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Involvement of inducible nitric oxide synthase (iNOS) in immune-functioning of *Paphia malabarica* (Chemnitz, 1782)

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ABSTRACT

In recent years, the role of inducible nitric oxide synthase (iNOS) isoform has been widely studied because of its immunological relevance in higher organisms as well as invertebrates including bivalves. However, little is known about the immunological role of iNOS in *Paphia malabarica* defense mechanism. In this study, we immunodetected the presence of iNOS in *P. malabarica* hemocytes using antibody N9657 monoclonal anti-nitric oxide synthase. In addition, increased iNOS activity was evident in response to a higher bacterial dosage (*Vibrio parahaemolyticus* and *V. cholerae*), highlighting the dose-dependent iNOS activity induction. Also, higher bacterial survivability was observed in the presence of iNOS inhibitor, i.e., S-methylisothiourea hemisulphate (SMIS) thus, validating the bactericidal role of iNOS. These findings implicate the involvement of iNOS in immune-functioning of *P. malabarica*. Future work should focus on elucidating the expression and regulation of pathogenesis in *P. malabarica*, involving iNOS.

1. Introduction

The immunological insight of an organism is quite necessary from ecological, economical as well as societal points of view. Past few years have been marked by intensive research done on molluscan immune effector mechanisms which determine their survivability in a given habitat [1–6]. Bivalve molluscs immune system is comprised of hemocytes (immune cells) and other humoral factors which protect the organism by activating a broad range of immune responses involving phenoloxidase system, phagocytosis, encapsulation, antimicrobial molecules release and, reactive oxygen species production [7,8].

Nitric oxide (NO) is another antimicrobial defense radical that is produced by concomitant conversion of L-arginine into citrulline by the cytosolic or membrane-bound isoenzymes nitric oxide synthases (NOS) [9, and references therein]. Previous studies have found out the immunomodulating role of NOS-mediated NO in several mollusc species such as clam, mussel, oyster, snail, and scallop [10–16]. In a recent study, Sahoo and Khandeparker [17] found the involvement of NOS in the cyprid metamorphosis of *Balanus amphitrite*. However, the functional roles of different NOS isoforms in vertebrates have been very well documented in the literature compared to invertebrates [18]. In vertebrates, three isoforms have been recognized based on their genetic origin, distribution in the tissues, and immune-functioning which are neuronal isoform (nNOS), inducible isoform (iNOS) and, endothelial isoform (eNOS) [19].

The immunological, sensory and neurological roles of iNOS in various invertebrate groups have been studied [9, and references therein]. There is enough evidence which shows that foreign epitopes (including lipopolysaccharides, silica beads, parasites, phorbol myristate acetate, laminarian and yeast) can stimulate iNOS mediated NO production [9,20,21]. Several studies on bivalves have also highlighted the role of bacteria in stimulating NO production [10,15,22–24]. However, most of the researchers have widely investigated the involvement of iNOS in bivalve's immune functioning by targeting only nitrate or nitrite concentrations as NO derivatives, and very few have considered enzymatic as well as molecular approach including the immunolocalization of iNOS or identification of iNOS genes.

The short-neck bivalve *Paphia malabarica* (Chemnitz, 1782) is one of the most valued shellfish in India. During monsoon season they are exposed to the freshwater influx as well as untreated sewage loaded with microbial contaminants which can have a potential effect on bivalve's biology including their immune functioning [25,26]. In a recent study, Khandeparker et al. [25] witnessed a high abundance of *Vibrio* species in a monsoon-influenced tropical estuary where this bivalve species inhabits. Moreover, *Vibrio* species are autochthonous to estuaries among which *V. parahaemolyticus* and *V. cholerae* are the most important pathogens known for causing diseases in shellfish, shrimps as well as humans [27–29]. Collectively, these findings can be translated

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to determine the bacteria-induced modulation in bivalve's immune response which was targeted in this study.

In the present study, immunodetection of iNOS enzyme (using antibody N9657 monoclonal anti-nitric oxide synthase) in *P. malabarica* hemocytes was carried out. The dose-dependent effect of *V. parahaemolyticus* and *V. cholerae* on iNOS activity was also evaluated by quantifying hemocytic iNOS immunofluorescence. Furthermore, we assayed the bactericidal activity of iNOS. This paper presents a new approach for understanding the immune response of *P. malabarica* towards the bacterial threat.

