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Research Article

Can Cinnamaldehyde Increase The Innate Immune System on Medaka Larvae?

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ABSTRACT

Fish larvae are the early stage of the life cycle and the key to the mass production of fish. Its nature, which is susceptible to invade by pathogens, especially viruses, it is an essential concern in increasing the innate immune system to against the invading virus. IFN-α and Mx are inducer genes that have the most role in enhancing the innate immune system. The study was conducted in September 2017 with the experimental method. The sample used was 1-day-old Medaka larvae in the amount of 30 fish larvae in each tank with triplicate in each concentration. The cytotoxicity results showed a 100% survival rate of medaka larvae at 72 h both in control (0 µg mL-1) and treatment group with 2.5 µg mL^{-1} cinnamaldehyde immersion. One-Way ANOVA results, the genes expression of IFN-α and Mx on 1-day-old Medaka larvae showed decreasing at 2.5 µg mL^{-1} concentration of cinnamaldehyde immersion for three days with several observation periods (12 h; 24 h; 48 h; and 72 h) compared to control. Based on the results of statistical analysis showed significant results at 12 h observation with the lowest IFNα expression level was $0.25x10^{-4} \pm 0.06x10^{-4}$ on cinnamaldehyde treatment group. Mx gene expression showed a significant difference at 48 h ($0.90x10^{-2} \pm 0.12x10^{-2}$) and 72 h ($0.90x10^{-2} \pm 0.05x10^{-2}$) on cinnamaldehyde treatment group compared to control. The cinnamaldehyde immersion with a concentration of 2.5 µg mL^{-1} is not effective as an immunostimulant in medaka larvae. More assay is needed to determine the mechanism of IFN-α and Mx gene expression on fish larvae as like using positive control and challenging with the virus.

Keywords: Cinnamaldehyde, Genes Expression, IFN-α, Innate Immune System, Medaka Larvae, Mx, *Oryzias latipes*

Introduction

Larvae are the beginning of a life cycle that requires more attention because of its vulnerability to disease and easily to mortality. The disease occurred by several agents, including virus, bacteria, and parasites. [1], Mortality caused by agents of disease is display on the juvenile stage are higher than adults. The second-largest pathogenic-agent after bacteria is a virus. The virus take 25% of aquaculture problems which have an impact on the economy in fisheries and aquaculture. For example, Nodaviruses, Aquabirnavirus, Hemorrhagic septicemia virus, infectious salmon anemia virus, Aparavirus spp. Crassostrea angulata iridoviruetc. [2;3]. Viruses are the most abundant and diverse pathogen on earth [4]. They are small obligate in tracellular parasites which often made up of nucleic acid molecules (DNA or RNA) within a protein shell. Fish viral diseases causes large-scale death in aquaculture and is very difficult to treat directly [5].

An immune system on the fish organism which is used to protect themselves from invading by various microorganisms. This immune system, starting in the early stage of embryogenesis [6]. The innate immune system naturally is the first defence needed by organisms in the face of environmental stresses. In the case of virus attack, IFN-α and Mx genes play a role in resistance to the virus. Interferons belong to the large class of protein termed as cytokines, which are produced by several host cells in response to various biological and synthetic stimuli. There are three classes of interferon in humans: IFN- alpha, IFN-beta, and IFN-gamma. As the treatment option, interferon-alpha (IFN-α) is the most effective. IFN-α has been

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proven effective as antiviral therapy and tumour antidote in the past two decades [7]. Mx is a pro tein with a 70-80kDa GTPases of dynamin superfamily among vertebrates which induced by IFN type-I (IFNα and IFNβ) is a type of antiviral protein which specifically prevents growth either in vivo or in vitro, of certain virus classes [8;9].

There is an urgent need to prevent the viral infection in fish. The use of immunostimulants for larval fish might propose as a potential method for improving larval survival by increasing the innate immune response until its adaptive immune response is increasing enough and sufficient to respond to the pathogen [10]. The usage of natural product, which is known to have a good impact on organisms for immunostimulants.

Cinnamaldehyde is one of the abundances of chemical compounds of cinnamon essential oil, which has various medical properties (anti-diabetes, neuroprotective, anti-oxidant) [11;12;13]. Cinnamaldehyde also has been reported to inhibit the toxin production by microorganisms and effective as anti-bacteria, anti-moulds, and anti-yeast [14;15;16;17;18] but there is no research about immunomodulatory function of Cinnamaldehyde for antiviral yet.

