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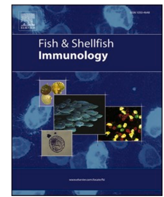

Short communication

Quantification of the inflammatory responses to pro-and anti-inflammatory agents in Manila clam, *Ruditapes philippinarum*

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ABSTRACT

Inflammation is a form of innate immune response of living organisms to harmful stimuli. In marine bivalves, inflammation is a common defense mechanism. Several studies have investigated the morphological features of inflammation in bivalves, such as hemocyte infiltration. However, the molecular and biochemical responses associated with inflammation in marine bivalves remain unexplored. Here, we investigated changes in nitric oxide (NO) levels, cyclooxygenase 2 (COX-2) activity, and allograft inflammatory factor-1 (*AIF-1*) gene expression levels in hemolymph samples collected from Manila clam (*Ruditapes philippinarum*) exposed to pro- and anti-inflammatory substances. These included the pro-inflammatory agent lipopolysaccharide (LPS), and the nonsteroidal anti-inflammatory drugs (NSAIDs) ibuprofen and diclofenac, all widely used in vertebrates. Our study showed that NO levels, COX-2 activity, and *AIF-1* expression increased in response to the treatments with LPS and decreased in response to the treatments with NSAIDs in a concentration-dependent manner. These results suggest that the mechanism of inflammatory responses in bivalves is very similar to that of vertebrates, and we propose that inflammatory responses can be quantified using these techniques and used to determine the physiological status of marine bivalves exposed to biotic or abiotic stresses.

1. Introduction

Inflammation refers to an innate immune response of a living organism against invading microorganisms or foreign substances and it facilitates the recovery of tissues damaged by infectious and non-infectious diseases [1–3]. In the early stage of inflammation, immune cells such as macrophages, mast cells, neutrophils, and eosinophils get activated; the inflammatory response is then further accelerated by the pro-inflammatory cytokines, growth factors, and enzymes produced by these cells to block injury or infection [4,5]. In invertebrates, cellular immunity is performed by hemocytes that circulate in the hemolymph system and mediate the inflammatory responses to both infectious and non-infectious stimuli [6–8].

In bivalves, inflammation is a common defense mechanism against pathogens or contaminants [9,10]. It involves several biochemical responses and is associated with morphological responses such as hemocytes infiltration or inflammatory encapsulation [11,12]. The biochemical responses include the secretion of various functional analogs of pro-inflammatory cytokines such as tumor necrosis factors, interleukins (ILs), and chemokines, and is associated with the release of other factors such as nitric oxide (NO), lysosomal enzymes, and bioactive peptides secreted by hemocytes [1]. Several studies have explored the role of NO and cyclooxygenase 2 (COX-2) in marine bivalves in relation to biotic and abiotic stressors [13–18]. Similarly, allograft inflammatory factor-1 (*AIF-1*), a gene that in vertebrates is stimulated by the T-cell-derived cytokine INF-γ, has been reported to be involved in various host immune responses against inflammatory stimuli [19–21]. For example, an elevation of *AIF-1* levels in response to bacterial infections has been reported in clams [22].

Histopathology is widely used in bivalves to evaluate the morphological features of inflammatory responses. Visual inspection of the morphological changes associated with hemocyte infiltration caused by

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microorganisms [23–25] and heavy metal exposure [26] has been carried out in many studies. For example, the intensity of inflammatory responses of the mussel *Mytilus galloprovincialis* was previously determined based on the intensity of hemocyte infiltration and on the aggregates found in histological sections [8].

Even though some studies have observed changes in the levels of pro-inflammatory cytokines following exposure to various stressors in bivalves [13–17], such changes in response to pro-inflammatory agents and anti-inflammatory drugs that are widely used in vertebrates have not been well studied in these animals.

In the present study, we investigated the inflammatory responses of hemocytes in Manila clam *Ruditapes philippinarum* in response to exposure to the pro-inflammatory agent lipopolysaccharide (LPS) and to the nonsteroidal anti-inflammatory drugs (NSAIDs) ibuprofen and diclofenac, and determined whether the mechanisms of the inflammatory response of bivalves are similar to those of vertebrates. Overall, we aimed to examine whether the quantification of specific markers of the inflammatory response will be a more useful and accurate method to determine the physiological state of marine bivalves than the visual inspection of the morphological features of inflammatory response.