2. Materials and methods

2.1. Experimental animal and bacterial culture

Clams (shell length: 3.5–4 cm; wet weight: 20–25 g) were hand-picked from their natural beds (15°49′80.8″N 73°81′79.8″E− Mandovi estuary, the central-west coast of India) during the month of October–November 2017. For minimizing transport-induced stress, clams were kept immersed in seawater (salinity of 35 PSU; collected from the sampling site) until further analyses.

For the bacterial culture of *V. parahaemolyticus* and *V. cholerae*, seawater collected from the sampling site was serially diluted and plated on freshly prepared Thiosulfate Citrate Bile Salt Sucrose Agar selective media (TCBS Hi-Media, Mumbai) for the growth of these bacteria [30]. Briefly, 10 ml of sampled seawater was serially diluted (3-fold) with filtered autoclaved seawater (FASW). Aliquots of diluted seawater (100 µl) were spread plated in triplicate on TCBS agar plates under surface-sterilized conditions followed by incubation at 37 °C for 24 h. The bacterial colonies of *V. parahaemolyticus* and *V. cholerae* grown on the TCBS agar were further confirmed using biochemical tests and MALDI-TOF MS biotyping method as described by Khandeparker et al. [30]. Post-incubation developed bacterial colonies (total viable counts) were suspended into FASW, and bacterial counts were determined using flow cytometer-FCM (FACS-VERSE, Becton Dickinson). For the dose-dependent experiment, the desired bacterial concentration was achieved by diluting with the FASW.

2.2. Hemolymph collection and immunodetection of inducible-NOS (iNOS) by immunofluorescence

Hemolymph (containing hemocyte cells) was aspirated (300 µl/clam) from the posterior adductor muscles of clams using a sterile needle (3 ml, 0.55 × 25 mm) and the samples were maintained on ice to avoid aggregation. Hemolymph was pooled (two clams/pool; 22×10^5 hemocytes/ml) for all the experimental treatments.

For iNOS detection, the immunolabeling protocol was adopted as described by Sahoo and Khandeparker with some modification [17]. All the steps were performed at room temperature. Briefly, 150 µl of pooled hemolymph was seeded on a poly-L-lysine (Sigma) coated glass coverslip placed in a disposable sterilized petri dish (35 MM). After the attachment of hemocytes to the coverslip (monolayer), fixation step was performed in 4% paraformaldehyde [dissolved in 5 mM phosphate buffer saline (PBS-Sigma) added with 2% NaCl; pH 7.4] for 30 min. The hemocyte monolayer was washed three times with PBS to remove excess fixative, and hemocytes were permeabilized for 30 min with 0.2% Triton X-100 (Sigma; prepared in PBS). After permeabilization, the monolayer was washed with PBS and treated with 5% bovine serum albumin blocking solution (BSA-Sigma; prepared in PBS) for 30 min to minimize any unspecific antiserum binding in hemocytes. Hemocytes were then incubated with the primary antibody N9657 monoclonal anti-nitric oxide synthase (inducible antibody produced in mouse, Sigma-Aldrich; 1:5000 dilution in PBS-BSA) for 90 min. After incubation, the monolayer was again washed three times with PBS-BSA for removal of excess antibody and incubated with goat anti-mouse IgG (H + L) cross-adsorbed secondary antibody (Alexa Fluor 488, 1:10000; Invitrogen) for 60 min. Finally, hemocyte monolayer was washed twice in PBS, and the coverslip was carefully mounted on a glass slide in PBS-glycerol. This protocol was repeated on three different pools of hemolymph samples. To analyze the inhibitory effect of S-methylisothiourea hemisulphate (SMIS) on iNOS activity as demonstrated by Sahoo and Khandeparker [17], hemolymph containing hemocytes was incubated with 10 µM SMIS (Sigma, prepared in FASW) for 180 min. Subsequently, hemocyte monolayers were prepared for immunodetection following the same protocol as mentioned above.

