A THESIS

FOR THE DEGREE OF MASTER OF SCIENCE

Flow cytometric characterization of the hemocytes of blood cockles

Anadara broughtonii* (Schrenck, 1867), *Anadara kagoshimensis

(Tocunaga, 1906), and *Tegillarca granosa* (Linnaeus, 1758)

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CONTENTS

국문 요약

해양 이매패류는 체액성 인자 (humoral factor)들과 혈구 (hemocyte)로 구성된 선천 면역 시스템 (innate immune system)을 보유하고 있으며, 혈구는 혈림프액 (hemolymph) 안에서 모든 조직세포 사이를 자유롭게 순환하며 생리적 안정상태를 유지하기 위해 다양한 반응을 보인다. 돌조개 과의 *Anadara*와 *Tegillarca* 종에 속하는 blood cockle들은 일반적인 이매패류들이 보유하고 있는 백혈구 외에도 헤모글로빈을 함유한 적혈구를 보유하고 있는 것이 특징이다. Blood cockle의 면역 시스템을 이해하기 위해서는 혈구 유형을 분류하고 세포별 면역 기능을 구명하는 것이 우선이나, 현재까지 blood cockle의 혈구세포 종류와 기능에 대한 연구는 미흡한 실정이다. 이 연구에서는 우리나라에 서식하는 대표적인 blood cockle인 피조개 (*Anadara boughtonii*), 새꼬막 (*A. kagoshimensis*), 꼬막 (*Tegillarca granosa*)을 대상으로 혈구세포의 형태와 기능을 유세포분석기 (flow cytometry)를 이용하여 비교 분석하였다. 세 종 모두 세포의 형태와 면역기능에 따라 erythrocytes type-I (erythrocytes-I), erythrocytes type-II (erythrocytes-II), granulocytes, hyalinocytes, 및 blast-like cells의 5가지 유형으로 동일하게 분류되었다. Erythrocytes는 혈림프액 내 혈구 세포 중 90% 이상을 차지하는 가장 많은 세포로 세포질 안에 많은 과립 (granules)을 가지고 있는 원반 형태였다. Erythrocytes는 세포의 크기와 면역 능력에 따라 erythrocytes-I와 erythrocytes-II의 2가지로 유형으로 분류되었다. Erythrocytes-II는 혈구세포 중 가장 크기가 컸으며, 세포질 내 많은 lysosome을 보유하고 혈구 세포들 중 가장 활발한 세포산화 능력 (oxidative capacity)을 보이는 등 erythrocytes의 세포성 면역능력을 보유하고 있는 것이 확인되었다. 그러나 erythrocytes-I와 erythrocytes-II는 식세포 작용 (phagocytosis)에는 관여를 하지 않았

다. Granulocytes는 긴 위족 (pseudopodia)과 세포질 내 많은 과립들이 있는 것이 특징이며, 혈구세포들 중 가장 많은 lysosome의 양과 가장 활발한 식세포 능력을 보였으며 erythrocytes-II 다음으로 활발한 세포산화 능력을 보였다. Hyalinocytes는 granulocytes 보다는 크기가 작은 구형의 세포로 세포질 내 과립이 없었다. Hyalinocytes는 세포질 내 일정 양의 lysosome을 보유하고 있으며 granulocytes 보다는 낮은 식세포 능력과 세포산화 능력이 확인되었다. Blast-like cells은 혈구세포들 중 가장 작은 크기와 얇은 세포질을 가진 세포로 식세포 능력과 세포산화 능력이 거의 없었다. 이번 연구를 통해 피조개, 새꼬막, 꼬막 3종의 혈구세포는 5가지 유형으로 분류되었으며, 그 중 erythrocytes와 granulocytes가 주된 면역세포인 것을 확인되었다.

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1. Introduction

Marine bivalves mollusks are widely used as sentinel species in coastal ecological monitoring because of its characteristics, as they are ubiquitous faunas, able to provide response spectrum to environmental stress, live a sedentary life in the habitat that may explain the spatial and temporal changes (Breitwieser et al., 2016; Goldberg, 1986; Grosell and Walsh, 2006). The direct and indirect stresses caused by environmental disturbance and pathogenic infection on organisms start at the cellular level and cascade up to the individual organism and population levels by altering the metabolic rates, survival, and other life-history traits (Fräzle, 2006). In marine bivalves, hemocytes are cells freely circulating in the hemolymph and are involved in various physiological responses to environmental, toxic, and disease stresses (Cheng, 1981; Donaghy et al., 2009a; Hine, 1999). Functional responses of hemocytes have often been used as a proxy for the physiological status of bivalves and, therefore, of the environmental quality of their habitat (Auffret, 2005; Fisher, 1988; Pipe and Coles, 1995; Renault, 2015). Numerous studies have reported the modification of hemocyte functions under stressful conditions. For instance, extreme temperature and salinity induced the alteration of hemocyte immune functions in marine bivalves (Chen et al., 2007; Fisher et al., 1987; Hégaret et al., 2003a, b; Liu et al., 2004; Matozzo et al., 2007; Monari et al., 2007; Yu et al., 2009). Bivalves exposed to a high level of heavy metals including mercury, zinc, cadmium, and copper exhibit decreased phagocytosis capacity of hemocytes (Brousseau et al., 2000; Fournier et al., 2001; Matozzo et al., 2001; Pipe, 1999). Exposure of marine bivalves to the persistent organic pollutant (POPs) including xenobiotics (Brousseau et al., 2000), polychlorinated biphenyl (PCBs; Liu et al., 2009), tributyltin (TBTs; Fisher et al., 1990), and polycyclic aromatic hydrocarbons (PAHs; Donaghy et al., 2010a, 2016; Dyrynda et al., 2000; Hong et al., 2016) caused sub-lethal effects on the hemocyte immune system.

Anadara and *Tegillarca* species of Arcidae, the blood cockles, are considered to be good sentinel species in monitoring coastal pollution and ecosystem health because they are

distributed widely in the subsurface of intertidal mudflats (Mirsadeghu et al., 2013; Sany et al., 2014). To understand the cellular response in blood cockles, the characterization of hemocyte types and immunological functions is crucial. One of the unique features of blood cockles is the presence of red blood cells in the hemolymph in addition to white blood cells found in common marine bivalves, although hemocyte types and functions of the blood cockles remain unclear (Bao et al., 2011). The primary role associated with erythrocytes is respiratory gas exchange however other functions including interaction with the immune system have been attributed to these cells (Bao et al., 2011; Dang et al., 2013). For example, the mRNA repression of hemoglobin in *Tegillarca granosa* hemocytes was significantly up-regulated against bacterial infection and exposure to bacterial pathogenic factors (Bao et al., 2011). Dang et al. (2013) observed an increase in circulating erythrocytes number over double in *Anadara trapezia* infected with trematode compared to non-infected cockle.

Despite the ecological importance of blood cockles, comparatively few studies have been carried out on the types and functions of hemocytes. Based on cell morphological features from microcopy, three major cell types including erythrocytes, granulocytes, and hyalinocytes have been described in *A. ovalis* (Rodrick and Ulrich, 1984), *A. kagoshimensis* (Zhihong et al., 2003), and *T. granosa* (Su et al., 2017; Zhu et al., 2011). According to these studies, the granulocytes were characterized by long pseudopodia and noticeable granules in the cytoplasm, while hyalinocytes were rather spherical with no distinctive pseudopodia or granules. In the *Anadara* species, the presence of granulocytes is not always confirmed. Granulocytes could not discriminate from hemocyte in *A. broughtonii* (Zhou et al., 2017), *A. inaequivalvis* (Kolyuchkina and Ismailov, 2007; Kolyuchkina and Milijutin, 2013), *A. trapezia* (Dang et al., 2013), and *A. kagoshimensis* (Kladchenko et al., 2020). In addition to erythrocytes, leucocytes were names as amoebocyte (Dang et al., 2013; Kladchenko et al., 2020; Kolyuchkina and Ismailov, 2007; Kolyuchkina and Milijutin, 2013) or white cell (Zhou et al., 2017).

