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Full Length Research Paper

Effects of N^G-monomethyl-L-arginine (NMMA) and catalase on intracellular survival of *Vibrio alginolyticus* in macrophages of large yellow croaker *Pseudosciaena crocea*

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The intracellular survival of *Vibrio alginolyticus* and *Vibrio parahaemolyticus* in large yellow croaker macrophages of *Pseudosciaena crocea* was investigated. In addition, the effects of N^G-monomethyl-L-arginine (NMMA) and catalase on the interaction of *V. alginolyticus* with macrophages from head kidney and on macrophages reactive nitrogen intermediate (RNI) and reactive oxygen intermediate (ROI) *in vitro* were determined. *V. alginolyticus* ND-01 was able to survive in macrophages from head kidney of large yellow croakers for at least 3 h, while *V. paraheamolyticus* 1.1614 could not survive in the macrophages for 1 h. Intracellular bacterial survival was affected by the addition of specific inhibitors of macrophage oxidative function. Exposure of macrophages to NMMA and catalase decreased the number of viable cells of *V. alginolyticus* inside large yellow croaker macrophages. Furthermore, a close correlation was observed between the number of intracellular survival bacteria with the amount of NO and H_2O_2 produced by macrophages.

Key words: Large yellow croaker, macrophages, *Vibrio alginolyticus*, N^G-monomethyl-L-arginine (NMMA), catalase.

INTRODUCTION

The large yellow croaker (*Pseudosciaena crocea*) is one of the most important cultured marine fish in China (Mai et al., 2005). In recent years, with the rapid expansion of intensive one-species aquaculture of large yellow croaker, infectious diseases caused by bacteria, mainly *Vibrio*, are spreading, resulting in great economic losses. *V. alginolyticus* is a Gram-negative short bacillus which widely distributed in the ocean and estuary environment (Molitoris et al., 1985). *V. alginolyticus* has been commonly associated with the epidemic vibriosis which leads to mass mortality of cultured large yellow croaker, and resulting in considerable losses (Yan et al., 2009).

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Macrophagesplay an important role in the early immune response by killing invading microorganisms through phagocytosis and release of bactericidal substances such as reactive oxygen intermediate (ROI) (Neumann et al., 2001). Given that the macrophage is one of the cell types which pathogens are likely to encounter soon after entry into the host and its prominent role as an effector cell in the immune response, it is not surprising that certain pathogens have adapted to live inside the macrophage for part of their life, using the cell as a shield against other cell-mediated and humoral immune responses (Kaufmann, 1993).

Intracellular survival is an important factor determining virulence of bacilli (Herdt et al., 1995). By surviving within phagocytes, pathogens, such as *Streptococcus pyogenes*, can also exploit the free-trafficking privileges of these cells within the host to systemically disseminate

from a local focus of infection (Medina et al., 2003). Several fish pathogens, such as *Mycobacterium* spp. (Chen et al., 1998), *Piscirickettsia salmonis* (McCarthy et al., 2008) and *Yersinia ruckeri* (Ryckaert et al., 2010) have been reported to resist killing by macrophages.

To survive within phagocytes, pathogens should withstand ROI and RNI such as superoxide (O^{2-}) and nitric oxide (NO) produced by the cells (Nathan and Shiloh, 2006). Chan et al. (1992) had previously demonstrated that intracellular survival of *Mycobacterium tuberculosis* in murine macrophages was effectively inhibited by IFN-γ and tumor necrosis factor alpha (TNF-α) or *Escherichia coli* lipopolysaccharide (LPS). The intracellular survival of *M. tuberculosis* in RNI-producing macrophages correlated with the amount of nitrogen oxides generated and was inhibited by NOS inhibitors (Chan et al., 1995). Virulent *Edwardsiella tarda* strains are able to adhere to, survive and replicate within blue gourami (*Trichogaster trichopterus*) phagocytes at least 6.5 h postinfection but fail to stimulate reactive oxygen intermediates (Rao et al., 2001). *Piscirickettsia salmonis* is capable of survival and replication within native salmonoid head kidney macrophages and its survival may depend on an ability to escape destruction within phagolysosomes, not utilise actin-based motility (ABM) as a means of evasion and intercellular spread (McCarthy et al., 2008). *Yersinia ruckeri* is able to survive inside macrophages in vitro as well as in vivo and is possessed by both SOD and catalase which can interfere with the ROI produced by the macrophages early after infection, thus conferring protection against early killing (Ryckaert et al., 2010).