2.3. Quantification of iNOS immunofluorescence in hemocytes

For quantification of iNOS immunofluorescence, hemocyte monolayers (after performing the above steps) were observed under the Olympus BX51 light-fluorescent microscope equipped with an Olympus DP71 digital imaging system. Randomly ten microscopic fields per pool (5 hemocytes/field) were observed after excitation with U-MNIB2 blue filter (excitation-480/20; emission-510LP, 120 W Olympus mercury light source) and projection images of hemocyte immunofluorescence were obtained on Image-Pro (8bit depth monochrome scale) software 6.2. Only non-aggregated hemocytes were taken into consideration. Profiles of all projected hemocyte images were stored in TIFF format. Pixel intensity was set between 0 and 255, and average fluorescent intensity (AFI) for each hemocyte was measured using the Image-Pro software [31].

2.4. Bacterial dose-dependent iNOS activity induction

After obtaining the suspension culture of viable bacteria as described previously (Section 2.1.), three bacterial concentrations (Dose I: 4.4×10^6 cell/ml, Dose II: 2.2×10^7 cell/ml, Dose III: 4.4×10^7 cell/ml) of *V. parahaemolyticus* and *V. cholerae* were achieved separately by diluting with the FASW. Subsequently, hemolymph (150 µl pooled) was incubated with each bacterial concentration (150 µl) corresponding approximately to Dose I 1:2; Dose II 1:10 and, Dose III 1:20 hemocyte/bacteria proportion for 180 min. After bacteria-hemocyte incubation, a hemocyte monolayer from each treatment was seeded on a poly-L-lysine coated glass coverslip, and immunodetection, as well as quantification of iNOS was carried out as mentioned in the previous sections (Section 2.2. and 2.3). The experiment was repeated three times.

2.5. Bactericidal activity of iNOS

After immunodetection of SMIS inhibitory effect on iNOS activity, we performed two experimental treatments representing positive and negative controls for iNOS bactericidal activity. Treatment 1 (positive control): SMIS (10 µM prepared in FASW) + hemolymph + bacteria and, Treatment 2 (negative control): FASW + hemolymph + bacteria. *V. parahaemolyticus* and *V. cholerae* were incubated separately with pooled hemolymph (1:10 hemocyte-to-bacteria ratio) for 180 min at room temperature. For each bacteria, three hemolymph pools per treatment were analyzed. After the incubation, hemolymph aliquots (100 µl) were four-fold serially diluted, and spread plating was done on selective media as mentioned earlier (Section 2.1). Viable bacterial enumeration (colony forming unit-CFU/ml) was done representing culturable bacteria survived in presence/absence of iNOS activity.

Separate samples were also prepared to detect the effect of SMIS inhibitor on hemocytes mortality. Briefly, hemolymph was incubated with SMIS (10 µM) and FASW separately for 180 min at room temperature. Subsequently, propidium iodide (PI, Molecular Probes; 10 µg/ml) which is commonly used to detect dead cells was added to the samples, and after 30 min of dark incubation, hemocyte events were analyzed (on FCM PE-channel-575/26) to evaluate hemocyte mortality. The direct effect of SMIS inhibitor on bacterial viability was also carried out by plating the bacterial populations in the presence and absence of the inhibitor.