Medaka is approved as one of the vertebrate model organisms with a small egglaying freshwater fish which allows genetic and embryological analysis like zebrafish and mouse [19;20]. This study aimed to find out whether Cinnamaldehyde effective in evoking the IFN-α and Mx genes as the innate immunerelated genes on medaka fish larvae as model fish.

Methods

Medaka Larvae Preparation

Medaka (*Oryzias latipes*) embryos were obtained from Taikong Corp, Taiwan. The 500 eggs (embryos) were maintained in modified medium [21] consisting of 1-L Ringer Solution 1x (NaCl 7.5g; KCL 0.2g; CaCl2 0.2g; NaHCO3 0.02g; 1L RO water) plus 3 mL methylene blue. Moreover, it kept on 28^{o}C with changing the water three times a week until hatching out and daily observa- tion. The dead embryos were showed blue colour and then were discarded immediately. The sur- vival larvae of medaka used in this study were 1- day-old.

Cinnamaldehyde (CA) Preparation

Cinnamaldehyde used in this study were using the immersion method as a consideration that the sample used was fish larvae which was not possible using other ways, such as Liposome-encapsulated cinnamaldehyde (LEC), or injection. The concentrations used in this study were 10 µg mL^{-1} ; 7.5 µg mL^{-1}; 5 µg mL^{-1} ; 2.5 µg mL^{-1}; and 0 µg mL^{-1} (control). Each set of treatment group contains Cinnamaldehyde (CA) (SigmaAldrich, China); DMSO 2%; Ringer Solution 1x; and 5 µg mL^{-1} of Kanamycin Sulfatefollowed 100 ml of total volume.

Short-Term Effects

Medaka larvae (1-day-old) were moved to 1-L tank, which was filled CA before. Each set of treatment was performed in triplicate, and in each repetition larvae from different hatches were used. Each set contained 30 Medaka larvae and was incubated on 72h without changing water and feeding of one drop artemia per day. The reason is the Medaka Larvae did not have an egg-yolk. The dead larvae were removed and were counted every day. The cinnamaldehyde concentration group, which showed a survival rate (SR) of 50% or more, would be used for further testing. SR (%) were followed [22],

$$\text{SR}\,(\%) = \frac{Total\ of\ survival\ fish\ larvae}{Total\ fish\ eggs} x100\%$$

RNA Extraction

Total RNA was extracted from the whole body of all alive Medaka larvae from selected Cinnamaldehyde (CA) concentration using Tripure Isolation Reagent (Roche, USA) followed by Trizol Reagent technical insert (Invitrogen).

Reverse Transcriptional Polymerase Chain Reaction (RT-PCR)

First copy complementary (cDNA) was synthesized from extracted RNA by using BIO-RAD iScript cDNA Synthesis Kit's protocol with RT-PCR tools (Bio-Rad Type T100) and then was processed on qPCR (ABI Type Step One Plus) to analyze the genes expression.

Innate Immune-Related Genes Expression

The cDNA was analyzed by real-time polymerase chain reaction also known as quantitative PCR (qPCR) was performed using KAPA SYBR FAST qPCR Master Mix (Kapa Biosystems, Inc., Wilmington, MA, USA) to check the innate immune-related genes expression. The gene expression was observed was IFN-α and Mx. The housekeeping gene was EF1α. The sequences of primers

used for the qPCR followed [23] are listed in Table 1.

Statistical Analysis

One-way analysis of variance (ANOVA) followed by Dunnet test each time point by using SigmaPlot 12.5 software. The standart error of the mean (SEM) was expressed in the data. Statistically, significant differences required that $p<0.05$.

Table 1. Primers of qPCR used in this study

Gene		Sequence (5'-3')
EF1a	F	5'-ATTTGCGGGGTTTGCAC-3'
	R	5'-TGGGAGCTTTTATACGGACTGG-3'
IFN-α	F	5'-GAGCTGAACAGCTGCCTGAA-3'
	R	5'-TTTCTTGCCCAGTTTTCTGC-3'
Mx	F	5'-CTATCATGAAACTGAAGGACATTGG-3'
	R	5'-AAGCTTGCTTGGGCCAGTAG-3'

Result and Discussion

Medaka Larvae Survival Rate (%)

The survival rate of Medaka larvae was used as the initial to determine safe concentration for the further step. Lethal concentration (LC50) was used in this study as guidelines in determining safe concentrations. [24], LC_{50} is a value in the statistical estimation of the dosage to kill 50% of the most population. The percentage of survival rate of Medaka larvae after 72 h exposure of Cinnamaldehyde showed in Table 2. The overall concentration of cinnamaldehyde exposure, 2.5 µg mL^{-1}, was the only concentration that showed a survival rate above 50%. In contrast, the other concentration were not indicated a survival rate of more than 50%, which means that the safe concentration of Cinnamaldehyde for the further assay was 2.5 µg mL^{-1}.