2. Materials and methods

2.1. Manila clam

The Manila clam (*R. philippinarum*) individuals used here were collected from Uljin-gun, Gyeongsangbuk-do Province, Korea, and then stored and transported to the laboratory at 4 °C. In the laboratory, *R. philippinarum* were maintained in 30-L (n = 30) or 50-L (n = 50) water tanks at 20 °C and salinity of 30 psμ until use, and were fed approximately 2.14×10^8 cells of *Isochrysis* sp. and *Chlorella* sp. mixture (1:1 ratio) twice a day per tank. The average shell length and height of the *R. philippinarum* used were 41.0 ± 2.8 and 30.1 ± 1.4 mm, respectively. The first 30 clams were used for NO or COX-2 assays, and the other 50 clams were used for the evaluation of the expression of *AIF-1*.

2.2. Quantification of NO levels using the Griess assay

Approximately 1 mL hemolymph was extracted from the posterior adductor muscle of each clam using a 1-mL insulin syringe. Then hemolymph collected from 10 individuals was mixed in a 20-mL beaker kept on ice. An aliquot 900 μL was added into each of ten 1.5-mL tubes, which were divided into three groups, with four tubes in the first group and three tubes in the other two groups. In the first group, the different hemolymph tubes were supplied with LPS (Catalog #L4515, Sigma, USA) dissolved in filtered seawater at one of the following final concentrations: 0, 1, 10, or 100 μg/mL. In the second and third groups, 10 μg/mL LPS was added to all tubes with ibuprofen or diclofenac at one of the following concentrations: 1, 10, or 100 μg/mL. Then, each tube was incubated for 24 h at 26 °C, centrifuged post-incubation at 12,225×*g* for 10 min, and the resulting supernatant was then used for NO quantification using the Nitrate/Nitrite Colorimetric Assay Kit (Catalogue # 780001, Cayman Chemical, USA). The method involved mixing 80 μL plasma with 10 μL nitrate reductase enzyme cofactor mixture and 10 μL of nitrate reductase enzyme; this mixture was then dispensed to a 96-well plate in 6 replicates and incubated for 1 h at room temperature to convert total NO to nitrogen dioxide ions (NO_2^-). In the same plate, standard NO_2 (Catalog #S2252, Sigma) was added in the control wells at concentrations ranging from 0 to 50 μM to generate a standard curve. To all the wells, 50 μL of Griess reagents R1 (1% Sulfonamide) and R2 (0.1% N-(1-naphthyl) ethylenediamine) were added, respectively, and the mixture was incubated for 10 min at room temperature. After incubation, the absorbance was measured at 540 nm using a UV spectrophotometer (NanoQuant Infinite M200, Tecan, Austria). The NO concentrations were determined for every sample using the standard curve obtained for NO_2. The measured results were expressed as concentrations of NO_2^- ions.

2.3. Quantification of COX-2 activity using the COX assay

The effect of the various treatments on COX-2 activity was also evaluated. Sample treatments and sample numbers were as described in section 2-2. COX-2 activity was measured using the COX Activity Assay Kit (Cat # PKCA577-K549, PromoCell, Germany). Briefly, each hemolymph sample was centrifuged (6000×*g*, 4 °C, 5 min, Vision, Korea). The resulting pellet was washed with phosphate buffered saline solution (pH 7.4), resuspended in 200 μL cell lysis buffer, and centrifuged (12,000×*g*, 4 °C, 3 min). The supernatant was collected and used for measurements. After mixing 20 μL of the supernatant with 150 μL of the COX assay buffer, 4 μL of COX cofactor, 2 μL of COX probe, and SC-560 (COX-1 inhibitor) in a 96-well plate, this was incubated for 10 min at room temperature, followed by the addition 20 μl of arachidonic acid/NaOH solution (except in the background wells) and a further incubation for 30 min. The absorbance was measured using a fluorescence spectrophotometer (Spectramax Gemini EM, Molecular Device, USA) with excitation and emission wavelengths of 535 nm and 587 nm, respectively; measurements were acquired for 1 min for three replicates. The COX-2 activity was estimated as relative fluorescence units per minute (RFU/protein mg/min) following the manufacturer's instructions.