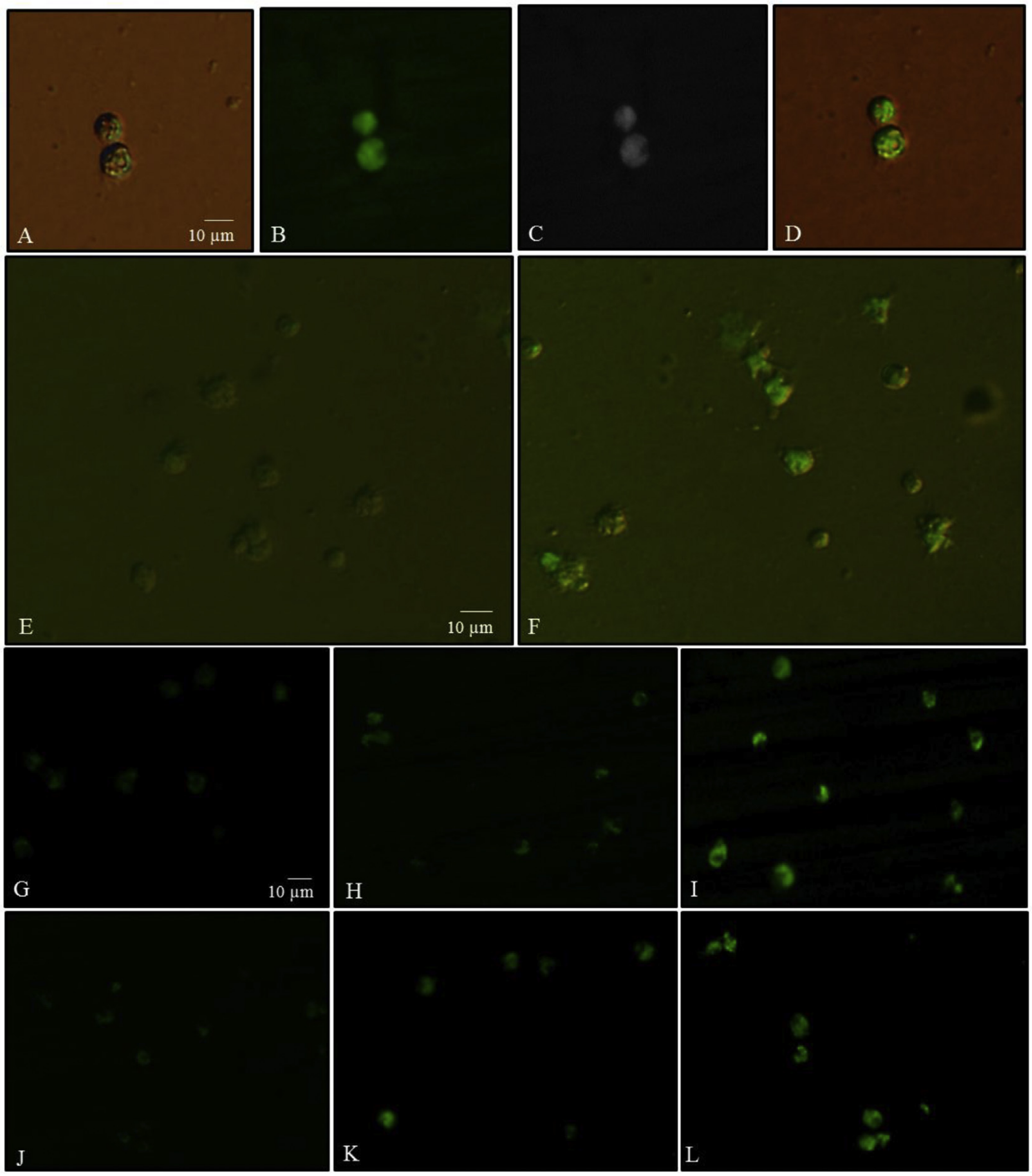

Fig. 1. Immunodetection of iNOS in *P. malabarica* hemocytes by immunofluorescence; the first lane represents- (A) DIC micrograph of the hemocytes, (B) Immunolabeled hemocytes, (C) Projection images of hemocyte immunofluorescence obtained on Image-Pro (8bit depth monochrome scale) and, (D) Composite image of hemocytes and immunofluorescence. The second lane shows immunolabeled hemocytes fluorescence in the presence (E) as well as absence (F) of SMIS inhibitor. The third and fourth lane represents the immunolabeled hemocytes after exposure to different *V. parahaemolyticus* (panel G-1:2, H-1:10 and, I-1:20 hemocyte-to-bacterial ratio) and *V. cholerae* (panel J-1:2, K-1:10 and, L-1:20 hemocyte-to-bacterial ratio) concentrations, respectively.

2.6. Statistical analysis

Before data analysis, all values were checked for normality and homogeneity. One-way ANOVA was performed using SPSS (16.0 version) to find significant relation among iNOS activity induced by different bacterial dosages and iNOS bactericidal effect. Significance was concluded at $p \leq 0.05$. Boxplots were generated using STATISTICA 8.0 software.

