Vol.66: e23220750, 2023
https://doi.org/10.1590/1678-4324-2023220750
ISSN 1678-4324 Online Edition

Article - Food/Feed Science and Technology

Evaluation of the Potential Use of Oxy-Hydrogen Gas for the Treatment of *Lactococcus garvieae* Infected-Zebrafish in Hydrogen-Rich Water Aquarium

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Editor-in-Chief: Bill Jorge Costa
Associate Editor: Marcos Pileggi

Received: 21-Sep-2022; Accepted: 16-Nov-2022

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HIGHLIGHTS

- Hydrogen-rich water can be applied as non-toxic compound for the treatment of bacterial infectious diseases of fish without any residual effects on fish or water.
- IL-1β and IL-6 gene expression levels decreased with the increase of hydrogen levels in the water
- HRW could reduce and prevent the death of zebrafish infected with *L. garvieae* (G5).

Abstract: This study aimed to evaluate the use of an oxy-hydrogen generator for preparing hydrogen-rich water (HRW) and studying its effects on the L. garvieae- affected zebrafish. 0, 10, 20, and 100% HRW were prepared to determine the effects of HRW on the mortality rate and gene expression levels of L. garvieae infected- zebrafish. After 48 hours of bacterial injection, the mortality rate of fish was 0, 0, 0, 66, 80, and 100% for the non-infected and non- HRW (G1), infected and 100% HRW (G5), non-infected and 100% HRW (G6), infected and 20% HRW (G4), infected and 10% HRW (G3), and infected without HRW (G2) groups. After 54 hours, there was a non-significant change in immunity-related gene expression levels (IL-1β and IL-6) between non-infected 100% HRW (G6) and non-infected non- HRW control (G1) groups. Gene expression levels were significantly upregulated for IL-1β (14, 13, and 9 times), IL-6 (48, 48, and 22 times), and SOD (9 times for each) genes in G2, G3, and G4 groups, respectively, but not for G5 comparing with control group G1. IL-1β and IL-6 gene expression levels decreased with the increase of hydrogen levels in the water. These results show that HRW could decrease (G3 and G4) and prevent (G5) the mortality of L. garvieae infected-zebrafish. This demonstrates the importance of the application of HRW for the treatment of bacterial infectious diseases of fish using a non-toxic compound as a green method without any residual effects on fish or water.

Keywords: Gene expression; Hydrogen-Rich Water; Lactococcus garvieae; Immunity; Oxy-hydrogen gas; Zebrafish.

INTRODUCTION

Zebrafish is one of the most frequently used model organisms in aquaculture and disease studies thanks to its circulatory system feature [1]. *Lactococcus garvieae*, the etiological agent of *Lactococcosis*, causes hyperacute and hemorrhagic septicemia in many fish species and causes important economic losses both in marine and freshwater aquaculture [2]. The losses produced from *Lactococcosis* exceed 50–80% of the total production [3].

Molecular hydrogen (H_2), the smallest molecule in the universe, can be absorbed from the digestive and respiratory systems and quickly enters the circulation system of living beings. H_2 can pass biomembranes and reach the cytosol, mitochondria, and nucleus and pass the blood-brain barrier [4].

Non-toxic effects have been reported at high doses as excess H_2 can be excreted through the lungs [5]. Non-known toxic effects of H_2 have been detected upon its ingestion or inhalation. Additionally, H_2 was characterized as a mild but effective reducing agent and selective antioxidant [6]. Hydrogen can selectively scavenge harmful free radicals i.e., hydroxyl radical and peroxynitrite anion without affecting the beneficial free radicals i.e. hydrogen peroxide and nitric oxide [7]. Furthermore, H_2 also helps to upregulate antioxidant enzymes such as glutathione peroxidase (GPx), superoxide dismutase (SOD), and catalase (CAT) [4,8]. H_2 has recently been recognized as a new therapeutic agent and suggested to be a possible treatment agent for certain neuromuscular disorders, cardio-metabolic diseases, and cancer types, and it mainly acts as an antioxidant, anti-inflammatory, and anti-apoptosis agent [9]. Hydrogen-rich water (HRW) is one of the different administration methods of H_2 including inhalation, hydrogen-rich saline, and hydrogen-eye drop, and hydrogen bath. Water electrolysis was considered a cheap method for producing hydrogen besides oxygen called commonly oxy-hydrogen gas. HRW showed many beneficial health properties in human and animal models [10,11,12,13]. Our team has recently revealed the ability of HRW to decrease the heavy metal-induced toxic responses such as inflammatory, oxidative stress, and DNA damage [14,15]. *L. garvieae* was considered as the disease agent of a hyperacute hemorrhagic septicemic infection that can infect different freshwater and marine fish leading to serious economic losses [2]. In the present study, the effects of HRW on the mortality rate and gene expression levels of *L. garvieae* infected- zebrafish were evaluated.