2.4. Quantification of AIF-1 expression

2.4.1. LPS exposure, RNA extraction, and cDNA synthesis

To determine the expression of *AIF-1* in Manila clam hemocytes, 10 μg LPS/200 μL of marine saline (MS) (12 mM $CaCl_2 \cdot 2H_2O$, 11 mM KCl, 26 mM $MgCl_2 \cdot 6H_2O$, 45 mM Tris-HCl, 6.45 mM NaCl, pH 7.4), or 10 μg LPS+10 μg ibuprofen/200 μL MS, were injected into the posterior adductor muscle of each specimen in the treatment groups, while 200 μl MS was injected into each specimen of the control group (five specimens per group). The specimens were maintained for 48 h at 20 °C in seawater. Hemolymph was then extracted from the posterior adductor muscle of each specimen and centrifuged (6000×*g*, 4 °C, 3 min) to separate the hemocytes. Tris reagent (Sigma, USA) was added to the collected hemocytes and stored at −80 °C. For RNA extraction, stored hemocytes were thawed at 4 °C and the cells were disrupted using a tissue homogenizer (Bioneer, Korea). RNA was isolated from these samples using the Trizol-chloroform method. The extracted total RNA was treated with DNase I (Takara, Japan) to remove any residual DNA, and 200 ng total RNA was used as template for cDNA synthesis following the protocol specified by the PrimeScript 1st strand cDNA Synthesis Kit (Takara, Japan). The reaction solution, containing 200 ng total RNA, 1 μL 50 mM oligo dT primer, and 1 μL dNTPs, was denatured for 5 min at 65 °C. After denaturation, 4 μL 5 × PrimseScript buffer, 20 units RNase inhibitor, and 100 units PrimeScript reverse transcriptase were added and the mixture was incubated for 1 h at 42 °C; the reaction was terminated by incubating the mixture for 15 min at 72 °C.

2.4.2. Real-time PCR

The expression level of *AIF-1* was identified using real-time PCR. The reaction mixture was prepared by mixing 5 μL template DNA, 10 pmol primer and probe set, and Dual-star qPCR preMix kit (Bioneer, Korea) in a total volume of 20 μL. The primer pairs used for *AIF-1* and *β-actin* amplification were those used in Refs. [22,27], respectively. Here, the *AIF-1* probe was 5′-CGAAGAGGAAATTGAGCCTGCACG-3′ (5′FAM-3′BHQ-1), as reported for the *AIF*-1 mRNA sequence (NCBI Accession number: GQ384410). PCR amplification was performed using Exicycler™ 96 Real-Time Quantitative Thermal Block (Bioneer, Korea). The reaction profile was as follows: 5 min pre-denaturation at 94 °C followed by 40 cycles of 30 s at 94 °C for denaturation, 30 s at 50 °C for primer annealing, and 30 s at 72 °C for extension. The expression level of *AIF-1* in each group was quantified as its copy numbers normalized against the cycle threshold value of *β-actin*.

2.5. Statistical analyses

Statistical analyses were performed using SPSS 12.0 (IBM, USA). One-way analysis of variance (ANOVA) was used to compare groups, and Duncan's multiple range test was used to confirm significant differences between mean values. Differences were considered significant at $p < 0.05$.

3. Results

3.1. Changes in NO levels in response to the different treatments

The changes in NO levels in clam hemolymph in response to exposure to LPS, ibuprofen, and diclofenac were determined using the Griess assay. Overall, NO concentration in hemolymph increased with increasing concentrations of LPS (Fig. 1), reaching 5.13 ± 0.40 (Mean ± SD, standard deviation), 6.57 ± 0.44, and 7.77 ± 0.08 μM in samples exposed to 1, 10, and 100 μg/mL LPS, respectively. Hemolymph that was not exposed to LPS showed only 3.26 ± 0.41 μM NO ($p < 0.05$; Fig. 1). Hemolymph samples in which the inflammatory response was induced using 10 μg/mL LPS showed that the NO concentration decreased when ibuprofen was added to the samples, and this decrease occurred in a concentration-dependent manner. NO concentrations of 6.41 ± 0.42, 4.93 ± 0.59, and 3.58 ± 0.23 μM were obtained following exposure to ibuprofen at concentrations of 1, 10, and 100 μg/mL, respectively ($p < 0.05$; Fig. 1). Similarly, the addition of diclofenac to samples activated with 10 μg/mL LPS also resulted in a dose-dependent decrease in the NO concentration, with levels reaching 5.81 ± 0.28, 4.98 ± 0.12, and 4.12 ± 0.31 μM in samples exposed to 1, 10, and 100 μg/mL, respectively ($p < 0.05$; Fig. 1).