3. Results

3.1. Immunodetection of iNOS by immunofluorescence

The first set of analyses immunodetected iNOS activity in *P. malabarica* hemocytes using primary antibody N9657 monoclonal anti-nitric oxide synthase (Fig. 1A–D). The intensity of hemocytes immunolabeled with the primary antibody ranged from 30.48 to 72.93 average fluorescent intensity (AFI)/cell. As a negative control, hemocyte monolayers incubated in the absence of primary antibody showed no labeled fluorescence thus, confirming the specificity of iNOS immunolabeling in hemocytes. The absence of immunolabeled fluorescence in SMIS treated hemocytes revealed the inhibitory effect of SMIS on iNOS activity (Fig. 1E and F).

3.2. Bacterial dose-dependent iNOS activity induction

Hemocytes showed varied iNOS activity after exposing to different bacterial dosages of *V. parahaemolyticus* and *V. cholerae* (Dose I: 4.4×10^6 cell/ml, Dose II: 2.2×10^7 cell/ml, Dose III: 4.4×10^7 cell/ml). The quantitative analysis of immunolabeled hemocytes confirmed a significant dose-dependent bacterial effect on induction of iNOS activity.

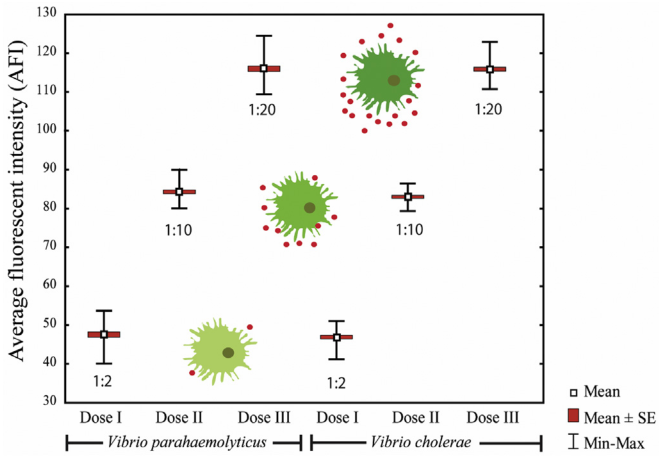

Fig. 2. The quantitative analysis of immunolabeled hemocytes showing the dose-dependent bacterial inducing effect on hemocyte iNOS activity (Dose I— 1:2, Dose II— 1:10 and, Dose III— 1:20 hemocyte-to-bacterial ratio).

In *V. parahaemolyticus*-hemocyte interaction, hemocytes were strongly immune-stained with increasing bacterial concentration (Fig. 1. G-I and Fig. 2). Hemocytes from dose I showed significantly lower immunolabeling [47.55 AFI/cell; with 95% confidence interval (CI) of 46.16–48.93] than the hemocytes from dose II [84.30 AFI/cell; 95% CI of 83.29–85.31]. In dose III, hemocytes were strongly immunolabeled [115.99 AFI/cell; 95% CI of 114.59–117.38], and the values were significantly higher compared to dose I and II.

Similar observations were also recorded in *V. cholerae*-hemocyte interaction (Fig. 1. J-L and Fig. 2). Hemocytes immunolabeling from dose III was significantly higher [115.86 AFI/cell; 95% CI of 114.82–116.89] than dose II [83.00 AFI/cell; 95% CI of 82.17–83.83]. Whereas, hemocytes from dose I were weakly immunolabeled [46.9 AFI/cell; 95% CI of 45.90–47.89].

3.3. Bactericidal activity of iNOS

The results of this experiment demonstrated the bactericidal role of iNOS (Fig. 3). In both bacterial pathogens, the presence of SMIS (inhibitor of iNOS) led to more bacterial survivability as compared with negative controlled samples. For *V. parahaemolyticus,* treatment 1 (with inhibitor) showed significantly higher bacterial survivability (average 1.08×10^6 CFU/ml; 95% CI of 1.03×10^6-1.13×10^6) than negative control i.e. treatment 2 (average 7.55×10^5 CFU/ml; 95% CI of 7.19×10^5-7.92×10^5). Similar results were also observed in the case of *V. cholerae* where the bacterial survival from treatment 1 (average 1.15×10^6 CFU/ml; 95% CI of 1.07×10^6-1.23×10^6) was significantly higher than treatment 2 (average 5.60×10^5 CFU/ml; 95% CI of 5.04×10^5-6.15×10^5).