MATERIAL AND METHODS

Ethical Statement

The study was carried out with the permission of the local ethics committee of animal experiments (No 2021/04-17 on 29/04/2021) of Van Yuzuncu Yıl University (Turkey).

Bacteria

L. garvieae strain isolated from infected rainbow trout was used in this study. The strain was grown at 21°C in MRS broth (Sigma) until reaching an optical density value of 1.0 at 600 nm.

Preparation of HRW

The water of the aquarium was bubbled with an oxy-hydrogen gas generator instrument (HB-33 Epoch, Taiwan) producing hydrogen (70%) and oxygen (30%) mixture by water electrolysis (Figure 1). The concentration of hydrogen in HRW aquariums and its stability were continuously controlled during the assay period, and its value was determined in preliminary experiments using the ORP electrode (Sensorex, USA). The levels of hydrogen were about 1.6 ppm in 100% HRW samples. 10% and 20% HRW waters were prepared by diluting 100% HRW with pure water at specific rates (See Figure 1 for specifications). The H_2/O_2 mixture was continuously provided to 100% HRW during the assay period by an oxy-hydrogen gas generator. The water of each application aquarium was continuously drained at 10 parts/min to a chlorinated waste tank to keep the level of water in each aquarium stable (Figure 1).

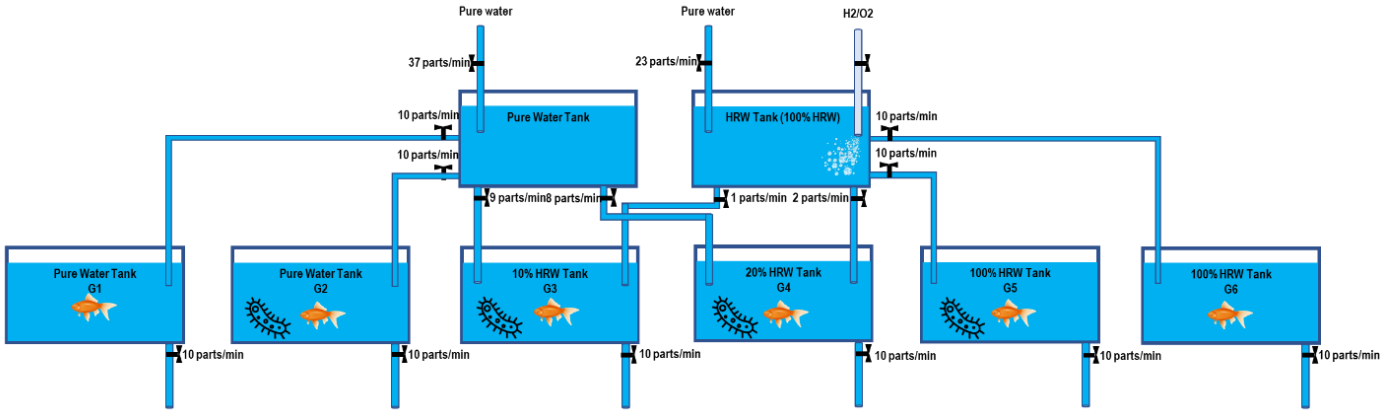

Figure 1. Schematic diagram of the experiment plan

Preparation of fish

Sixty healthy adult zebrafish (*Danio rerio*) (4-5 cm) were provided by the Aquatic Life Experimentation Unit (SUCAN) of Van Yuzuncu Yil University (Turkey). Fish were exposed to adaptation for 7 days. The fish was fed with commercial fish pellets twice a day and wastes were removed every day. Water criteria were determined and the same environmental conditions (pH 7.0-7.5, conductivity of 400 mS, and temperature of 24-25 °C) were provided for each group. Ten fish in 50-liters aquariums with different HRW percentages were used. *L. garvieae* was inoculated to the application fish groups.