3.2. Changes in COX-2 activity in response to the different treatments

Results showed that mean COX-2 activity was 0.084 ± 0.009, 0.089 ± 0.014, 0.103 ± 0.013, and 0.159 ± 0.018 RFU/protein mg/min in response to LPS addition at concentrations of 0, 1, 10, and 100 μg/mL, respectively. The difference in COX-2 activity between the untreated samples and those treated with LPS was demonstrated by a gradient increase; however, no statistically significant differences were observed between controls and the samples treated with the two lowest LPS concentrations ($p > 0.05$); however, the difference was statistically significant for samples treated with 100 μg/mL LPS ($p < 0.05$; Fig. 2).

Further, we investigated the effects of the NSAIDs ibuprofen and diclofenac in hemolymph samples treated with 10 μg/mL of LPS. COX-2 activity decreased with increasing concentrations of ibuprofen; mean COX-2 activity was 0.103 ± 0.013, 0.106 ± 0.027, 0.078 ± 0.005, and 0.078 ± 0.002 RFU/protein mg/min in response to treatment with 0, 1, 10, and 100 μg/mL concentrations of ibuprofen, respectively. This decrease was statistically significant ($p > 0.05$) for treatment with 10 and 100 μg/mL concentrations. Similarly, COX-2 activity also decreased significantly upon treatment of the samples with 10 and 100 μg/mL concentrations of diclofenac ($p < 0.05$; Fig. 2). No further effects were found when ibuprofen and diclofenac concentrations increased from 10 to 100 μg/mL.

3.3. Regulation of AIF-1 in response to LPS and ibuprofen

A significant three-fold increase in relation to controls was found for *AIF-1* expression in hemocytes exposed to LPS for 48 h. However, the injection of ibuprofen in conjunction with LPS caused a significant reduction of *AIF-1* expression (p < 0.05; Fig. 3).

We further verified the homology between the sequence of *AIF*-1 mRNA obtained from Manila clam individuals used in the present study and previously reported sequences, and a 100% homology to *AIF*-1 mRNA obtained from Chinese Manila clam (*R. philippinarum*) (Accession number: GQ384410) was found.

4. Discussion

In the present study, we investigated changes in NO levels, COX-2 activity, and *AIF-1* expression levels in hemolymph samples isolated from Manila clam in response to treatments with a pro-inflammatory agent (LPS) and two NSAIDs (ibuprofen and diclofenac), all of which being widely used in vertebrates. Results showed changes in all measured parameters in response to the treatments, and these changes were observed to be dependent on the concentrations of the pro- and anti-inflammatory agents used. These results suggest that the mechanism of inflammatory response in Manila clam is very similar to that of

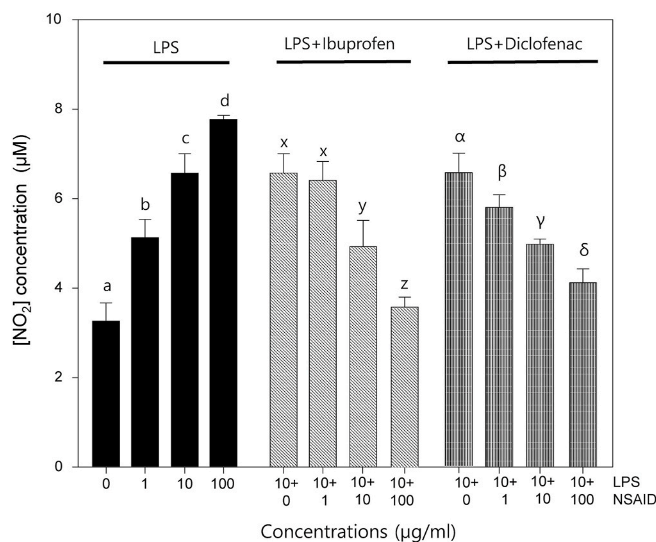

Fig. 1. NO levels (Mean ± SD) in the hemolymph of *R. philippinarum* treated with the pro-inflammatory agent lipopolysaccharide (LPS), and the nonsteroidal anti-inflammatory drugs (NSAIDs) ibuprofen and diclofenac. One-way ANOVA was performed in triplicate. Different letters (a, b, c, and d, or x, y, and z, or α, β, γ, and δ) indicate significantly different levels between treatments within each group (Duncan's post-hoc test, p < 0.05; n = 6 per group).