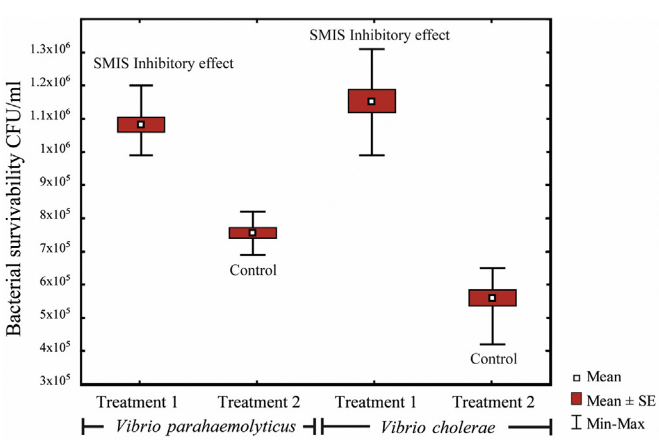

Fig. 3. The graph represents bacterial survivability to hemocytes iNOS activity, in the presence/absence of SMIS inhibitor (Treatment 1: SMIS + hemolymph + bacteria and, Treatment 2: FASW + hemolymph + bacteria).

The presence of SMIS inhibitor did not have any effect on hemocyte's mortality (Fig. 4). Moreover, we did not observe any significant difference between negative control and inhibitory treatment of SMIS on bacterial viability (data not shown).

4. Discussion

Several biochemical studies have established the role of iNOS-derived NO in invertebrate's immune functioning. However, the knowledge of *P. malabarica* immune functioning and their immune response to bacterial pathogens remains unclear and less studied. The results of our study demonstrated the presence of iNOS activity in *P. malabarica* hemocytes using antibody N9657 monoclonal anti-nitric oxide synthase. Also, the inhibitory effect of SMIS on iNOS activity confirmed the presence of iNOS in the hemocytes. Surprisingly, a considerable level of iNOS activity was witnessed in the hemocytes even without exogenous bacterial stimulation. This could be due to basal bacterial load in the hemolymph of *P. malabarica* while sampling which might have induced iNOS activity in the hemocytes. Similar kind of observations was also made by Yeh et al. [32] in crayfish hemocytes. There is also a high possibility that unstimulated iNOS activity could be attributed to iNOS continual responsiveness. In a study carried out on untreated *Mytilus edulis* immunocytes, Stefano et al. [33] demonstrated that basal release of NO happens in a cyclic continuous manner which can be regulated by exogenous stimulation. Clearly, in natural conditions, bivalves are relentlessly exposed to diversified bacterial load. Therefore, it can be conceivably assumed that there is a continuous expression of iNOS in bivalve's hemocyte revealing the perpetual role of iNOS in immune defense.

In bivalves, increased NO production by hemocytes have been observed after exposure to exogenous immune stimulants such as bacteria [23]. In the present work, we also observed an apparent bacterial dose-dependent excitatory iNOS activity after exposing to higher bacterial concentrations of *V. parahaemolyticus* as well as *V. cholerae.* Jeffroy and Paillard [10] made similar observations in *Ruditapes philippinarum* hemocytes where they found a 10-fold dose-dependent increase in the NO production after exposure to *V. tapetis*. Yeh et al. [32] also demonstrated an elevation in iNOS activity of crayfish hemocytes after lipopolysaccharide treatment. During post-monsoon season, bacterial abundance of *Vibrio* species increases owing to higher salinity as well as dissolved nutrients in a monsoon-influenced estuary [25]. These findings combined with our results suggest a definite probability of high bacterial mediated iNOS activity in wild population of *P. malabarica*, especially in a monsoon-influenced tropical estuary.