However, intracellular survival of *V. alginolyticus* in macrophages and the effects of drugs on the intracellular survival of *V. alginolyticus* have not been reported. In the present study, we examined the effects of N^G-monomethyl-L-arginine (NMMA) and catalase on the intracellular survival of *V. alginolyticus* within macrophages and on the production of RNI and ROI, for a better understanding of the intracellular survival of *V. alginolyticus*.

MATERIALS AND METHODS

Bacterial strain and culture conditions

*V. alginolyticus*ND-01 was isolated from the spontaneously infected large yellow croakers and confirmed as the pathogen by artificial infection (Yan et al., 2001). *V. parahaemolyticus* 1.1614 was obtained from China Center for Type Culture Collection .Both strains were grown on beef extract-peptone agar with 2% NaCl at 28°C. After incubation for 18 h, bacterial cells were harvested and resuspended in phosphate-buffered saline (PBS, pH7.4). The density of bacterial suspension was adjusted according to the value of OD_{550}.

Preparation of macrophages suspension

Head kidney macrophages were collected according to the procedures of Bayne (1986) with some modifications. The tissue overlaying the pronephros of each side was removed, and the organ was placed in 2 ml ice-cold Leibovitz L-15 medium (Biological Industries, Israel) supplemented with 10 IU heparin per ml 100 IU S/P/ml and 2% fetal calf serum. All head kidneys were sheared, and pushed through 100-order nylon net. The cell suspensions from the two organs were then loaded onto a 34/51% discontinuous Percoll (Amersham Pharmacia Biotech, UK) density gradient with a syringe and centrifuged at 400×g for 30 min at 4°C. The band of cells at the 34/51% interface was collected, then washed twice by resuspending in L-15 medium and centrifugated at 400×g for 10 min at 4°C, and the living and dead cells were counted by trypan blue staining. And then the cells were counted with hemocytometer, and adjusted to 2.4×10^7 cells/ml in L-15 medium with 10% FCS, 100IU S/P/ml and 10 IU heparin/ml and transferred to six pore plates at 1 ml/well.