Table 2. Survival rate (%) of Medaka larvae after 72 h cinnamaldehyde exposure with triplicate

CA Concentration (µg mL^{-1})	Larvae Alive			Survival Rate (%)
	1	2	3	
Control	30	30	30	100%
10 µg mL^{-1}	0	0	0	0%
7.5 µg mL^{-1}	5	5	9	21.1%
5 µg mL^{-1}	5	5	12	24.4%
2.5 µg mL^{-1}	30	30	30	100%

Innate Immune-related Genes Expression

Many kinds of literature state the effectiveness of Cinnamaldehyde as an antimicroorganism through various study [25], but there is no research states that Cinnamaldehyde can be used for virus problem. Unlike other pathogens, viruses have a more complicated infection rate than other pathogens. A virus needs a suitable host cell for self-replication to be able to do the infection [26].

Interferon α (IFN-α) is a kind of type I interferon,plays a role against viruses infection [27]. Interferon existence in vertebrate from fish to mammals [28]. Through the JAK-STAT pathway, IFN will lead to the formation of various ISGs (Interferon Stimulated-Genes) as the innate antiviral response in suppressing viruses and other effects caused by IFN [29]. Mx proteins are best known for inhibiting negative-stranded RNA viruses; besides that, Mx also inhibits other virus families [30].

Based on the explanation above, it can be concluded that IFN-α and Mx will be upregulated if an infection occurs in the host cells so that the use of IFN-α and Mx as agents that play a role in antiviral therapy was more developed. However, in this study, researchers wanted to try to increase the expression of IFN-α and Mx genes, by immersing Cinnamaldehyde in order to become immunostimulants.

The expression of IFN-α in 1-day-old Medaka larvae showed in Figure 1. The fluctuation expression of IFN-α genes occurs at each time point, graphically. However, despite expression fluctuations, there was no significant differences between control and cinnamaldehyde treatment sample except at 12 h observation. The significantly decreased of IFN-α gene expression occurred at 12 h exposure of Cinnamaldehyde ($0.25x10^{-4}\pm 0.06 \times 10^{-4}$), which compared to control ($2.16x10^{-4} \pm 0.12 \times 10^{-4}$).

Cinnamaldehyde has a strong antipathogenic ability. The expression of IFN-α gene at 0 h as a first-time point did not experience a significant difference between control and treatment group, which means that at 0h the cells in the fish larvae still had not received the effects of Cinnamaldehyde. However, at 12 h, the expression level of IFN-α on cinnamaldehyde treatment was decreased dramatically. It was suspected because there was the worst cell damage that causes IFN-α production to decrease. IFN-α is one type of Interferon type I which belongs to the cytokine group. Cytokines are small protein with the low molecular weight with low life span [31]. Cytokines are released by one cell to regulate the function of

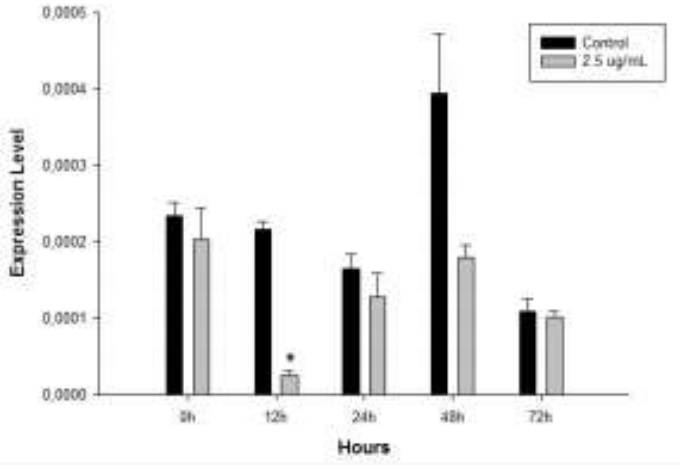

Figure 1. The expression of IFN-α on 1-day-old Medaka larvae with various time point observations. Each time point data were compared to each control and standard error of the mean (SEM) as the error bars <0.05.