Identification and quantification of hemocyte populations based on microscopic observation can be led to numerous inconsistencies and misinterpretations, partially due to the subjectivity of the visual analysis (Donaghy et al., 2017). To understand hemocyte types and their involvement in cellular responses, it is necessary to determine their functional activities (Donaghy et al., 2017). For this reason, flow cytometry has successfully been applied in hemocyte characterization based on their morphology and functions from marine mollusks (Ashton-Alcox and Ford, 1998; Donaghy et al., 2009a; Goedken and DeGuise, 2004). To date, only two studies have applied flow cytometry to characterize hemocyte populations of blood cockles including *T. granosa* (Zhu et al., 2011) and *A. kagshimensis* (Kladchenko et al., 2020). However, these studies described only two hemocyte populations without functional characterization. Therefore, further investigations are necessary to understand the hemocyte populations of blood cockles and their respective functions. In the present study, we applied flow cytometry and light microscopy combined assays to characterize the types and cellular immune activities of the circulating hemocytes of blood cockles including *A. broughtonii*, *A. kagoshimensis*, and *T. granosa*.

Materials and Methods

2.1. Sampling effort

To characterize the hemocyte types and functions of blood cockles, in July 2018, *A. broughtonii* ranging 5.9-7.0 cm in shell length (i.e., length of the longest axis of the shell), was collected from an intertidal mudflat area in Gamak Bay, off the south coast of Korea (Fig. 1 and 2). At the same period, *A. kagoshimensis*, 3.2-3.7 cm shell length, and *T. granosa*, 3.4-4.1 cm shell length were harvested from an intertidal mudflat area at Yeoja Bay on the southern coast of Korea (Fig. 1 and 2). The harvested cockles were transported and placed in a tank with aerated seawater (water temperature of 20˚C and salinity of 33 PSU) over 48 h to minimize physiological stresses induced during sampling and transportation.
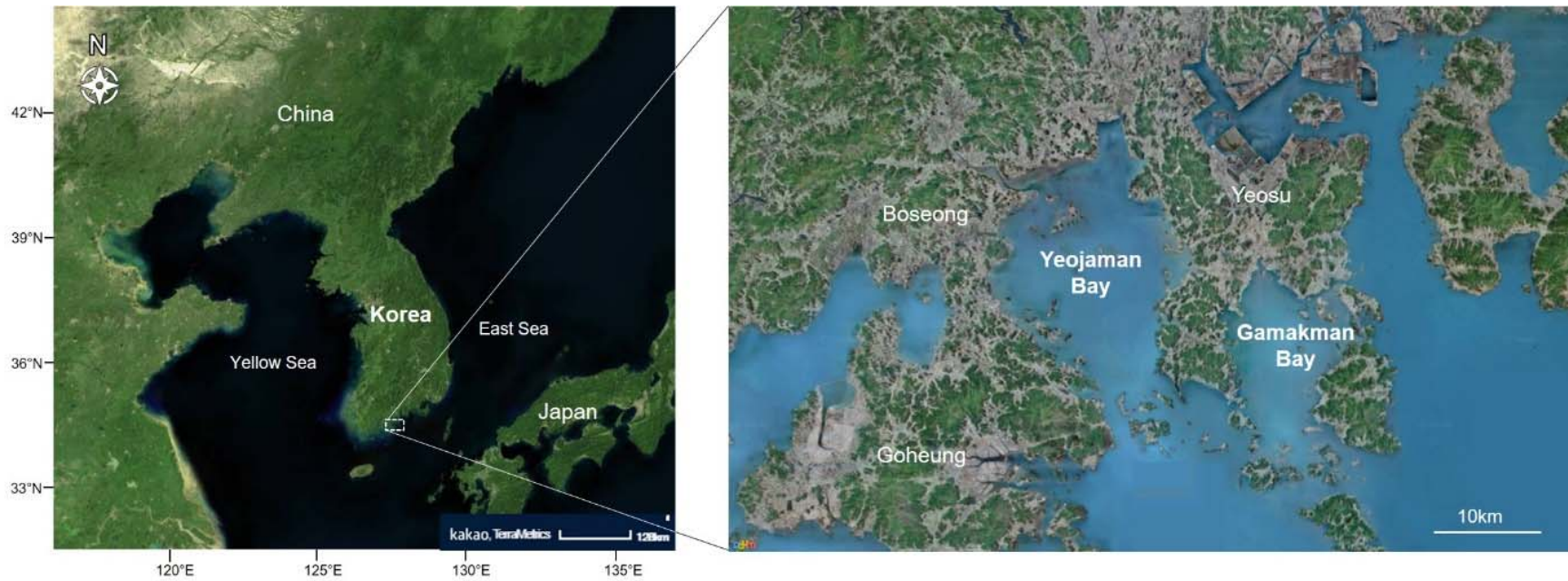
Fig. 1. Location map of the sampling site of *Anadara broughtonii*, *A. kagoshimensis,* and *Tegillarca granosa*. *A. kagoshimensis* and *T. granosa* were harvested from an intertidal mudflat area at Yeoja Bay on the southern coast of Korea. *A. broughtonii* was collected from an intertidal mudflat area in Gamak Bay, off the south coast of Korea.
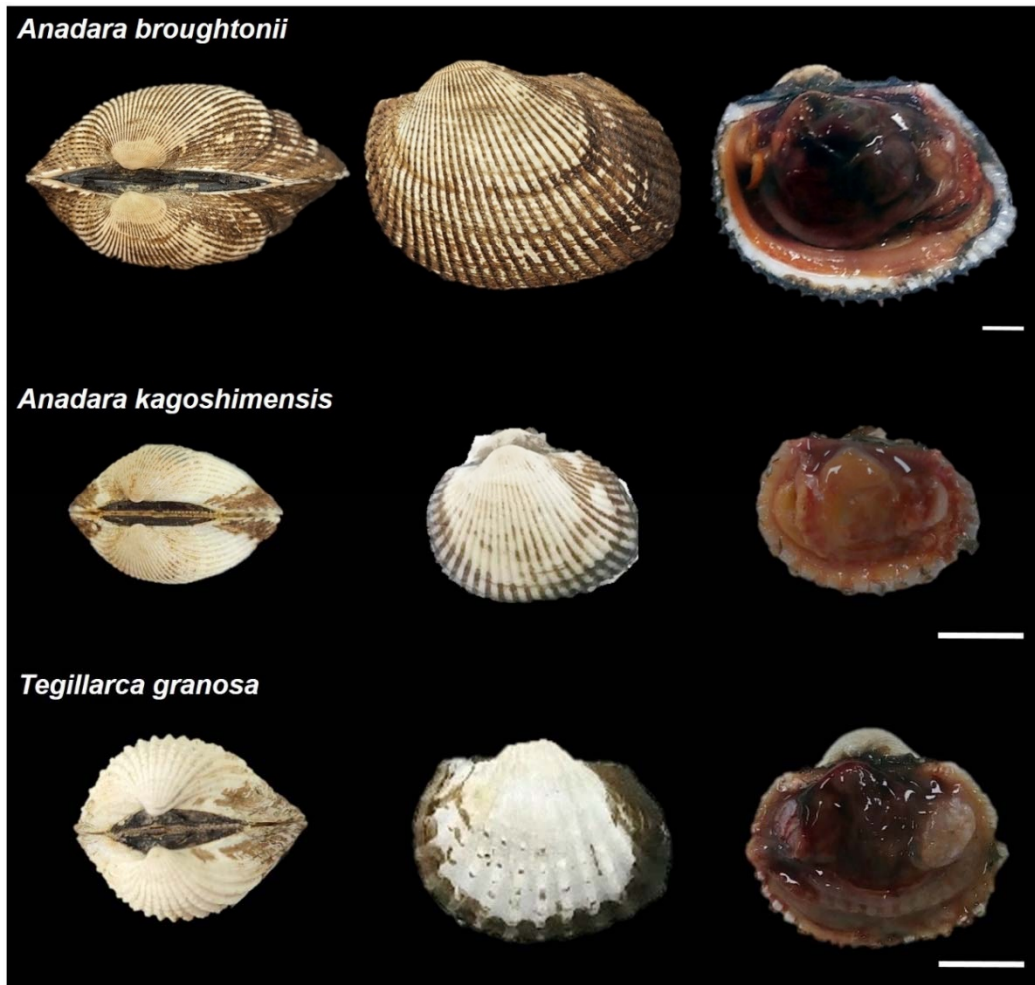
**Fig. 2.** Morphology of shell and somatic tissue of *Anadara broughtonii*, *A. kagoshimensis*, and *Tegillarca granosa* used in this study. Scale bar = 1cm.