Transmission electron microscopy (TEM) analysis

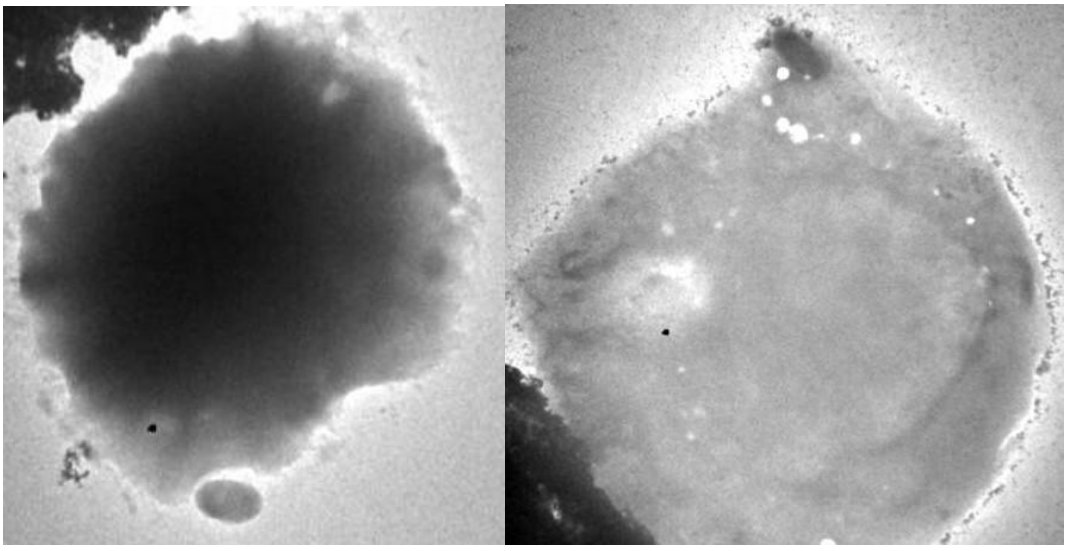
The cells were transferred to six pore plates at 1 ml/well before the bacteria were added. The bacterial suspension [multiplicity of infection (MOI) =100(100 bacteria per macrophage added)] was added to each well and incubated at 28°C. After 0.5 h of incubation, the cells were pooled in sterile tubes. The tubes were centrifuged at 400×g for 10 min at 28°C and the supernatant was discarded, then 2.5% glutaraldehyde in PBS was added for 30 min at 4°C to fix the macrophages. Then was rinsed twice in PBS for 5 min each time and macrophages were resuspended in PBS by gentle rocking. A 300-mesh Formvar-coated copper grid was placed on a drop of macrophages suspension for 5 min. Duplicates were performed for each type of bacteria and the images were obtained using JEOL 1010 transmission electron microscope (JEOL, Ltd., Tokyo, Japan) at an acceleration voltage of 80 kV with calibrated magnification.

In vitro infection assay

The *in vitro* infection assay was performed as described by Larsen et al. (2001) with some modifications. The cells were transferred to six pore plates at 1 ml/well before the bacteria were added, denoted as time point -2 h. 1 ml of the bacterial suspension [multiplicity of infection (MOI) = 100 (100 bacteria per macrophage added)] was added to each well and incubated at 28°C. After 0.5 h of incubation, the cells were pooled in sterile tubes. The tubes were centrifuged at 100×g for 5 min at 28°C and the supernatant was carefully removed without disturbing the packed cells. After washing twice by resuspending in 3 ml PBS and centrifuged for 5 min at 100×g at 28°C, the packed cells were resuspended in 2 ml PBS. Then the cell suspensions were treated with 3000 U Gentamycin/ml for 20 min at 4°C and were washed twice as above. The supernatant fluid was withdrawn for sterility test by plate counting. The packed cells were resuspended in fresh L-15 medium with 10 IU heparin/ml, 10% FCS and 100 units S/P, and the time point was denoted as zero. The cells suspension was allowed to be incubated at 28°C in 5% CO_2 for 0, 30, 60, 120, and 180 min, respectively. After incubation, the cells were centrifuged for 5 min at 100×g at 28°C and the supernatant was aspirated, then 1 ml of sterile distilled water was added for 30min to lyse the cells. The CFU number of the cell lysate was determined by plate counting (McCarthy et al., 2008; Ryckaert et al., 2010).

The effects of timing of NMMA and catalase treatment on the intracellular fate of *V. alginolyticus*

The effects of timing of NMMA and catalase treatment on the intracellular fate of *V. alginolyticus* were performed as described by



A B

Figure 1. Transmission electron micrographs of macrophages phagocytosis. (A) A bacteria was phagocytosing by the macrophage (×6000); (B) a bacteria had been devoured by a macrophage (×8200).

