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New Insights into the Role and Mechanism of Macrophage Migration Inhibitory Factor in *Epinephelus awoara* Infected with *Vibrio alginolyticus*

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ABSTRACT

Background: The cytokine Macrophage migration inhibitory factor (MIF) is involved in inflammatory diseases. The objective of the present study was to measure the expression levels of MIF in the tissues of *Epinephelus awoara* (*E. awoara*). mRNA levels of MIF were investigated in the tissues including spleen, liver, brain, head kidney, stomach, gill, heart, intestine and muscle of *E. awoara.*

Methods: In the experiment, *E. awoara* were infected with *V. alginolyticus and* then they were analyzed by ELISA to confirm the mRNA levels of MIF, TNF-α and IL-1 in the many tissues in diseased fishes were higher than those in healthy fishes.

Result: The mRNA levels increased significantly in treatment groups. Likewise, *E. awoara* with *V. alginolyticus* infections showed significant increases in IL-1 and TNF-α tissues content of intestine, head kidney, liver, muscle and spleen, respectively. There was evidence that MIF stimulates the release of TNF-α and IL-1. The results have the potential to perpetuate that MIF is connected with the inflammatory process in *V. alginolyticus* infectious diseases.

Key words: Alphainterleukin-1, *Epinephelus awoara*, Macrophage migration inhibitory factor, Tumor necrosis factor, *Vibrio alginolyticus*.

INTRODUCTION

V. alginolyticus is a common pathogen in marine environments and is dominant in the intestinal tracts of aquatic animals (Yoshida *et al*., 2022; Zhu *et al*., 2022). *V. alginolyticus* causes chronic skin ulcers in cultured groupers (Wang *et al.,* 2016). As an important economic species, the *E. awoara* was farmed abundantly in Southeast Asia and China. Unfortunately, bacterial-borne diseases have occurred in teleostean and bivalve cultivation, resulting in economic losses (Harikrishnan *et al.,* 2010; Laith *et al*., 2020). *V. alginolyticus* has caused mortality and affected the aquaculture production of *E. awoara* (Petchimuthu *et al*., 2021). Therefore, a thorough understanding on the defense mechanism depend on the immune system in *E. awoara* can be useful in the control of the disease. MIF is associated with innate immune responses in the disease (Zhang *et al.,* 2022; Petralia *et al.,* 2020). MIF cDNAs were found previous in fishes, amphibians and birds (Jiang *et al.,* 2020; Wang *et al.,* 2016). MIF was related to inflammation-associated diseases, including systemic infections, sepsis, autoimmune diseases, cancer and rheumatoid arthritis (Muniyan *et al*., 2022; Wu *et al*., 2020; Zhu *et al*., 2020; Zhang and Lu, 2017). The molecular mechanisms regulating the expressions of the MIF genes (MIF) are still largely unknown. MIF genes were found in sea bass (Xu *et al.,* 2019), North Atlantic hagfish (Sato *et al.,* 2003) and many bony fishes (Zhang *et al.,* 2022). The molecular mechanisms and the biological activity of MIF are still largely unknown. North Atlantic hagfish has MIF genes (Sato *et al.,* 2003) and in different teleost species (Zhang *et al.,* 2022). MIF was detected in rat (Lan *et al.,* 2018). MIF was involved in pathogenic factors and therapeutic targets for diseases. Elevation of MIF serum levels have been detected in some disease (Günther *et al.,* 2019), such as sepsis (Toldi *et al.,* 2021). MIF has been associated with worsening pathologic conditions and some clinical diseases courses (Fouda *et al.,* 2022).