2.2. Hemolymph collection

Using a 1-mL syringe equipped with a 22G × 1 1/4″ needle, hemolymph was withdrawn from the posterior adductor muscle. Collected hemolymph was immediately transferred into microtubes and kept on ice to minimize cell aggregation. Twenty individual cockles for each species were used to characterize hemocyte types and functions using flow cytometry, light microscopy, and scanning electron microscopy (SEM). All subsequent analyses were carried out individually.

2.3. Flow cytometric analyses

Hemocyte type and count, lysosome contents, and phagocytosis and oxidative capacities were analyzed using CytoFlex flow cytometry (Beckman Coulter, USA) equipped with two active lasers (488- and 639-nm) and four channels for fluorescence detection.

2.3.1. Hemocyte type and count

Hemocyte type and count were determined using SYBR green I (Invitrogen, USA), a green-fluorescent dye that binds to double-stranded DNA. Each 30 µL of hemolymph from *A. broughtonii* and *T. granosa* were fixed with 570 µL of a 3% formalin solution. For *A. kagashimensis*, a 50 µL of hemolymph was fixed with 150 µL of a 3% formalin solution. Fixed hemocytes were incubated with 1,000x SYBR green I (final concentration = 10x) for 30 min in the dark at room temperature. After selecting hemocytes only stained by SYBR green I, hemocyte types were discriminated based upon their relative cell size (forward scatter) and granularity (side scatter). THC was calculated as the number of cells per mL^{-1} of hemolymph.

2.3.2. Intracellular lysosome quantification

The lysosome content in hemocyte populations was determined using LysoTracker

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Red (Invitrogen, USA), a red-fluorescent dye that accumulates within lysosomal compartments in live cells. Each 30 µL of hemolymph from *A. broughtonii* and *T. granosa* were diluted with 570 µL of filtered seawater (FSW). For *A. kagashimensis*, a 50 µL of hemolymph was diluted with 150 µL of FSW. Diluted hemolymph was incubated with Lysotracker Red (final concentration = 1 mM) for 60 min in the dark at room temperature. The relative intracellular lysosomal quantity was expressed as the level of red fluorescence of the flow cytometer in arbitrary units (A.U.).

2.3.3. Phagocytosis capacity

Phagocytosis was induced using fluorescent beads (2.0 µm in diameter; Polysciences Inc., USA). Each 30 µL of hemolymph from *A. broughtonii* and *T. granosa* were diluted with 570 µL of FSW. For *A. kagashimensis*, a 50 µL of hemolymph was diluted with 150 µL of FSW. Diluted hemolymph was incubated with beads (final concentration at 2%). Phagocytosis capacity of hemocytes was induced for 10, 30, 60, 90, 120, and 180 min in the dark at room temperature. Phagocytosis capacity was expressed as the percentage of hemocytes that engulfed beads among all the hemocytes.

2.3.4. Oxidative capacity

Oxidative capacity was measured using 2'7'-dichlorodihydrofluorescein diacetate (H_2DCFDA; Invitrogen, USA). Intracellular H_2DCFDA is oxidized to a highly fluorescent 2'7'-dichlorofluorescein (DCF) by reactive oxygen species (ROS) and reactive nitrogen species (RNS) and quantified by the green fluorescence detector of the flow cytometer. Oxidative capacity of hemocytes was stimulated by the addition of phorbol 1,2-myristate 1,3-acetate (PMA; Sigma–Aldrich, USA). A 100 µL of hemolymph diluted in the same volume of FSSW was incubated with DCFH-DA (final concentration = 10 µM) for 10, 30, 60, 90, 120,

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and 180 min in the dark at room temperature. The oxidative capacity was expressed as fluorescence arbitrary units (A.U.) on the green fluorescence detector.

2.4. Microscopic observation

2.4.1. Light microscopy

Because the concentration of hemocytes in the hemolymph is too high for the analyses, the hemolymph was diluted with FSW. Each 30 μL of hemolymph from *A. broughtonii* and *T. granosa* were diluted 20 times with 570 μL of FSW. For *A. kagoshimensis*, a 50 μL of hemolymph was diluted 4 times with 150 μL of FSW. The diluted hemolymph was placed onto glass slides coated with poly-L-Lysine (MAS-11; Matsunami Glass Ind., Ltd., Japan), and hemocytes were allowed to adhere for 30 min in a humidity chamber at room temperature. The attached hemocytes were fixed with absolute methanol and then stained with Hemacolor reagent (Merck, Germany). The morphology of hemocytes was examined under a light microscope, and hemocyte images were captured using a digital camera. Cell and nucleus diameters of hemocytes were measured from the captured cell images using image-analysis software (Image-Pro Plus; Media Cybernetics, USA).

2.4.2. Scanning electron microscopy (SEM)

The hemolymph was attached to a glass slid coated with poly-L-Lysine (MAS-11; Matsunami Glass Ind., Ltd., Japan) for 60 min at room temperature. The adherent hemocyte was fixed with 2 % glutaraldehyde (Sigma-Aldrich, USA) for 60 min at room temperature. Fixed hemocytes were washed three times for 5 min in 1X Phosphate buffered saline (PBS; 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 2 mM $KHPO_4$, pH 6.8) and then dehydrated in graded series ethanol (50, 70, 90, 95, and 100%). Hemocytes were dried using a freeze dryer after substrate in graded series isoamyl acetate (30, 50, 70, and 100%). The slides were

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mounted on aluminum stubs and coated with gold using a sputter coater (Q150RS; Quorum Technologies, UK). Finally, morphological features of hemocytes were observed using a scanning electron microscope (MIRA3; TESCAN, Czech Republic).