Zhao et al. (1997) with some modifications. Macrophages in the wells of six-pore plates were divided into 8 groups. Group C and D were pre-incubated in 1 ml L-15 culture medium with 250 µg/ml NMMA, and group G and group H were incubated in 1 ml L-15 culture medium with 1 mg/ml catalase. Groups A, B, E, F, I, J and K were pre-incubated in 1 ml L-15 culture medium without NMMA or catalase. After 2 h of pre-incubation, the macrophages were subjected to infection by *V. alginolyticus* at a MOI of 100. After 1 h of infection, the cell suspensions were treated with 3000 U Gentamycin/ml for 20 min at 4°C and were washed twice as above. 1 ml fresh L-15 medium was added to every pore. Groups B, C and J was cultured in 1 ml L-15 medium with 250 µg/ml NMMA, and Groups F, G and K was cultured in 1 ml L-15 medium with 1 mg/ml catalase. In addition, Groups A, D, E, H and I was not exposed to NMMA or catalase. All groups of macrophages were cultured for 3 h after infection, and the numbers of intracellular *V. alginolyticus* cells were then assessed by plate counting.

Measurement of NO_2^-

To determine the production of nitric oxide by macrophages, its stable end product, NO_2^-, was analyzed by the Griess reaction (Green et al., 1982). Briefly, conditioned media were collected and centrifuged (400 × g) for 10 min. Aliquots (100 µl) of the conditioned media were then distributed in a 96-well microtiter plate, and then equal volumes of the Griess reaction solutions (0.1% naphthyl-ethylenediamine dihydrochloride and 1% sulfanilamide in 2.5% phosphoric acid) were added. The reaction was allowed to proceed for 10 min at room temperature, and the absorbance at 550 nm was measured by a microplate reader (BIO-RAD). The analysis was conducted in triplicate for each sample. The amounts of NO_2^- in the samples were calculated by extrapolation from a sodium nitrite standard curve prepared for each experiment.

Measurement of hydrogen peroxide

The production of hydrogen peroxide by macrophages was determined according to hydrogen peroxide kit (Nanjing Jiancheng Bioengineering Institute) based on the oxidative polymerization of molybdic acid to a complex compound reaction product. Briefly, Aliquots (0.1 ml) of conditioned media were collected in 5 ml centrifuge tube, homogenized and centrifuged (10000 × g) for 10 min. 0.1 ml supernatant and 1 ml reagent I (pre-heated at 37°C) were incubated in 4 ml centrifugal tube at 37°C for 1 min, then 1 ml reagent II was added. The absorbance at 405 nm was measured by a microplate reader (BIO-RAD). The bank and standard group was done as below. The analysis was conducted in triplicate for each sample. The amounts of hydrogen peroxide in the samples (gprot/L) were calculated by $[(A_{experiment} - A_{blank})/A_{standard} - A_{blank})] \times$ standard concentration (163 mmol/L)÷ proten concentration of the sample.

Statistical analysis

The results were expressed as mean ± standard deviation. Student's t-test was used to determine the difference between two groups. Values of $P<0.05$ and $P<0.005$ were considered significant difference and extremely significant difference, respectively.

RESULTS

Phagocytosis micrographs

As visualized using TEM, the cell membrane of a macrophage invaginated and formed the pseudopod and *A. hydrophilia* was phagocytosing by the macrophage (Figure 1A). Then the bacteria had been devoured by a macrophage (Figure 1B).

Intracellular survival ability of 2 strains

V. alginolyticus exhibited considerable intracellular

Table 1. Survival of two strains of *Vibrio* in cultured macrophages from head kidney of the large yellow croaker.

Strains	Bacteria survive (CFU/ml）				
	0 min	30 min	60 min	120 min	180 min
V. alginolyticus	2755000 ±145000	560000 ±150000	365000 ±85000	109167±4922	66567±5406
V. parahaemolyticus	10±5	14±6	0	0	0

Table 2. Effects of NMMA treatment on the intracellular fate of *V. alginolyticus.*

Treatment ways	Number of *V. alginolyticus* viable (CFU/ml)		
	0 h	1 h	3 h
A(no NMMA)	3480000±11314	292000±8485	48300±4667
B(NMMA added after infection)	3480000±11314	118667±18148	10267±2715
C (NMMA added before and after infection)	3230000±4243	52000±11314	15600±3292
D(NMMA added before infection)	3230000±4243	251000±15556	62900±4384