MIF was involved in tissue inflammation and facilitated the production of TNF-α and IL-1(Howait *et al.,* 2019). The genetic reduction of MIF in invertebrates has led to a parallel decrease in monocyte/macrophage-derived mediators, such as IL-1 and TNF-α. MIF's upstream regulator function in the inflammatory cascade has been confirmed (Leng *et al.,* 2003; Varughese *et al.,* 2019). Monocyte/macrophage-derived inflammatory cytokines such as IL-1, interferon gamma (IFN-c) and TNF-α have physiological roles in the

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adult-onset of Still's disease. This mechanism has already been a therapeutic target. The immune functions of MIF genes in animals and teleost fishes have been verified (Xu *et al.,* 2015; Qin *et al.,* 2016). To date the function of MIF genes in groupers is unknown. In our study, we examined MIF, IL-1 and TNF-α tissue levels of *V. alginolyticus*-affected *E. awoara* by RT-PCR and these were over expressed in all the tissues. MIF maybe a diagnostic marker of *V. alginolyticus* disease severity in *E. awoara*.

MATERIALS AND METHODS

Bacterial strains

V. alginolyticus was isolated and verified by live fishes which were infected in our laboratory. *V. alginolyticus* was inoculated onto thiosulfate citrate bile salts sucrose agar (TCBS, Difco) at 28°C, was used for fish challenge experiments in Fisheries College, Jimei University from 2020 to 2022.

Fish pathogenicity experiments

E. awoara were procured from Zhangpu (Fujian, China) for *V. alginolyticus*-affected fish experiments. Fishes were determined by routine examination, including the head, fins, skin, body and other morphological markers. 30 fishes *were* raised *indoors* in 500 L aquaria tanks for 1 week. Then the fishes were moved and subdivided into 2 equal groups (1.35±10 kg). 6.0×10^7 CFU/mL*V. alginolyticus* were injected into healthy fishes. The control group was injected with 20 µL of Phosphate Buffered Saline. The test fishes were injected with 0.1 mL of 7.0×10^7 CFU/mL *V. alginolyticus*. The bacterium disease signs and mortality were detected for 14 d.*Organ* specimens were obtained from diseased fishes and healthy fishes. These organs included head kidney, spleen, liver, brain, intestine, gill, heart, stomach and muscle tissues in *E. awoara* (n=5). All the tissues need PBS washing. The same tissue samples were pooled, such as all the livers were pooled together, RNA extraction was collected and frozen at -80°C.

RNA extraction and reverse transcription

Total RNA from the frozen head kidney, spleen, liver, brain, intestine, gill, heart, stomach and muscle tissues was extracted using TRIzol (Invitrogen, Carlsbad, CA) (Xu *et al.,* 2016). First-strand of cDNA was isolated with a synthesis kit.

Real-time PCR

Five fishes from each replicate were pooled. RNA was extracted as described above. MIF gene expression in the tissue were detected by qPCR. The experiment was performed in volumes of 10 µL reactions. PCR primers sequences were 5′-TMATGCCGATGTTYRTCVGTGARCAC-3′ and 5′-CTGGAATGATCTTYTDTTTC-3′. β-actin was as used a reference gene. The primers of β-actin were 5′-CGAGAAATCGTTCGTGAC-3′(forward) and 5′-TGCTGTTGT AGGTGGTCTCGT-3′ (reverse).

Quantification of MIF, TNF-α and IL-1 tissue levels

Diseased and healthy fishes tissue specimens were collected as those in 2. 3. Commercial kits (Wuhan EIAab Science Co., Ltd., PR China) were used for the detection of MIF, IL-1 and TNF-α levels in the tissues, *Epinephelus* Macrophage migration inhibitory factor, MIF ELISA Kit (Catalog Number. E0698f), *Epinephelus* Tumor Necrosis Factor α (TNF-α) ELISA KIT (Catalog Number CSB-E13254Fh), *Epinephelus* Interleukin 1β (IL-1β) ELISA Kit (Catalog Number CSB-E13259Fh). Anti-TNF-α antibody and anti- *Epinephelus* Interleukin 1β antibody were used and the MIF, IL-1 and TNF-α assay sensitivity of the antibodies was 50 pg/mL, 1.22 pg/mL and 6.7 pg/mL, respectively.