Bacteria re-isolation and identification

Liver and kidney samples were cultured on Columbia blood agar (Sigma) and incubated at 21°C for 48 hours. Identification of the grown bacteria was confirmed by Real-Time PCR (Rotor-Gene Q 9000, country) using the 27F-1492R primer set (Primer-Premium, UK), and the amplicons were confirmed by the Sanger method. Confirmation was considered when the infection was found in the pure culture obtained from *L. garvieae* infected zebrafish tissues only.

Anesthesia and bacteria inoculation

The bacterial inoculation (injection) was carried out separately for each fish. For each fish in each group, the average time of anesthesia of zebrafish was 55 seconds, and the exit time from anesthesia was 110 seconds. Hemorrhage and mortality were checked in the first 15 minutes after intraperitoneal injection of 2×10^6 CFU *L. garvieae* in anesthetized zebrafish. No death was observed after PBS and bacterial injection in any groups.

Mortality rate

During the trial period i.e., 54 h, the daily mortality percentage was determined using the following equation:

Mortality (%) = ((initial fish number-final live fish number)/initial fish number) × 100

Gene expression analysis

During the trial, the total RNA isolation from the muscle tissues of fish was performed using the RNeasy mini kit (Qiagen-Germany). The cDNA process was performed with the RT2 first strand kit (Qiagen-Germany) according to the manufacturer's instructions. ACTB, IL-1β, IL-6, and SOD gene expression levels of different groups were examined in terms of regulatory changes on the 54^{th}-hour phase. Results were evaluated by volcano plot analysis at a $p<0.05$ significance level. The primers used in the study and their properties are given below (Table 1) [16].

Table 1. Genes and primer properties for gene expression analysis [16,29].

Gene	Primer sequence (5'-3')	Accession number
β-actin	ATGGATGAGGAAATCGCTGC ATGCCAACCATCACTCCCTG	NM131031.1
IL-1β	TGGACTTCGCAGCACAAAATG GTTCACTTCACGCTCTTGGATG	NM212844.2
IL-6	AGACCGCTGCCTGTCTAAAA TTTGATGTCGTTCACCAGGA	NM001261449.1
SOD	TGAGACACGTCGGAGACC TGCCGATCACTCCACAGG	BC055516

Statistical analysis

All statistical analyses were performed using Qiagen-Web software and One Way ANOVA method. Data were expressed as mean ± SD and the differences were considered significant when $p<0.05$. Graphs were generated by Qiagen-Webs software and the "ggplot2" package (v. 3.1.0) [17].

RESULTS

Mortality

Fish mortality was checked at 0, 6, 12, 24, 36, 48, and 54 hours after bacterial inoculation. The number of dead fish and the death time (hours) were recorded for each group. The mortality increased along the time for the infected without the HRW group (G2). The same tendency was also observed for G3 and G4 groups, while G5 (infected 100%HRW) did not show any mortality at any time. No mortality has been observed after 6 hours of the injection phase for all groups ($p<0.05$). On the 12th hour, no mortality has observed in G1, G5, and G6 groups, while 33, 16, and 16% were found for G2, G3, and G4 groups ($p<0.05$). On the 24th hour, no mortality has observed in G1, G5, and G6 groups, while 50, 33, and 33% has been shown for G1, G5, and G6 groups ($p<0.05$). On the 36th hour, no mortality has observed in G1, G5, and G6 groups, while 83, 50, and 50% has been observed for G1, G5, and G6 groups ($p<0.05$).

On the 48th hour, the mortality was 100, 83, and 66% for G2, G3, and G4, respectively, whereas it was null for G1, G5, and G6 ($p<0.05$) (Figure 2). At the end of assay time i.e. 54 hours, no death was observed in the non-inoculated control group (G1) and non-inoculated 100% HRW (G6), and *L. garvieae* infected 100% HRW group (G5). However, all fish were died in the infected without HRW group (G2), infected 10% HRW and infected 20% HRW ($p<0.05$) (Figure 2).