Table 3. Effects of catalase treatment on the intracellular fate of *V. alginolyticus.*

Treatment ways	Number of *V. alginolyticus* viable (CFU/ml)		
	0 h	1 h	3 h
E(no catalase)	2500000±42426	162000±8485	42800±2828
F(catalase added after infection)	2500000±42426	102000±9165	38400±5103
G (catalase added before and after infection)	2410000±15556	39267±1701	18900±707
H(catalase added before infection)	2410000±15556	73330±8080	36267±1617

survival ability in macrophages of large yellow croaker, while *V. parahaemolyticus* showed very low intracellular survival ability. Despite the numbers of viable *V. alginolyticus* in macrophages from head kidney of the large yellow croaker decreased from 0 to 180 min after infection, the number of viable cell still remained on a high level (66567±5406 CFU/ml). However, no viable cell of *V. parahaemolyticus* was detected at 60, 120 and 180 min postinfection (Table 1).

Intracellular survival of *V. alginolyticus* in durg-treated macrophages

The number of intracellular viable *V. alginolyticus* in macrophages pre-treated with NMMA and pre-treated without NMMA at 0 h were 3480000±11314 and 3230000±4243 CFU/ml, respectively. There was no significant difference (P>0.05) between different groups. In macrophages pre-treated with NMMA, *V. alginolyticus* in group D at 1 and 3 h exhibited stronger intracellular survival ability than group C ($P<0.05$). In macrophages pre-treated without NMMA, *V. alginolyticus* in group A at 1 and 3 h exhibited stronger intracellular survival ability than group B ($P<0.05$). There was no significant difference between group A and D and between group B and C at 1 and 3 h (P>0.05). In macrophages without treatment with NMMA, the number of intracellular viable *V. alginolyticus* was 48300±4667 CFU/ml at 3 h postinfection (Table 2, treatment A). In macrophages exposed to NMMA after infection with *V. alginolyticus*, the percent decline of intracellular *V. alginolyticus* was 78.7% at 3 h after infection (Table 2, treatment B). In macrophages pretreated with NMMA and continuously incubated with NMMA thereafter the percent decline of viable *V. alginolyticus* was 67.7% at 3 h after infection (Table 2, treatment C). In macrophages which pretreated with NMMA, there was a 30.2% increase at 3 h postinfection (Table 2, treatment D).

The number of intracellular viable *V. alginolyticus* in macrophages pretreated with catalase and pre-treated without catalase at 0 h were 2500000±42426 and 2410000±15556 CFU/ml, respectively, exhibiting no significant difference (P>0.05). In macrophages pre-treated with catalase, *V. alginolyticus* in group H at 1 and 3 h exhibited stronger intracellular survival ability than group G ($P<0.005$). In macrophages pre-treated without catalase, the number of viable *V. alginolyticus* in group E at 1 h was greater than group F ($P<0.05$) and exhibited no significant difference with group F at 3 h. In macrophages without treatment with catalase, the number of viable intracellular *V. alginolyticus* was 39867±5460 CFU/ml at 3 h postinfection (Table 3, treatment E). In macrophages exposed to catalase after

Table 4. The intracellular fate of *V.alginolytics* in macrophages pretreated with NMMA and catalase and continuously incubated with NMMA and catalase.