Statistical analysis

SPSS 13.0 were used for the results of tissue MIF, TNF-α and IL-1. The Kruskal-Wallis H test was used to compare independent samples of control groups and treatment groups. Statistical significance was compared with ANOVA.

RESULTS AND DISCUSSION

Clinical symptoms

Lethargy, anorexia and other clinical disease signs were detected in infected *E. awoara* (Fig 1). Skin lining, hemorrhagic spots were also found. Congestion with ulceration was the common features.

Expression of MIF in fish tissues in response to *V. alginolyticus* infection

MIF has been detected in all healthy and diseased fish organs (Fig 2). The sampled tissues consisted of head kidney, spleen, liver, brain, intestine, gill, heart, stomach and muscle. PBS and *V. alginolyticus* were injected into fishes and served as control groups and treatment groups. MIF mRNA levels were low in the tissues sampled in control groups. The MIF mRNA levels of the head kidney, liver, brain and spleen in diseased *E. awoara* increased compared with those in control groups (P<0.05) (Fig 2).

MIF, TNF-α and IL-1tissue levels

ELISA was used for detecting MIF, TNF-α and IL-1 levels. The tissues consisted of spleen, head kidney, muscle, intestine and liver. Tissue levels of MIF (Fig 3A), TNF-α (Fig 3B) and IL-1 (Fig 3C) were found higher in infected *E. awoara* compared to the levels in the untreated infected fish tissues. These average values were from all 5 fishes. The MIF levels of liver were 6.280±0.110 ng/mL in diseased *E. awoara*, while 5.309±0.031 ng/mL in that of control. Tissue MIF levels of spleen, head kidney, intestine and muscle infected fishes were 6.095±0.070 ng/mL, 6.027±0.035 ng/mL, 6.115±0.046 ng/mL and 6.075±0.116 ng/mL higher than the MIF levels in untreated infected fishes (5.737±0.039 ng/mL, 5.478±0.047 ng/mL, 5.478±0.047 ng/mL and 4.988±0.037 ng/mL). There



Fig 1: (1) *E. awoara* was seen with deep and superficial ulcers. (2) Ulceration of skin were observed in diseased *E. awoara*.