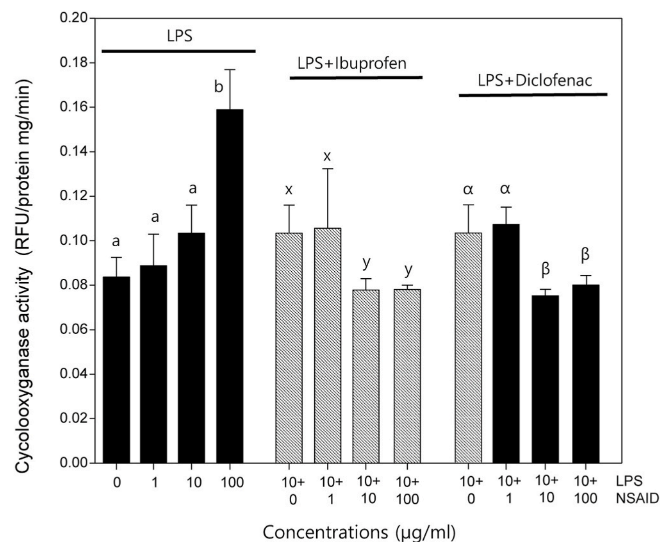

Fig. 2. COX-2 activity (Mean ± SD) in the hemolymph of *R. philippinarum* treated with the pro-inflammatory agent lipopolysaccharide (LPS), and the nonsteroidal anti-inflammatory drugs (NSAIDs) ibuprofen and diclofenac. One way ANOVA was performed in triplicate. Different letters (a and b, or x and y, or α and β) indicate significantly different levels between treatments within each group (Duncan's post-hoc test, p < 0.05; n = 3 per group).

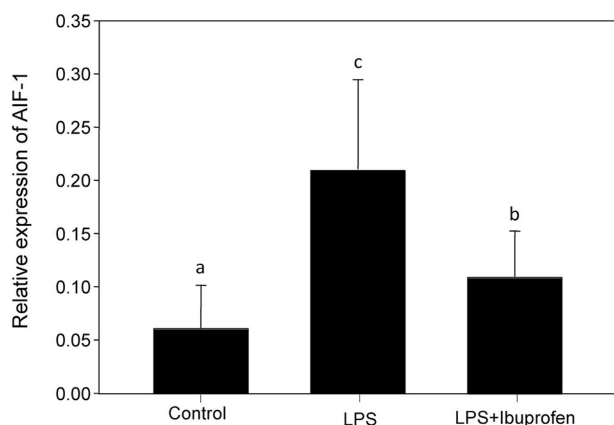

Fig. 3. *AIF-1* expression levels in relation to those of *β-actin*, the reference gene, in hemocytes of *R. philippinarum* treated with the pro-inflammatory agent lipopolysaccharide (LPS) and the nonsteroidal anti-inflammatory drug ibuprofen. Different letters indicate significantly different levels among groups (Duncan's post-hoc test, $p < 0.05$) ($n = 5$ per group) (Mean ± SD).

vertebrates.

To determine the inflammatory responses of Manila clam, we analyzed changes in the NO levels of hemolymph. According to Ref. [28], NO concentration increases when nitric oxide synthase (NOS) production in Manila clam hemolymph is activated by L-arginine, whereas NO concentration decreases significantly when NOS activity is inhibited by N^G-nitro-L-arginine methyl ester. Similar observations have been reported in carpet shell clam (*R. decussatus*) and eastern oyster (*Crassostrea virginica*) [13,29]. Further, a recent study has shown that bivalves have inducible NOS (iNOS) [30]. Altogether, these results strongly suggest that the changes observed in the NO levels of Manila clam are mediated by iNOS activity.