Treatment ways	0 h	1 h	2 h	3 h
I(control)	2245000 ±10500	150000 ±56569	107500±10607	44250±354
J(NMMA) added after infection	2245000 ±10500	405000 ±91924	192500±10607	22500±2828
K(catalase) added after infection	2245000 ±10500	75000 ±7071	55000 ±7071	24833±4481

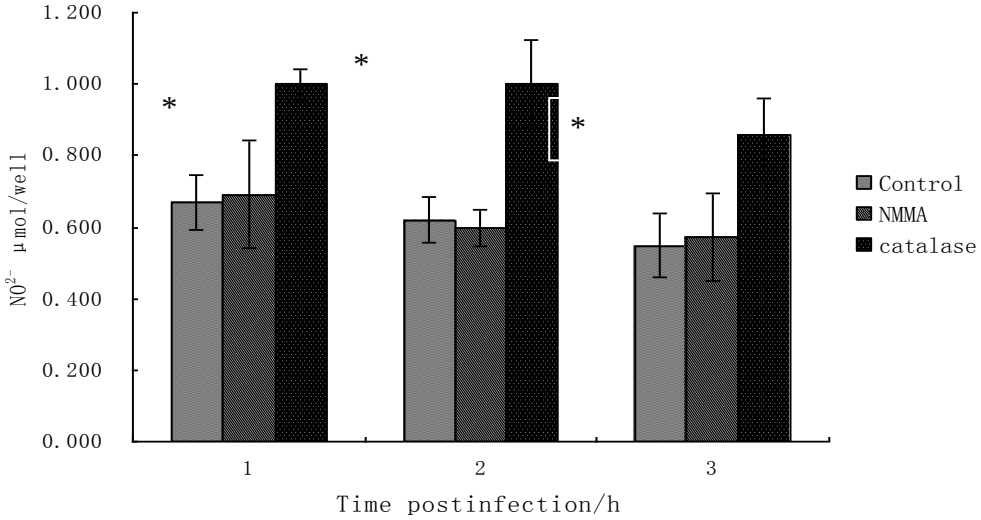


Figure 2. Effects of NMMA and catalase on macrophages reactive nitrogen intermediate.

infection with *V. alginolyticus*, the percent change of intracellular *V. alginolyticus* was 10.3% at 3 h postinfection (Table 3, treatment F, $P<0.05$). In macrophages pretreated with catalase and continuously incubated with catalase thereafter the percent decline of viable *V. alginolyticus* was 8.3% at 3 h after infection (Table 3, treatment G, $P<0.05$). In macrophages that were pretreated with catalase but not exposed to catalase after infection, there was a 15.3% decline at 3 h postinfection (Table 3, treatment H, $P<0.05$).

Effects of NMMA and catalase on intracellular survival of *V. alginolyticus*

The number of intracellular viable *V. alginolyticus* in macrophages treated with catalase or NMMA or untreated with drugs at 0 h were 2245000±10500 CFU/ml. *V. alginolyticus* in group J at 1 h reflected stronger intracellular survival than group I and K ($P<0.05$), and there was not significant difference between group I and K ($P>0.05$). *V. alginolyticus* in group J at 2 h still showed stronger intracellular survival than group I and K ($P<0.05$), and there was significant difference among the three groups ($P<0.005$). At 3 h, group J and H exihibited the same intracellular survival ($P>0.05$), and group I showed the stronger survival compared with the other groups. In control group, the number of viable intracellular *V. alginolyticus* was 44250±700 CFU/ml at 3 h postinfection (Table 4, treatment A). In macrophages pretreated with NMMA and continuously incubated with NMMA, the percent decline of viable *V. alginolyticus* was 49.2% at 3 h postinfection (Table 4, treatment B). In macrophages pretreated with catalase and continuously incubated with catalase, the percent decline of viable *V. alginolyticus* was 43.9% at 3 h postinfection (Table 4, treatment C).

Effects of NMMA and catalase on macrophages NO^{2-}

Treatment of macrophages with catalase increased the production of NO^{2-} significantly (Figure 2, $P<0.005$), while the treatment with NMMA did not show significant effect on the production of NO^{2-} (Figure 2, $P>0.05$). NO^{2-} produced by infected macrophages of control group (without treatment with NMMA or catalase) was increased somewhat in infected macrophages treated with catalase (Figure 2, $P<0.005$), while was not influenced when NMMA was added to macrophages (Figure 2, $P>0.05$). NO_2^- produced by infected macrophages treated with catalase was 1.0 00±0.043 μmol/well at 1 h after