2.5. Statistics

One-way analysis of variance (ANOVA) followed by Duncan's range test was conducted to compare the hemocyte parameters among three species using the Statistical Package for the Social Sciences (SPSS, IBM, USA). The percentage data were transformed as arcsine of the square root before ANOVA. The difference was considered statistically significant for $P < 0.05$. For the ANOVA, THC was log-transformed, while the percentage of hemocyte mortality and phagocytosis capacity were transformed as the arcsine of the square root.

3. Results

3.1. Hemocyte types

Based on the morphometric characteristics of hemocytes from flow cytometry and light microscopy, five types of cells could be discriminated identically in the three blood cockles: erythrocytes type-I (erythrocytes-I), erythrocytes type-II (erythrocytes-II), granulocytes, hyalinocytes, and blast-like cells (Fig. 3). Erythrocytes were round cells containing hemoglobin with granules in the cytoplasm. Erythrocytes were divided into two types (erythrocytes-I and erythrocytes-II) depending on the relative cell size. Granulocytes characterized by numerous granules in the cytoplasm and long pseudopodia on the cell surface. Contrary to granulocytes, hyalinocytes were comparatively small and round cells and exhibited no granules in the cytoplasm. Blast-like cells characterized by small round cells with a very thin cytoplasm.

SEM allowed the characterization of the ultrastructure of erythrocytes, granulocytes, and hyalinocytes (Fig. 4). Erythrocytes had a discoid or biconcave disc shape with very shallow centers (Fig. 4A-C). The granulocytes were characterized by their toughened surface membranes and the presence of many long pseudopodia (Fig. 4D-F). Compared to granulocytes, the hyalinocytes were comparatively smaller in size, smooth cell surface and spherical (Fig. 4G-I).
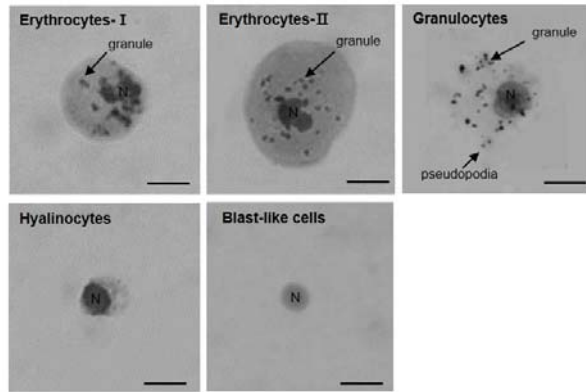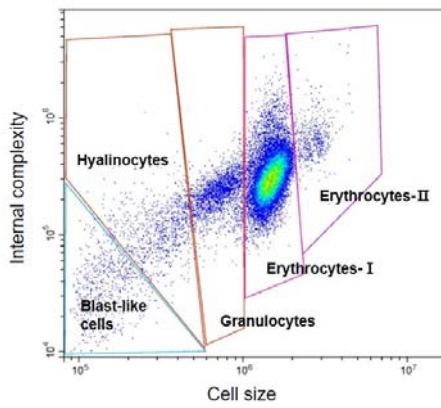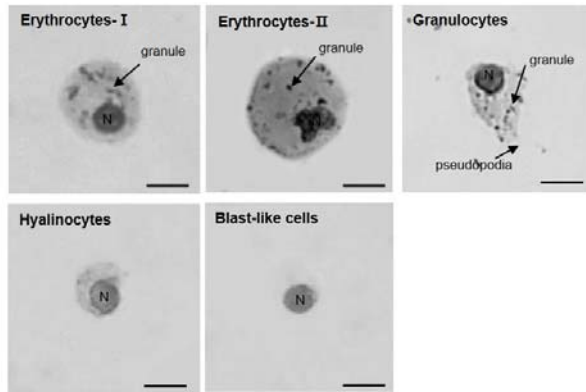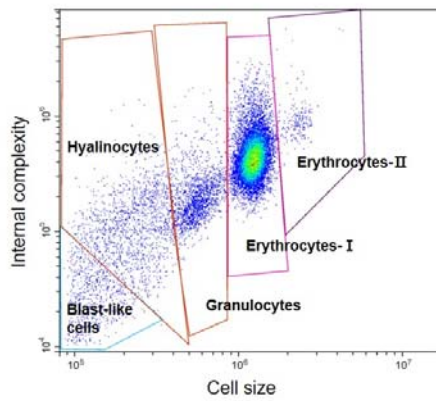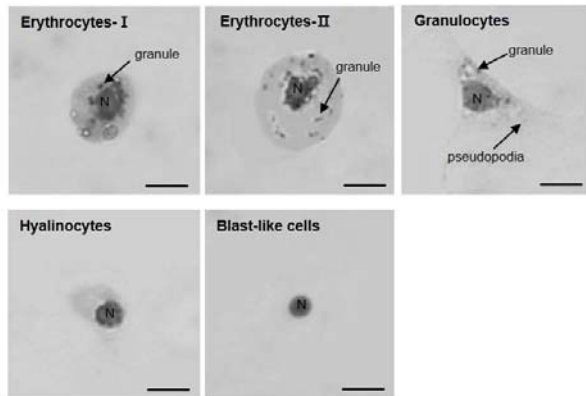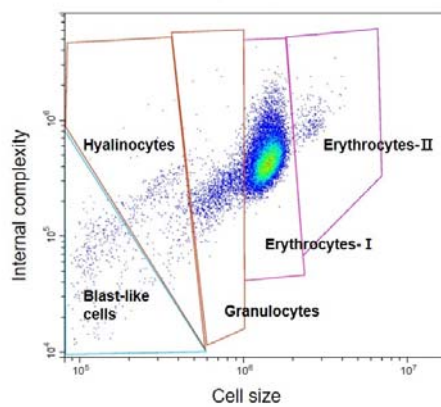
Fig. 3. Five hemocyte types from *Anadara broughtonii*, *A. kagoshimensis*, and *Tegillarca granosa* were determined using a combination of flow cytometry and light microscopy. Scale bar = 5 μm.
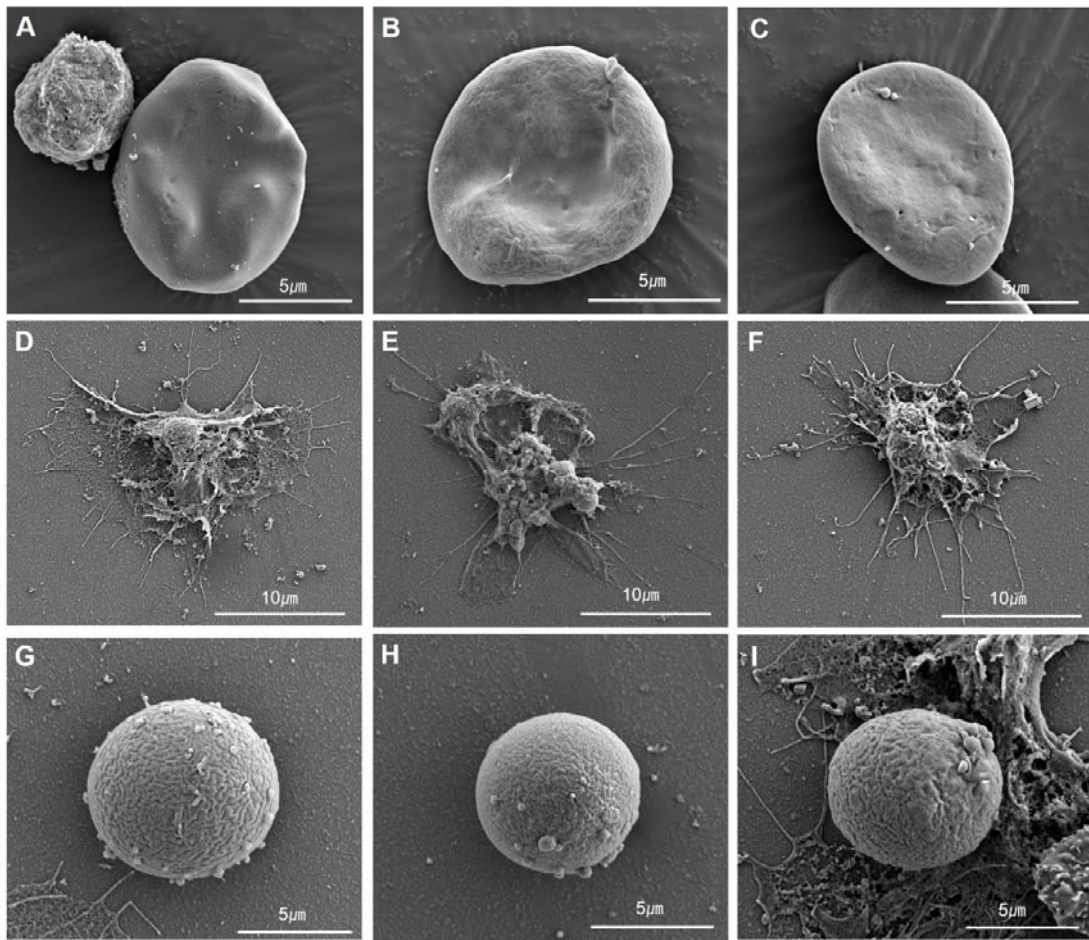
Fig 4. . Scanning electron micrograph of hemocyte sub-populations of *A. broughtonii*, *A. kagoshimensis,* and *T. granosa*. Erythrocytes in *A. broughtonii* (A), *A. kagoshimensis* (B), and *T. granosa* (C). Granulocytes in *A. broughtonii* (D), *A. kagoshimensis* (E), *T. granosa* (F). Hyalinocytes in *A. broughtonii* (G), *A. kagoshimensis* (H), *T. granosa* (I).