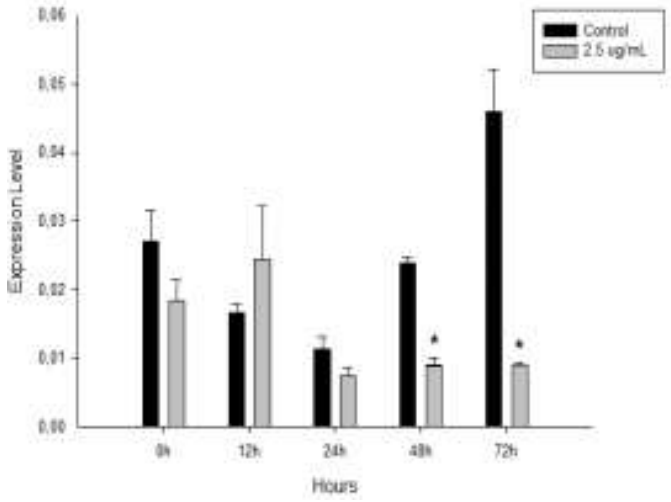

Figure 2. The expression of Mx on 1-day-old Medaka larvae with various time point observations. Each time point data were compared to each control and standard error of the mean (SEM) as the error bars <0.05.

other cells. On the immune system, cells communicate through cytokines, so that cytokines have a role in regulating the nature, intensity and duration of the immune response through their effects on lymphocytes and other cells. In other words, cytokines are messenger or intercellular chemical signalling or signalling molecules that play a role in cellto-cell communication in inflammation, infection and trauma, also helps inter-cel communication in specific and non-specific immune response [32]. Therefore, when a cell is damaged, the amount of IFN-α expressed will decrease as well. At the 24 h to 72 h, the IFN-α expression on the cinnamaldehyde treatment group returned to normal, which is presumably because the cell has regenerated, so that can reproduce the IFN-α gene.

Related to IFN-α, Mx protein will immediately accumulate in the cytoplasm or nucleus by forming oligomers, which can interfere with the virus replication [33;34]. Allegedly, the antiviral mechanism in the Mx gene is by inhibiting the transport of viral nucleocapsids, inhibiting the transcription or translation of viral RNA, and targeting viral elements, such as the virus polymerase complex [35;36] whereas IFN can inhibit cell replication and spread of various viruses [37;38].

The expression of Mx genes was showed in Figure 2**.** In line with IFN-α, the Mx gene showed higher expression results in entire control groups

when compared to treatment group (2.5 µg mL^{-1} of Cinnamaldehyde). The highest expression of Mx gene occurred at 72 h of the control group with a value of $0.05 \pm 0.62x10^{-2}$, and the lowest expression was at 24 h of the cinnamaldehyde treatment group was $0.75 \times 10^{-2} \pm 0.11 \times 10^{-2}$.

Meanwhile, the highest IFN-α gene expression was $3.95 \times 10^{-4} \pm 0.78 \times 10^{-4}$ at 48 h for the control group and the lowest gene expression was at 12 h at cinnamaldehyde treatment group ($0.25 \times 10^{-4} \pm 0.06 \times 10^{-4}$). The expression of Mx gene at 48 h and 72 h occurred a significant difference between cinnamaldehyde and control treatment group. Although there were significant differences, in terms of value, the cinnamaldehyde treatment is lower than the control treatment. The decreasing of Mx gene expression level thought to be due to the weakening of Mx activity originating from within the cell.

The low gene expression of both Mx and IFN-α genes indicated that there was no signifi- cant threat of virus in threatening or disrupting the immune system in the bodies of medaka larvae, especially in the group treated with an addition of Cinnamaldehyde. [39] reported that Interferon (IFN) involved in innate immunity against viruses and Mx shown the possess antiviral properties. The IFN-α and Mx were upregulated by Infectious pancreatic necrosis virus (IPNV) in salmon fish [40]. This phenomenon suspected that Cinnamaldehyde could not trigger an innate immune system, especially IFN-α and Mx in the Medaka larvae body.

Conclusion

Cinnamaldehyde (CA) with 2.5 µg mL^{-1} concentration is not able to induce the Innate Immune-related (IFN-α and Mx) genes expression on Medaka larvae. More examination like addition of positive control using virus or substance that induce innate immune-related genes, pretreatment with CA and then challenge with virus or co-treatment of both will be needed to understand this phenomenon.

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