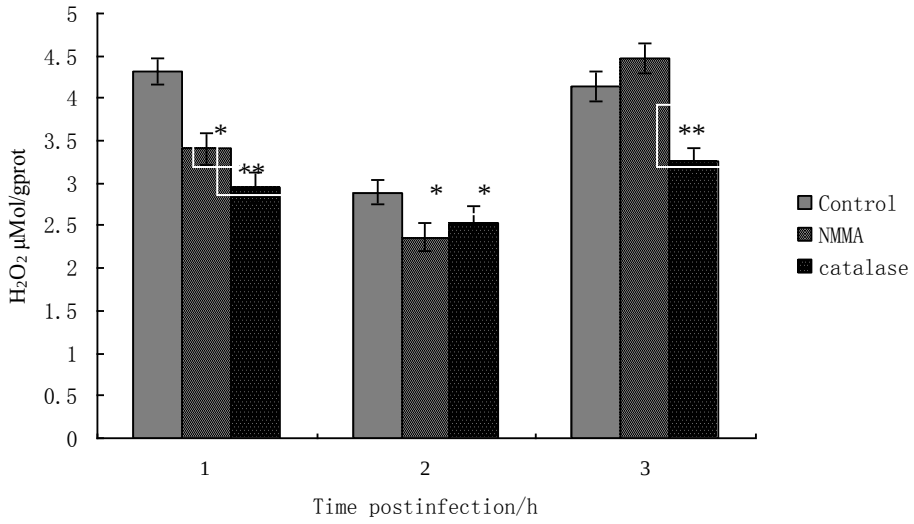


Figure 3. Effects of NMMA and catalase on macrophages reactive oxygen intermediate.

infection. NO_2^- produced by infected macrophages treated with NMMA was no significant difference with the control group (P>0.05), while there was a significant difference with the group treated with catalase (P<0.005).

Effects of NMMA and catalase on macrophages hydrogen peroxide

Hydrogen peroxide produced by infected macrophages treated with NMMA or catalase decreased compared to that of infected macrophages without treatment of NMMA or catalase (Figure 3, P<0.05). Hydrogen peroxide produced by infected macrophages treated with NMMA or catalase was significantly difference with the control group (Figure 3, P<0.05). Hydrogen peroxide produced by infected macrophages treated with NMMA decreased during 1 and 2 h, followed by an increase and the value was 4.468 μmol/gprot at 3 h after infection (Figure 3, P<0.05). Hydrogen peroxide produced by infected macrophages treated with catalase decreased during 1 and 2 h, followed by an increase and the maximum value was 3.253 μmol/gprot (Figure 3, P<0.05). The trend of hydrogen peroxide produced by infected macrophages treated with no NMMA and no catalase was the same with that of hydrogen peroxide produced by infected macrophages treated with catalase.

DISCUSSION

The capability of survival inside the host phagocytes is considered to be a bacterial virulence factor as it facilitates the spreading and infection of the pathogens. A virulent *E. ictaluri* strain was killed by channel catfish (*Ictalurus punctatus*) macrophages (Shoemaker et al., 1997), while virulent *E. tarda* strains were capable of survival and replication in head kidney phagocytes of blue gourami (*Trichogaster trichopterus*) at least 6.5 h postinfection (Rao et al., 2001). In the present study, *V. alginolyticus* ND-01 was able to survive in macrophages from head kidney of large yellow croakers for at least 3 h, while *V. paraheamolyticus* 1.1614 could not survive in the macrophages for 1 h, which indicated the virulent of *V. alginolyticus* ND-01 to large yellow croakers.