The Griess assay is widely used for the quantification of NO levels and it is used to quantify inflammation by measuring changes in NO concentration [31–35]. In the Griess assay, the NO produced by cells is oxidized to NO_2^- and nitrate ions (NO_3^-). The NO_3^- ions are then converted to NO_2^-, and the NO concentration is estimated by measuring NO_2^- concentration [36–38]. The 4,5-diaminofluorescein diacetate (DAF) assay directly measures the NO levels using a fluorescence probe, and the fluorescence of each individual hemocyte of Manila clam can be determined using a fluorescence microscope and image analysis [28]. The Griess assay is therefore more useful than the DAF assay when sample sizes are large, whereas for smaller sample sizes of marine bivalves the DAF assay is more suitable.

The COX assay is the most commonly used technique for the quantification of inflammation in vertebrates [39,40]. COX is also known as prostaglandin G/H synthase and it is involved in the conversion of arachidonic acid to various prostaglandins (PGs) such as PGG_2. The COX assay quantifies the COX enzyme activity based on the PGG2 levels in the sample as the PG produced from arachidonic acid can induce inflammation via conversion to various prostanoids such as prostacyclin [41,42]. Inflammation was successfully quantified in the hemolymph of the freshwater mussel *Elliptio complanata* exposed to sewage effluent by using the COX assay [14,43]. Our study also showed that inflammatory responses in Manila clam can be determined using the COX assay. COX-2 activity also showed changes in response to the treatments with pro- and anti-inflammatory agents in Manila clam, suggesting that the mechanism of inflammatory response in Manila clam might be very similar to that in vertebrates.

Our study also identified changes in the expression levels of *AIF-1* in response to pro- and anti-inflammatory agents in Manila clam. The *R. philippinarum AIF*-1 mRNA sequence was first obtained from specimens collected in China, in which the gene was highly expressed in the hemocytes, gills, hepatopancreas, and mantle [22]. This previous study also reported that *AIF-1* expression increased in hemocytes when the clam was exposed to *Vibrio anguillarum*, a known pathogen of shellfish, which also causes hemorrhagic septicemia in fish. Our observations agree with this previous study and further confirm that the evaluation of the inflammatory response in the hemolymph is more simple and practical than the time-consuming and qualitative histopathological observations.

It is well documented that NSAIDs such as ibuprofen and diclofenac are widely present in marine environments in concentrations ranging from few ng/L to several μg/L, and that these chemicals accumulate in bivalves at several ng/g dry weight [44,45]. Although several studies reported that NSAIDs induce various kinds of physiological disturbances in marine bivalves under laboratory conditions [45], the effects of NSAIDs on the inflammatory responses of marine bivalves have not been adequately addressed so far except for the effect of diclofenac on COX modulation in *M. galloprovincialis* via the inhibition of PG E2 synthesis [46]. However, in the present study, the inflammatory response of *R. philippinarum* was significantly regulated by both ibuprofen and diclofenac, suggesting that NSAIDs may regulate the inflammatory response of several, if not all, marine bivalves.

Overall, our study showed that inflammatory responses in the hemolymph of Manila clam are regulated by pro- and anti-inflammatory agents, suggesting that the pathogenesis of inflammation in marine bivalves likely occurs via a mechanism similar to that of vertebrates, as suggested by Ref. [47]. In vertebrates, inflammation is regarded as a physiological status of the animal [48,49]. Similarly, the quantification of inflammatory responses in marine bivalves can be used to determine their physiological status upon exposure to biotic or abiotic stresses. Our study has shown that inflammatory response markers, namely NO levels, COX-2 activity, and *AIF-1* expression, can be used to evaluate the inflammatory status of Manila clam. However, further studies are needed to probe how these mechanisms are orchestrated to respond to a broad range of mutualistic, commensal, or pathogenic waterborne microbes.

CRediT authorship contribution statement

Seung-Hyeon Kim: Methodology, Formal analysis, Writing – original draft. **Ki-Woong Nam:** Methodology, Formal analysis, Writing – original draft. **Bassem Allam:** Validation, Writing – review & editing. **Kwang-sik Choi:** Validation, Writing – review & editing. **Kwan-Ha Park:** Conceptualization, Data curation. **Kyung-Il Park:** Conceptualization, Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of competing interest

The authors declare no conflicts of interest. The funders had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript; or in the decision to publish the results.

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