The antimicrobial activity of iNOS is attributed due to the iNOS-derived nitric oxide radical [34] as well as reactive nitrogen intermediates including NONOates, S-nitrosothiols, peroxynitrite, nitrite, and nitrous acid [35]. Their reactivity to structural elements, nucleic acids, metabolic enzymes as well as virulent molecules of infectious pathogens forms the basis for their antimicrobial as well as antiviral activity [34]. In the present study, the addition of SMIS which is a potent and selective iNOS inhibitor [36] yielded a conspicuous difference in the bacterial survivability. Higher *V. parahaemolyticus* and *V. cholerae survivability* after blocking iNOS activity clearly indicate the involvement of iNOS in bacterial killing. In addition, earlier studies have substantiated the role of iNOS mediated NO as well as its derivatives in promoting bacterial attachment to hemocytes [14,32,37]. In *R. philippinarum*, Jeffroy and Paillard [10] witnessed a correlation between declined NO production with inhibited iNOS activity, which also affected the hemocyte ability to form pseudopods. These studies suggest the role of iNOS or NO in alteration of bacteria-hemocyte adhesion,

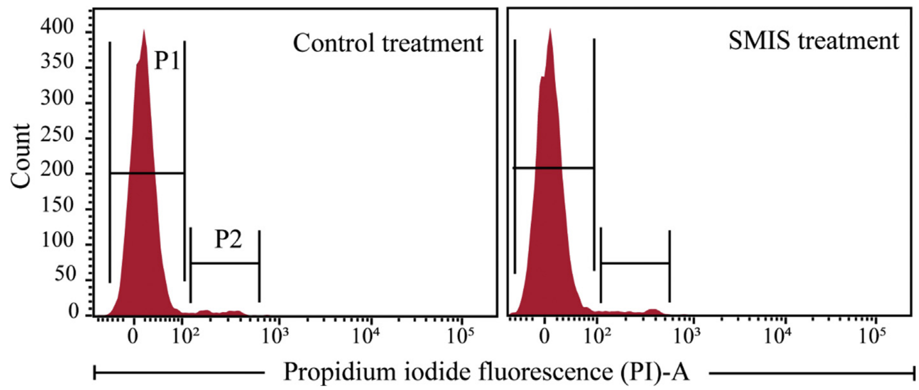

Fig. 4. The flow-cytometer histogram represents the effect of SMIS inhibitor on hemocytes mortality. P1 population representing healthy hemocytes (unstained by PI), whereas P2 population represents dead hemocytes (stained by PI).

possibly making the bacteria less accessible or vulnerable to hemocyte cytotoxicity (such as encapsulation, phagocytosis, reactive oxygen species, lysozyme and, esterase activity) which might explain the iNOS-inhibited high bacterial survivability in the present study.

P. malabarica is a commercially exploited bivalve species of India and understanding their immune-functioning will be very supportive especially in a monsoon-influenced tropical estuary where lower salinity can inflict immune-compromised state in this bivalve [26]. The immuno-depressed state could also make bivalves more susceptible to bacterial infection and disease development [38–46]. The presence of iNOS in *P. malabarica* hemocytes as is the case in macrophages of vertebrates highlights highly conserved immune effector mechanism against pathogens during evolution [47,48]. Also, the ubiquity and generality of iNOS/NO enable a very efficient and potential defensive response in the host [49]. Given this, we propose the necessity for understanding the iNOS expression in *P. malabarica*, which can be a potential immunomarker for monitoring the effect of different biotic as well as abiotic elicitors of the bivalve immune system.

5. Conclusion

The present study has gone some way towards understanding the involvement of iNOS in *P. malabarica* immune-functioning. Our work is the first immunological demonstration of iNOS existence in *P. malabarica* hemocytes using a monoclonal antibody against iNOS. Our results also highlighted the bacterial dose-dependent augmentation in iNOS activity and iNOS involvement in the bactericidal activity. Based on these findings, we can conclude the pivotal as well as perpetual role of iNOS in *P. malabarica* immuno-surveillance against bacterial pathogens. Therefore, further studies on the expression and regulation of iNOS enzyme are needed for a clear understanding of the pathogenesis in *P. malabarica*.

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