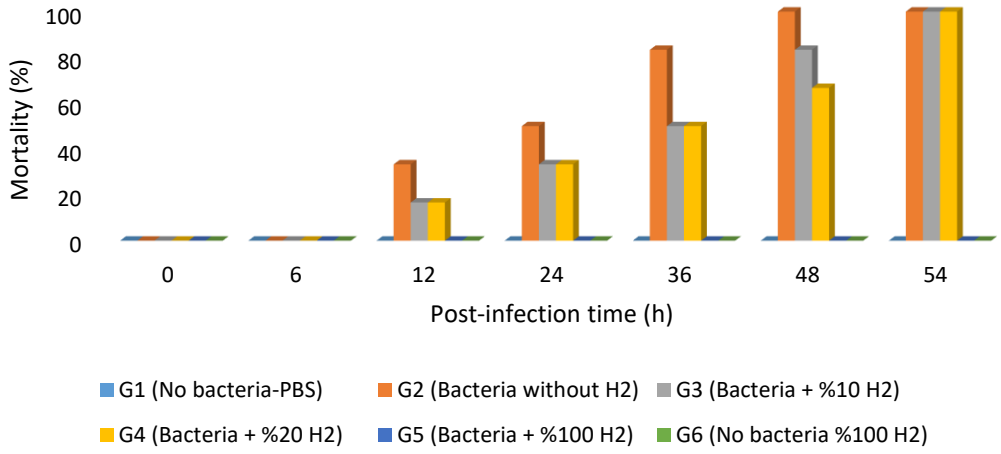

Figure 2. Effect of HRW treatment on mortality of *L. garvieae infected- zebrafish*

Gene expression

On the 54th hour, zebrafish were chosen from all groups for conducting gene expression studies. Total RNA isolation was performed from 25 mg muscle tissues. cDNA synthesis was performed from 1ng/ul RNA. After Real-Time PCR analysis, the difference in the expression levels between IL-1β, IL-6, and SOD genes and the reference gene (ACTB) was examined (Figure 3).

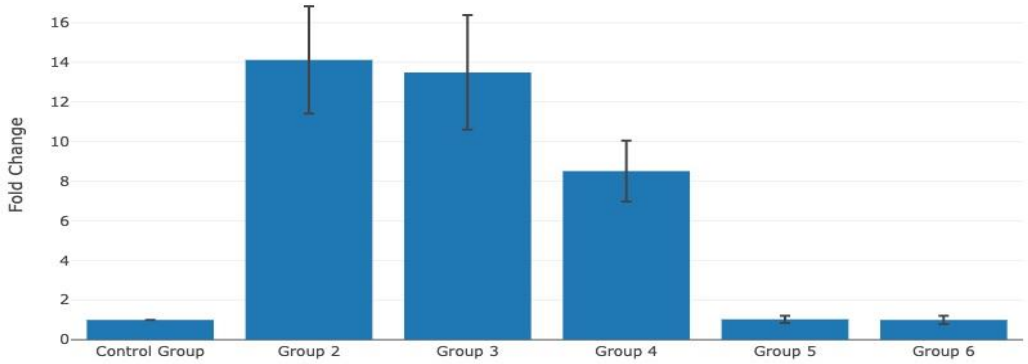

Figure 3. Effect of HRW treatment on IL-1β gene expression of *L. garvieae* infected- zebrafish

According to gene expression, the highest upregulation levels of IL-1β and IL-6 were observed for the *L. garvieae* infected- non-HRW (G2) and 10% HRW groups ($p<0.05$) (Figures 3 and 4).

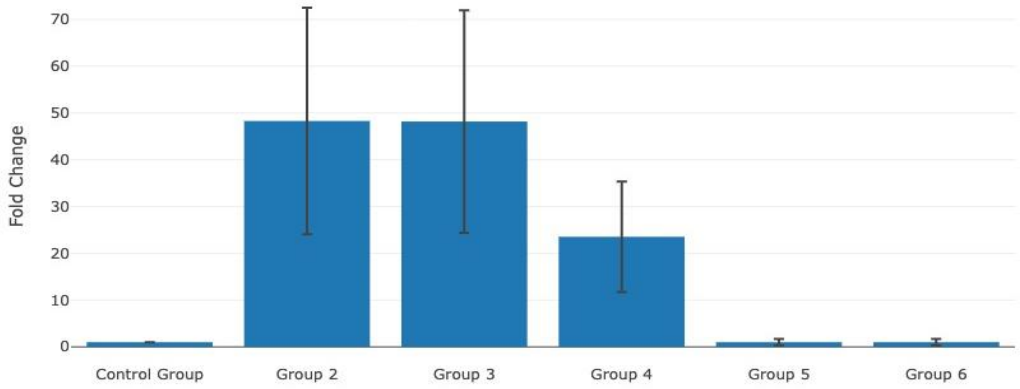

Figure 4. Effect of HRW treatment on IL-6 gene expression of *L. garvieae* infected- zebrafish

