRW treatment with the innate immune

system of zebrafish, our data might shed lights on hydrogen's antimicrobe effects and further application in aquaculture fish species.

Conflict of interest

The authors have declared that no conflict of interest exists.

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