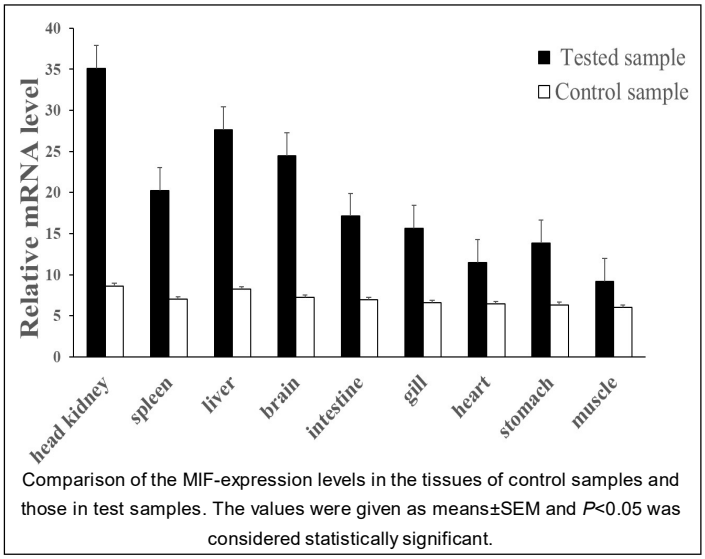


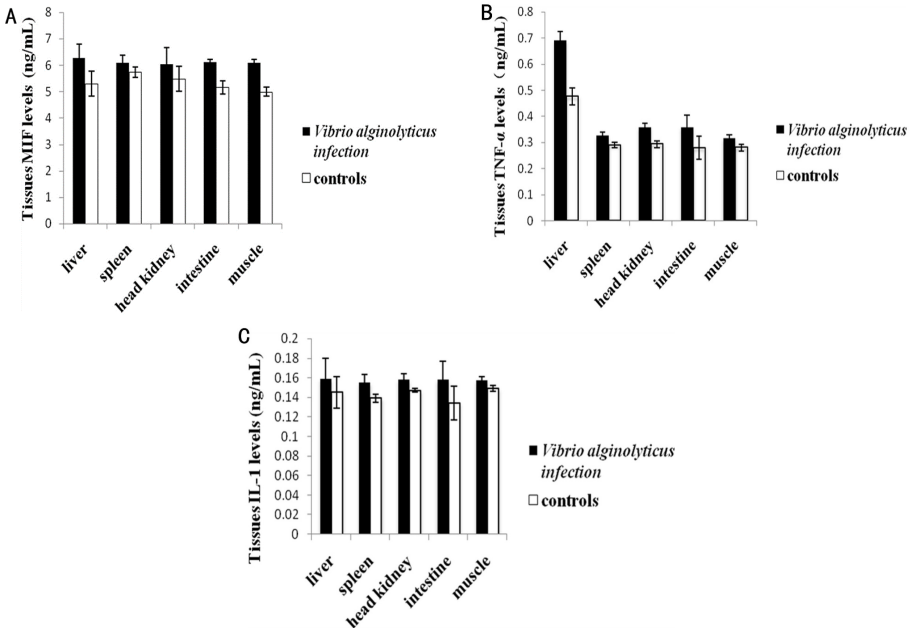
Fig 2: MIF expressions in 9 selected tissues and organs (head kidney, spleen, liver, brain, intestine, gill, heart, stomach and muscle tissues) detected by qRT-PCR.

was a significant difference between uninfected, untreated control groups and infected (P<0.05, Fig 3A).

Likewise, TNF-α levels in all the tissues were higher in treatment groups than those in controls. With regard to the tissues of *E. awoara*, TNF-α levels of liver, spleen, head kidney, intestine and muscle in the infected groups were 0.690±0.005 ng/mL, 0.326±0.003 ng/mL, 0.358±0.005 ng/mL and 0.316±0.002 ng/mL respectively and 0.476±0.007 ng/mL, 0.291±0.004 ng/mL, 0.294±0.008 ng/mL, 0.279 ±0.005 ng/mL and 0.281±0.007 ng/mL in controls. TNF-α levels in the tissues were increased in treatment groups compared to controls (P<0.05, Fig 3B).

The IL-1 levels in spleen, head kidney, intestine, muscle and liver of diseased fishes were 0.155±0.001 ng/mL, 0.158±0.001 ng/mL, 0.158±0.002 ng/mL, 0.157±0.004 ng/mL and 0.159±0.004 ng/mL, respectively. Infected fishes displayed higher tissue levels of IL-1 detected by ELISA than those found in the non-infected groups. The IL-1 levels of spleen, head kidney, intestine, muscle and liver of untreated infected fishes were significantly lower (0.139±0.001 ng/mL, 0.147±0.002 ng/mL, 0.134±0.002 ng/mL, 0.149±0.003 and 0.145±0.002 ng/mL, respectively). The two groups had statistically significant differences (P<0.05, Fig 3C). These results show MIF, TNF-α and IL-1 play a role in diseased fishes and the levels in the spleen, head kidney, intestine and muscles were much higher than those in controls (Fig 3A, B, C). MIF, TNF-α and IL-1 levels in *V. alginolyticus* -affected fishes were positively correlated with bacteria in the fishes. The MIF, TNF-α and IL-1 could be detected in controls.