Furthermore, there was a non-significant difference between the infected 100% HRW (G5) and non-infected 100% HRW (G6) groups compared to the control group (G1) ($p<0.05$). The gene expression of IL-1β and IL-6 decreased with the increase of HRW concentration in aquarium water. The healthy uninfected non-HRW fish group showed the lowest levels of IL-1β and IL-6, which is similar to 100%HRW groups i.e. G5 and G6. Regarding the SOD gene expression, we noticed that the highest level was observed for G2, G3, and G4 groups without significant differences among them ($p<0.05$) (Figure 5)

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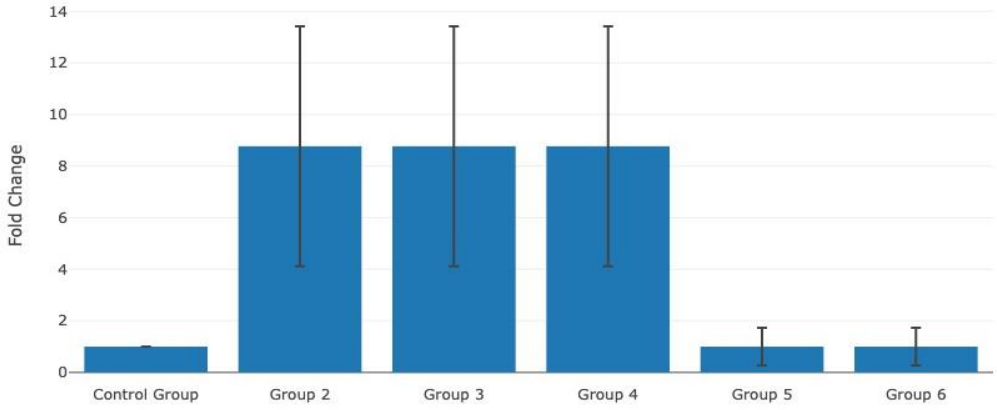

Figure 5. Effect of HRW treatment on SOD gene expression of *L. garvieae* infected- zebrafish

However, the lowest level was observed for G1, G5, and G6 groups without significant differences among them ($p<0.05$). This shows that only the infected non- HRW and both infected 10% and 20% HRW groups upregulated SOD expression but not 100% HRW.

DISCUSSION

Zebrafish are among the most used experimental animals because it is the human denier model and their genetic characteristics are well known compared to other aquatic animals. Zebrafish have been used as a model to study the inflammation and the innate immune response to different infectious diseases [18]. On the other hand, *L. garvieae* is the common bacterial disease agent found in aquaculture that causes economic losses. Although the disease agent and fish species used in this study are commonly studied in the field of aquaculture, non-studies were carried out on the impact of hydrogen-rich water on *L. garvieae* infected- zebrafish.

Our results showed a decrease in mortality rate with the increase of HRW level, which means that molecular hydrogen dissolved in HRW healed better the infected zebrafish when its level increased in water. The absence of mortality in both G1 and G6 groups reveals that full hydrogen-saturated water i.e. 100% HRW had no inhibition effect on the healthy (non-infected) zebrafish. Hu and coauthors [16] found that 1%

and 4% HRW increased the survival rate of *A. hydrophila* infected- zebrafish at 48th hours injection phase with better protection effect at 1% HRW compared with 4%HRW. The difference in the infectious agent (*L. garvieae* vs *A. hydrophila*) and the gas type (H_2/O_2 vs H_2) between our study and that of Hu and co-workers could explain this difference in results. Regarding the results of gene expression, in immunity-related academic studies, the most frequently used genes are interleukin-related. These genes are directly used in health studies as immunity-related genes. The ACTB gene is one of the reference genes used in genetic studies from tissues. Our results showed that on the 54th hour, gene expression levels were significantly upregulated for IL-1β (14, 13, and 9 times), IL-6 (48, 48, and 22 times), and SOD (9 times for each) genes in infected groups i.e. G2, G3, and G4 but not G5 comparing with control group G1; and this increase in interleukin gene expression in infected groups was inversely proportional with the level of molecular hydrogen dissolved in water. These results show the ability of molecular hydrogen dissolved in HRW to downregulate the gene expression levels of the cytokines IL-1β and IL-6 in *L. garvieae*-infected zebrafish, and this ability increased with the increase of dissolved hydrogen level in the water.