In order to survive within the macrophage, pathogens must avoid being killed by the cell's numerous defense, and have evolved a variety of survival or escape tactics (McCarthy et al., 2008). NMMA is a nonspecific iNOS inhibitor which affects the L-arginine-dependent cytotoxic pathway mediated the potent antimicrobial function of macrophages, decreasing the generation of NO and RNI in mammalian (Chan et al., 1995). Conflicting results have been reported about the effect of NMMA on the survival of pathogens inside host phagocytes. NMMA was reported to exacerbate murine listeriosis (Beckerman et al., 1993; Boockvar et al., 1994), suggesting that macrophages treated with NMMA was not able to ineffectively kill or inhabit intracellular *Listeria*. Cross et al. (1999) demonstrated that exposure of ferret macrophages to NMMA did not significantly affect the intracellular survival and growth of *Mycobacterium bovis*. However, Gregory et al. (1993) demonstrated that when NMMA was used in another murine listeriosis model in which treatment was administered once at the time of infection, it appeared to decrease tissue listerial burden. In the present study, treating macrophages of large yellow croaker with NMMA inhibited the intracellular survival of *V. alginolyticus*. The results indicate that NMMA is unsuitable for the cure of vibriosis of large yellow croaker.

Catalase facilitates bacteria to survive inside host

macrophage by catalyzing the breakdown of hydrogen peroxide to water and oxygen (Day et al., 2000). The results of Day et al. (2000) indicated that catalase provided resistance to hydrogen peroxide in vitro and contributed to the survival of *Campylobacter jejuni* in BALB/c mice macrophage, suggesting a novel mechanism of intracellular survival. Cross et al. (1999) demonstrated that exposure of ferret macrophages to catalase did not significantly affect the intracellular survival and growth of *M. bovis*. The results of our study showed that catalase inhibited the intracellular survival of *V. alginolyticus*. Since there is no report about survival of pathogen inside fish phagocyte treated with NMMA and catalase, it is still unknown whether the pathogens or the hosts lead to the conflicting results.

Free soluble nitrite is an indicator of nitric oxide synthesis. Ferret macrophages (Cross et al., 1999) and human monocyte-derived macrophages (Cameron et al., 1995; Murray and Teitelbaum, 1992) did not produce measurable levels of free soluble nitrite. However, oyster hemocytes produce considerable ROI and RNI after bleeding even without stimulation by zymosan or PMA (Lambert et al., 2007). In the present study, fish macrophages activated in vitro can produced some amounts of NO_2^-.

Different results have been reported on the effect of NMMA and catalase on RNI production of mammalian and bivalve. The release of nitric oxide from monocytes infected by *Mycobacterium avium* was found to be inhibited by NMMA (Zhao et al., 1997). Similar results were recorded from mice (Chan et al., 1995) and rats (Upchurch et al., 2001). However, Goedken et al. (2004) suggested that NMMA failed to inhibit the production of a respiratory burst in oyster haemocytes. Lambert et al., (2007) demonstrated that NMMA was a potent inhibitor of hyalinocyte ROI/RNI production (27 to 33% decreases) and had no significant effect on granulocytes in *Crassostrea gigas*. In the present study, NMMA did not significantly inhibit macrophages infected with *V. alginolyticus* from producing the RNI, identifying with the results of Goedken et al. (2004) and Lambert et al. (2007). The results in the present study showed that catalase can lead to the suppression of H_2O_2, which in accord with Cross et al. (1999), who reported that catalase caused a mean of 82% suppression of H_2O_2 in ferret macrophages. NMMA also caused the suppression of H_2O_2, it is possible that NO reacts very rapidly with oxygen radicals (Oliveira et al., 2006), resulting in the decrease of H_2O_2.

In conclusion, *V. alginolyticus* can survive in large yellow croaker macrophages for at least 3 h. The intracellular survival of *V. alginolyticus* was inhibited by NMMA and catalase. Catalase promoted the production of NO^{2-} by macrophages. NMMA and catalase resulted in the decrease on the amount of H_2O_2 in macrophages. And this study provided a better understanding of the role of ROI and RNI production and suggested that there is a correlation between intracellular survival bacteria with the amount of RNI and ROI produced by macrophages.

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