3.2. Morphology of the hemocytes

Table 1 summarizes the size of the cells (C) and nucleus (N) as well as the ratio of nucleus to cells (N/C) of the five types of hemocytes, as determined by light microscopy observation. In all species, erythrocytes-II were the largest cells (11.77-13.56 μm) in the circulating hemocytes followed by erythrocytes-I (8.97-11.38 μm), Granulocytes (8.16-8.74 μm), hyalinocytes (6.47-7.29 μm), and blast-like cells (3.15-3.82 μm). The sizes of the cell (3.15-3.82 μm) and nucleus (2.44-3.06 μm) of blast-like cells were significantly ($P<0.05$) smaller than those of other hemocyte types. Consequently, the N/C ratio of blast-like cells (0.79-0.80) was significantly ($P<0.05$) higher than the ratios of other hemocyte populations (0.29-0.58).

Among the three species, the erythrocytes-I of *A. broughtonii* was larger than those of *A. kagoshmensis* and *T. granosa* (Table 1). The erythrocytes-II of *A. kagoshimensis* was smaller than those of *A. broughtonii* and *T. granosa* (Table 1). The cell size of granulocytes, hylinocytes, and blast-like cells was not significantly different among the three species (Table 1).

3.3. THC and percentage of each hemocyte types

The mean of THC in *A. broughtonii* and *T. granosa* were 2.8 x 10^8 cells mL^{-1} and 2.3 x 10^8 cells mL^{-1}, respectively, without significant difference (Table 2). On the other hand, mean THC in *A. kagoshimensis* (0.2 x 10^8 cells mL^{-1}) was 10 times lower than those of *A. broughtonii* and *T. granosa*. (Table 2). The percentage of each hemocyte types in the hemolymph was similar between the three blood cockles. In all species, erythrocytes-I was the most abundant cell in the hemolymph accounting for 79-89 %, followed by granulocytes (4.3-8.4 %), hyalinocytes (0.9-4.8 %), erythrocytes-II (2.2-2.8 %), and blast-like cells (0.7-4.7 %).

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Table 1. Cell and nucleus diameters and nucleus/cell (N/C) ratio of *Anadara broughtonii*, *A. kagoshimensis,* and *Tegillarca granosa* stained with the Hemacolor staining solution. Values are presented as mean ± standard error. N: Number of analyzed cells.

	N	*A. broughtonii*					*A. kagoshimensis*					*T. granosa*				
		Erythrocytes-I	Erythrocytes-II	Granulocytes	Hyalinocytes	Blast-like cells	Erythrocytes-I	Erythrocytes-II	Granulocytes	Hyalinocytes	Blast-like cells	Erythrocytes-I	Erythrocytes-II	Granulocytes	Hyalinocytes	Blast-like cells
Cell (μm)	10	11.38 ± 0.76	13.56 ± 1.35	8.48 ± 1.26	7.29 ± 0.77	3.21 ± 0.46	9.00 ±0.65	11.77 ±1.42	8.74 ±1.32	7.29 ±0.77	3.82 ±0.67	8.97 ±0.65	12.81 ±1.64	8.16 ±1.02	6.47 ±1.17	3.15 ±0.60
Nucleus (μm)	10	3.67 ± 0.38	3.94 ± 0.57	4.24 ± 0.86	4.25 ± 0.72	2.50 ± 0.33	3.42 ±0.31	3.57 ±0.44	4.40 ±1.07	4.11 ±0.63	3.06 ±0.57	3.51 ±0.54	3.90 ±0.47	3.80 ±0.82	3.72 ±0.66	2.44 ±0.27
N/C ratio	10	0.32 ± 0.04	0.29 ± 0.05	0.50 ± 0.09	0.58 ± 0.10	0.79 ± 0.14	0.38 ±0.04	0.30 ±0.06	0.50 ±0.08	0.56 ±0.08	0.80 ±0.10	0.39 ±0.05	0.31 ±0.05	0.47 ±0.08	0.58 ±0.06	0.79 ±0.10

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Table 2. The total hemocyte count (THC) and percentage of each hemocyte types of *Anadara broughtonii*, *A. kagoshimensis*, and *Tegillarca granosa*.

	A. broughtonii	*A. kagoshimensis*	*T. granosa*
THC (cells mL^{-1})	$2.8 \times 10^8 \pm 2.3 \times 10^7$	$0.2 \times 10^8 \pm 0.4 \times 10^7$	$2.3 \times 10^8 \pm 1.8 \times 10^7$
Erythrocytes-I (%)	89.2 ± 1.1	79.5 ± 2.2	88.1 ± 0.8
Erythrocytes-II (%)	2.8 ± 0.3	2.2 ± 0.5	2.5 ± 0.2
Granulocytes (%)	4.3 ± 0.6	8.4 ± 1.1	7.6 ± 0.8
Hyalinocytes (%)	1.5 ± 0.3	4.8 ± 0.6	0.9 ± 0.1
Blast-like cells (%)	2.0 ± 0.5	4.7 ± 1.0	0.7 ± 0.7

3.4. Immunological activities

The cellular immune activities of blast-like cells were excluded from the result, due to their undetectable levels of phagocytosis and oxidative capacities.