Hu and coauthors [16] evaluated the effect of HRW (1 and 4%) on the expression of immune-related genes (IL-1β, IL-6, and NF-κB) in different organs of zebrafish i.e. spleen, kidney, and liver. The authors found that HRW treatment had a significantly decreased trend on the IL-1β and IL-6 expression levels in *A. hydrophila*- infected zebrafish compared with the control. While the authors studied the evaluation of the expression of immune-related genes with the time every 6 hours during 24 hours in spleen, kidney, and liver; we have studied the levels of these genes at the 54th -hours phase in muscle. Furthermore, in the present study, we have used H_2/O_2 mixture, while the previous authors used pure H_2. The decrease of pro-inflammatory production in bacterial-infected zebrafish found inside the HRW aquarium means that molecular hydrogen enhanced immunity against bacterial infection [16]. Our results agree with many previous studies reporting that molecular hydrogen can reduce pro-inflammatory cytokines such as TNF-α, IL-1β, IL-6 and IL-8 in animal models [19,20,21] and humans [22,23,24].

Regarding the oxidative marker i.e. SOD, our results showed that both infected non-HRW group (G2) and infected 10% HRW(G3) and 20% HRW (G4) groups increased the gene expression of SOD but not infected 100% HRW (G5) (Figure 5). We can assume that at 100% HRW and due to the low levels of IL-1β and IL-6 gene expression that was similar to the healthy control group G1, there was no need to up-regulate the SOD gene expression that stayed similar to healthy non-HRW and healthy 100% HRW groups. This observation could be related to the antioxidant property of molecular hydrogen that is similar to that of SOD. Furthermore, molecular hydrogen has been proved for its ability to suppress superoxide (O_2^-) generation in the mitochondrial complex I [25]. As superoxide forms the substrate of SOD, the low levels of the substrate in the medium don't require further synthesis of SOD that can explain the low level of SOD gene expression in the 100% HRW group.

Generally, many reports revealed the up-regulation of SOD in the presence of molecular hydrogen in animals [26,19], plant [27], and humans [7].

Molecular hydrogen has been reported to play an immunomodulatory role by its ability to modify gene expression. In this study, we have revealed that HRW can modify the responses of cytokine gene expression in *L. garvieae*-infected zebrafish.

Carnovali and coauthors [28], reported that HRW levels below 15% were safe for zebrafish embryos and did not affect vitality or growth rate. However, toxicity in zebrafish embryos was found at levels starting from 25% HRW. Taking into account the previous observations of Carnovali and co-workers, we have designed our experimental plan to include low levels of HRW i.e. 10 and 20%. However, in our study, we have used an H_2/O_2 mixture (2/1, v/v), which provides an additional advantage over the previous study because dissolved oxygen is necessary for the respiration process. Our study is the first work showing the beneficial use of the electrolysis technique as a cheap source to produce H_2 from water besides O_2 (oxy-hydrogen gas) for the treatment of bacterial infectious diseases of fish. The results of this study show the potential application of HRW for the treatment of bacterial diseases of fish. Further studies are needed to optimize the conditions of HRW application in large-scale and commercial aquaculture diseases.

CONCLUSION

Molecular hydrogen has been proved for its different physiological activities such as antioxidant, anti-inflammatory, and anti-apoptotic properties in animal models and human trials. Different studies reported the ability of molecular hydrogen to alleviate the proinflammatory symptoms in different diseases. *L. garvieae* was known as a disease agent of a hyperacute hemorrhagic septicemic infection that can infect different freshwater and marine fish leading to serious economic losses. The treatment of infected fish needs generally costly drugs and methods with potential risks to contaminate the waters with hazardous chemical residuals.

This shows the need to find green and non-toxic methods to treat the bacterial diseases of fish. The use of the water electrolysis technique for producing both the therapeutic molecular hydrogen and the respiration agent molecular oxygen together exhibits the importance of this study for fishery and aquaculture fields thanks to its cheapness and eco-friendly properties. The study is the first work showing the beneficial use of the oxy-hydrogen gas produced by the water electrolysis technique as a cheap source to produce H_2 besides O_2 for the treatment of bacterial infectious diseases of fish. It also confirms the previously found anti-inflammatory properties of HRW and extends its potential applications to cover the commercial aquaculture of fishes.

Acknowledgments: Authors thank Epoch company (Taiwan) for providing the oxy-hydrogen instrument**.**
Funding: The authors declare that no funds, grants, or other support were received during the preparation of this manuscript
Conflict of Interests: The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper**.**
Ethics Approval and Consent to Participate: The study was carried out with the permission of the local ethics committee of animal experiments No 2021/04-17 on 29/04/2021.

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