V. alginolyticus had been isolated from some diseased marine invertebrates, including *E. awoara* (Mohamad *et al.,* 2019), shrimp (Yin *et al.,* 2022), *Epinephelus coioides* (Wang *et al.,* 2021) and *Charybdis japonica* (Zhang *et al.,* 2015). *E. awoara* fishes infected with *V. alginolyticus* had anorexia, local hemorrhagic ulcers in head and body and other clinical signs of *V. alginolyticus* caused mortality in *E. awoara.*



A. Comparison of MIF tissue levels by study groups. B. Comparison of TNF-α tissue levels by study group. C. Comparison of IL-1 tissue levels by study group. Data are represented as means±standard deviation (SD). These results were averaged from 5 independent experiments. Statistically significant differences between control groups and treatment groups: *p<0.05.

Fig 3: Tissue levels of MIF, TNF-α and IL-1 of *E. awoara* ainfected with *V. alginolyticus* and controls (healthy fishes) were determined by ELISA.

MIF plays an important role in the immunity system. MIF is ancient in its evolutionary basis and is a highly conserved cytokine with multiple functions. MIF promotes colorectal cancer and tumour invasion. MIF deficiency reduced chronic inflammation in the tissue.

We reported that MIF levels were higher in tissues of *E. awoara* infected with *V. alginolyticus* than those in controls. MIFmRNA levels of the examined tissues have been detected by qPCR analysis in other teleo steans (Xu *et al.,* 2016; Xu *et al.,* 2019) and mammals (Poulsen *et al.,* 2021; Wang *et al*., 2021). MIF levels of the tissues in infected with *Edwardsiella tarda* groups were significantly lower than those from fishes in the untreated infected groups. MIF could be determined in various cells and tissues of the endocrine system.The system includes lung, skin and gastro-intestinal tissues in mammals. In vertebrate species (Howait *et al.,* 2019) and in sea bass (Xu *et al.,* 2019), the thymus had the highest MIF mRNA levels. However, the MIF mRNA levels in the gut and spleen were lower than those in gills and head kidney in *Tetraodon nigroviridis*. MIF mRNA levels of the tissues in *V. alginolyticus*-affected fishes were detected higher than those of untreated healthy fish groups. Brain, head kidney, spleen and liver have higher levels of MIF mRNA than those in other tissues, including muscle, gill, heart, stomach and intestines in diseased fishes. MIF was relevant to *E. awoara* immune defence against invading *V. alginolyticus*. MIF played a role in fish susceptibility to in flammatory diseases. High levels of MIF expressions were associated with autoimmune diseases (Baños-Hernández *et al.,* 2019). As a cytokine, MIF also played a critical role in inflammatory and infectious diseases.

In this study, the qRT-PCR analysis results were consistent with the results of ELISA analyses. In infected *E. awoaras*, MIF levels in head kidney and liver were higher than in other tissues. Statistically significant differences in TNF-α and IL-1 expressions were observed in treated organs compared to control organs. This result was similar to previous reports that an overreaction in host response can occur that results in IL-1 and TNF-α and leading to the loss of periodontal attachment (Werber *et al.,* 2021). MIF could induce IL-1 and TNF-α (Howait *et al.,* 2019; Jiao *et al.,* 2020). MIF increased tumour cell proliferation by releasing cytokines including TNF-α, IL-1b and IL-6 (Kolostova *et al.,* 2022). In our study, tissue levels of MIF, TNF-α and IL-1 were up-regulated in infected *E. awoara*; these results were similar to previously reported results (Sam *et al.,* 2021).These results may offer a novel therapeutic strategy for treating bacterial disease.

CONCLUSION

In this study, our result displayed that MIF has been detected in all healthy and diseased fish organs, including head kidney, spleen, liver, brain, intestine, gill, heart, stomach and

muscle. The MIF mRNA levels of the head kidney, liver, brain and spleen in diseased *E. awoara* increased compared with those in control groups ($P<0.05$). Moreover, these results show MIF, TNF-α, and IL-1 play a role in diseased fishes, and the levels in the spleen, head kidney, intestine and muscles were much higher than those in controls. MIF, TNF-α and IL-1levels in V. alginolyticus-affected fishes were positively correlated with bacteria in the fishes. The MIF, TNF-α, and IL-1 could be detected in controls.

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Conflict of interest: None.

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