3.4.1. Lysosomal contents

In all species, intracellular lysosomal content of granulocytes (0.9-1.2 x 10^5 A.U.) was the highest followed by erythrocytes-II (0.5-0.8 x 10^5 A.U.), hyalinocytes (0.3-0.7 x 10^5 A.U.), and erythrocytes-I (0.1-0.2 x 10^5 A.U.) in all species (Fig. 5). Among the three species, the lysosomal content of erythrocytes was significantly ($P<0.05$) higher in *A. broughtonii* (0.2 x 10^5 A.U. of erythrocytes-I and 0.7 x 10^5 A.U. of erythrocytes-II) than *T. granosa* (0.1 x 10^5 A.U. of erythrocytes-I and 0.5 x 10^5 A.U. of erythrocytes-II, Fig. 5). The lysosomal content of granulocytes was not significantly different among the three species. Hyalinocytes of *A. kagoshimensis* (0.7 x 10^5 A.U.) contained significantly ($P<0.05$) more lysosome than that of *A. broughtonii* (0.3 x 10^5 A.U.) and *T. granosa* (0.3 x 10^5 A.U., Fig. 5).
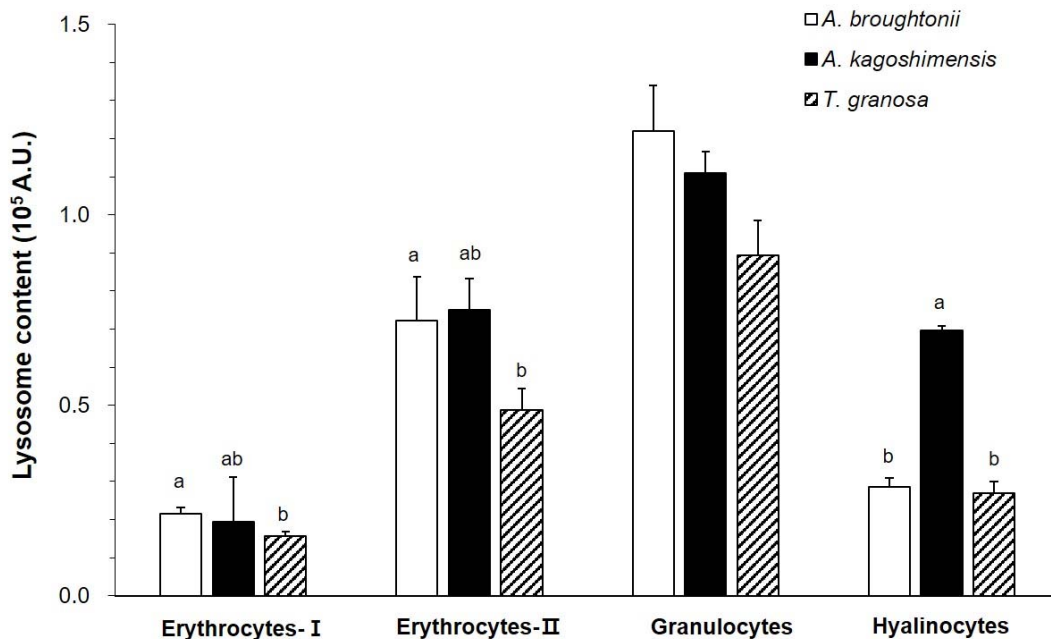
Fig. 5. Relative quantity of lysosomes in the hemocytes populations of *Anadara broughtonii*, *A. kagoshimensis,* and *Tegaillarca granosa*.

3.4.2. Phagocytosis capacity

Flow cytometry revealed that erythrocytes and blast-like cells did not show any phagocytosis activity, while the granulocytes and hyalinocytes appeared to be involved in phagocytosis. In all species, granulocytes (24.4-34.2%) were 2-4 times more active in phagocytosis than hyalinocytes (7.1-18.0%, Fig. 6). The development of the phagocytosis capacity of granulocytes of all species was similar from 10 min to 180 min, without significant difference. The phagocytosis capacities of granulocytes of *A. broughtonii*, *A. kagoshimenesis*, and *T. granosa* increased to 24.4, 33.3, and 34.2% at 180 min, respectively (Fig. 6). The phagocytosis capacity of hyalinocytes did not significantly increase over time with a low phagocytosis capacity of less than 20% in all species. The phagocytosis capacity of hyalinocytes of *A. kagoshimensis* (7.1%) was significantly ($P<0.05$) higher than that of *A. broughtonii* (18.0%) at 180 min (Fig. 6). The phagocytosis capacity of hyalinocytes of *T. granosa* increased to 12.2% at 180 min (Fig. 6).
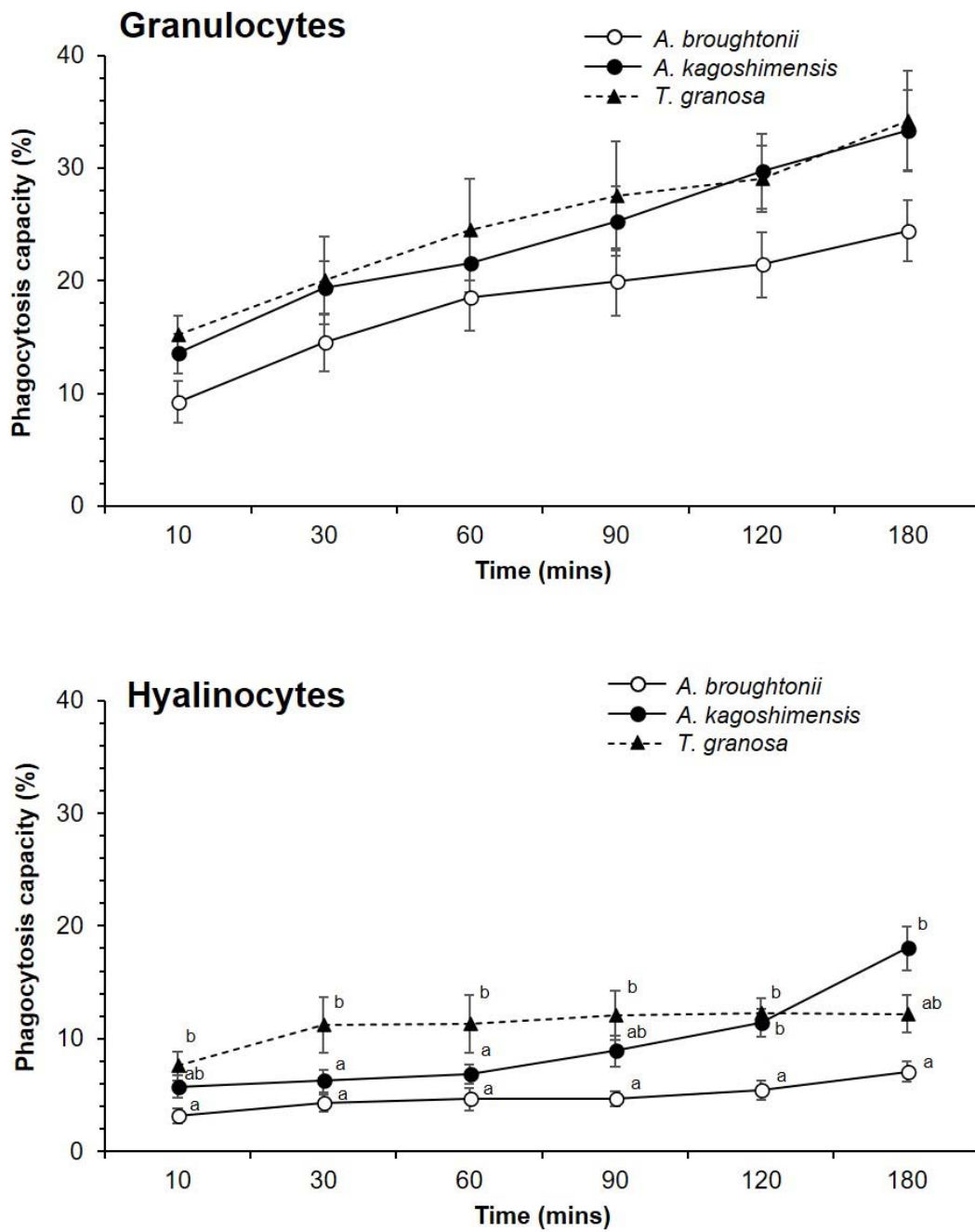
Fig. 6. Phagocytosis capacity of the granulocyte and hyalinocyte of *Anadara broughtonii*, *A. kagoshimensis*, and *Tegillarca granosa*.

3.4.3. Oxidative capacity

Flow cytometry demonstrated that erythrocytes, granulocytes, and hyalinocytes have the ability to produce ROS and RNS. In all species, Erythrocytes-II and granulocytes were major hemocytes engaged in the oxidative activity, although erythrocytes-I and hyalinocytes also exhibited a certain level of oxidative activity (Fig. 7). Erythrocytes-II was 2-3 times more active in oxidative capacity than granulocytes in *A. kagoshimensis* and *T. granosa* ($P<0.05$). In *A. broughtonii*, however, the oxidative capacity of erythrocytes-II was similar to that of granulocytes from 10 min to 180 min.

Among the three species, the oxidative capacity of each hemocyte types from 10 min to 180 min was quite different (Fig. 7). Interestingly, the relative of the amount of ROS and RNS occurring due to PMA stimulation was considerably higher (9- to 52-fold) in *T. granosa* hemocytes than in *A. broughtonii* and *A. kagoshimensis* hemocytes (Fig. 7). The oxidative capacities of erythrocytes-I of *A. broughtonii*, *A. kagoshimensis*, and *T. granosa* were increased to 0.03×10^6 A.U., 0.04×10^6 A.U., and 1.5×10^6 A.U. at 180 min, respectively. The oxidative capacities of erythrocytes-II of *A. broughtonii*, *A. kagoshimensis*, and *T. granosa* were increased to 0.09×10^6 A.U., 0.5×10^6 A.U., and 4.4×10^6 A.U. at 180 min, respectively. The oxidative capacities of granulocytes of *A. broughtonii*, *A. kagoshimensis*, and *T. granosa* were increased to 0.1×10^6 A.U., 0.2×10^6 A.U., and 1.8×10^6 A.U. at 180 min, respectively. Finally, the oxidative of hyalinocytes of *A. broughtonii*, *A. kagoshimensis*, and *T. granosa* were increased to 0.04×10^6 A.U., 0.07×10^6 A.U., and 0.7×10^6 A.U. at 180 min, respectively.
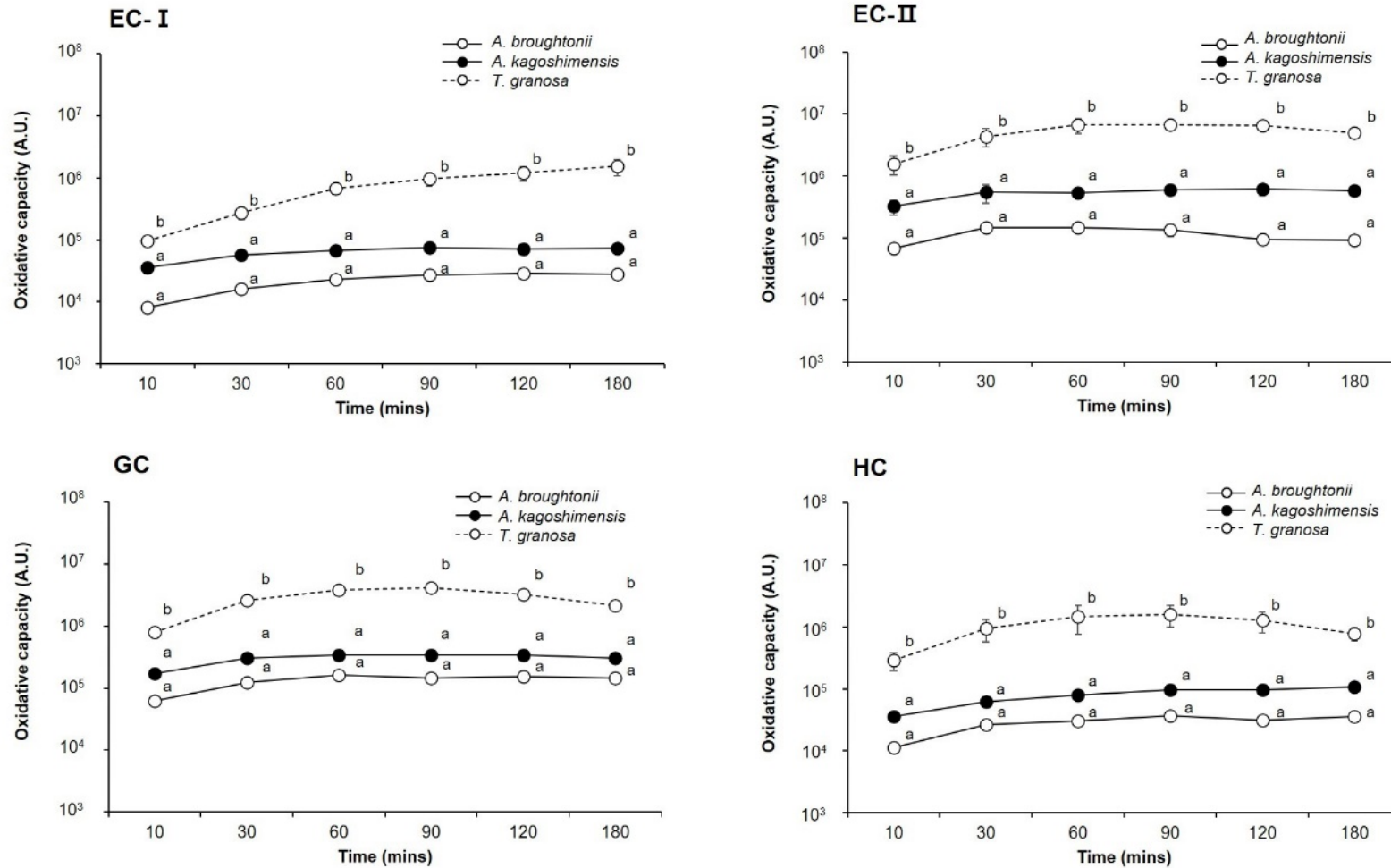
Fig. 7. Intracellular oxidative capacity of the different hemocyte populations of *Anadara broughtonii*, *A. kagoshimensis*, and *T. granosa*.

4. Discussion

Hemocyte activities of marine bivalves are highly sensitive to the variation in the environmental parameters including, water temperature, salinity, nutrients, and toxicants (see the review of Donaghy et al., 2009). Accordingly, the immune functions of marine bivalves have been used extensively as biomarkers to understand the impacts of environmental and biological changes. (Auffret, 2005; Cajaraville et al., 1996; Donaghy et al., 2009a; Pipe and Coles, 1995; Renault, 2015). For the last two decades, the flow cytometry has been adapted and used widely in marine bivalve immunology, as the flow cytometry allows fast, accurate, quantitative, and simultaneous analyses of the cell morphology and the functions involved in the defensive activities (Donaghy et al., 2009a; Nguyen and Alfaro, 2019). In particular, numerous studies applied the flow cytometry on understanding immune functions of oysters (Aladalieh et al., 2007; Donaghy et al., 2009b; Hégaret et al., 2003a, b; Hong et al., 2013, 2014; Xue et al., 2001), mussels (Andreyeva et al., 2019; Donaghy et al., 2011; Parrino et al., 2019; Rolton and Ragg, 2020; Yang et al., 2015), and clams (Allam et al., 2002; Cima et al., 2000; Hong et al., 2014, 2016; Jauzein et al., 2013). In contrast, a few studies used the flow cytometry in the immunology of the blood cockles (Dang et al., 2013; Kladchenko et al., 2020; Zhu et al., 2011).

Like oysters and mussels, blood cockles are considered to be a sentinel species in surveillance of the coastal ecosystem because they distribute widely in the subsurface of intertidal mudflats with a high density (Mirsadeghu et al., 2013; Sany et al., 2014). One of the unique features of blood cockles is the presence of erythrocytes in the hemolymph in addition to the leukocytes commonly founded in marine bivalves. While several studies differentiated the blood cockle erythrocytes based on their morphological features (Kolyuchkina and Ismailov, 2007; Kolyuchkina and Milijutin, 2013; Kladchenko et al., 2020; Rodrick and Ulrich, 1984; Su et al., 2017; Zhihong et al., 2003, Zhou et al., 2017; Zhu et al., 2011), few studies have investigated immunological functions of the erythrocytes. Using flow cytometry, Dang

et al. (2013) confirmed the oxidative capacity of the red blood cells in *A. trapezia* occurring in Queensland, Australia. In the present study, we successfully applied flow cytometry to discriminate different types of the hemocyte of *A. broughtonii*, *A. kagoshimensis*, and *T. granosa*. The flow cytometry revealed that all three blood cockle species have five types of hemocytes, erythrocytes-I, erythrocytes-II, granulocytes, hyalinocytes, and blast-like cells.

Although the presence of the red blood cells in blood cockles and their functions as suppling oxygen during anoxic condition has been well known, cellular defensive activities of the erythrocytes remain poorly understood. As the flow cytometry indicated, the erythrocytes-II and the granulocytes are mainly responsible for reactive oxygen species production, and the erythrocytes-II of the three blood cockle species show a higher level of lysosome and exhibiting higher oxidative capacity than the granulocytes. Several studies have reported that the granulocytes of marine bivalves such as oysters and clams or abalones are deeply engaged in the phagocytosis and production of the reactive oxygen species (Donaghy et al., 2009a, 2010b; Hong et al. 2013, 2019). Accordingly, it is believed that the blood cockles may have a superior cellular defensive capacity than other marine bivalves since the blood cockles have two types of hemocytes, the erythrocytes-II and the granulocytes which are actively engaged in production of the reactive oxygen species, which is considered to be a unique feature of the blood cockles.

The hemolymph of *A. broughtonii*, *A. kagoshimensis*, and *T. granosa*, also contained the white blood cells that are involved in various physiological functions such as nutrient digestion and transport, excretion, wound repair and the internal defense. From the three blood cockles, we identified two different types of hemocytes, mainly engaged in the cellular defense, the granulocytes, and the hyalinocytes. The scanning electron microscopy revealed that the granulocytes have many long pseudopodia and numerous granules in the cytoplasm. In contrast, the cytoplasmic granules and the pseudopodia are absent in the hyalinocytes. The flow cytometry indicated that the granulocytes are more actively involved in the cellular

defensive activities, including phagocytosis and production of reactive oxygen species.

Table 3 summarizes the types of hemocytes and THC of different blood cockle species previously reported. As was observed in this study, several studies have reported the presence of the hyalinocytes and the granulocytes in *T. granosa* (Liu et al., 2016; Shi et al., 2017, 2018; Su et al., 2017; Zhu et al., 2011) and *A. kagashimensis* (Zhihong et al., 2003, 2005). It is noticeable the granulocyte was not identified from the hemolymphs of *A. broughtonii* (Zhou et al., 2017), *A. kagoshimensis* (Kladchenko et al., 2020), and *A. trapezia* (Dang et al., 2013; Table 3), suggesting that types of the hemocyte in blood cockles are not fully understood yet, and highlighting the need for further studies of the mechanisms involved in hematopoiesis in blood cockles.

We also identified the smallest agranular cells with a small quantity of cytoplasm from the hemolymphs of three blood cockle species and named as blast-like cells. The flow cytometry demonstrated that the blast-like cells lacked phagocytosis activity and exhibited extremely low levels of oxidative activity, indicating that the blast-like cells may not be directly involved in the cellular immune response. Such morphological features and the absence of cellular defensive activities are a typical characteristic of undifferentiated blood cells. The blast-like cells were reported from several marine bivalves such as oysters (Aladaileh et al., 2007; Donaghy et al., 2009b; Hong et al., 2013), mussel (Yang et al., 2015), and clams (Cima et al., 2000), and the present study first reports the presence in the blood cockle hemolymphs.

The mean of THC of *A. broughtonii* (2.8 x 10^8 cells mL^{-1}) and *T. granosa* (2.3 x 10^8 cells mL^{-1}) was statistically higher than those of *A. kagoshimensis* (1.9 x 10^7 cells mL^{-1}), and we first report the THC of *A. kagoshimensis,* and *A. broughtonii* determined using flow cytometry. As summarized in Table 3, several studies determined THC of *T. granosa* in Yueqing Bay, Zhejiang province of China by counting the cells using a hemocyte counter under light microscope (Liu et al., 2016; Shi et al., 2017, 2018; Su et al., 2017; Zhu et al., 2011). The

number of total hemocytes of *T. granosa* (2.3×10^8 cells mL^{-1}) measured in this study using flow cytometry was somewhat comparable to that the numbers reported by Shi et al. (2016, 2018, as 1.3×10^8 cells mL^{-1} and 1.2×10^8 cells mL^{-1}, respectively). In contrast, the THC of *T. granosa* determined in this study is comparatively higher than the numbers from Zhou et al. (2017, 3.29×10^6 cells mL^{-1}), Liu et al. (2016, 7.61×10^6 cells mL^{-1}), and Su et al. (2017, 8.7×10^7 cells mL^{-1}). Such differences in the THC of *T. granosa* reporting from the present study and other studies could be in part, explained by different habitats of the *T. granosa*, and the methods used.

Lysosomes are organelles involved in digestion and decomposition of unnecessary substances through the release of various hydrolytic enzymes (Donaghy et al., 2009a, 2017). Lysosome of marine organisms plays a vital role in detoxification and defense. In the present study, we confirmed that the intracellular lysosomal content of the granulocytes is the highest, followed by erythrocytes-II, hyalinocytes, and erythrocytes-I in all three blood cockle species. Among the species, *T. granosa* showed the least amount of lysosome in the erythrocytes-I, erythrocytes-II, and the granulocytes. On the other hand, the lysosomal content of the hyalinocytes of *A. kagoshimensis* was significantly higher than *A. broughtonii* and *T. granosa*. Such differences in the amounts of lysosomes among different hemocyte types and the species might be responsible for the observed differences in the hemocyte capacities, such as the oxygen species production and the phagocytosis activities.

Phagocytosis is the most widely investigated cellular activity of marine bivalves hemocytes, which is deeply involved in the immune defense against invading foreign materials (Donaghy et al., 2009a). In this study, flow cytometry demonstrated that the granulocytes and the hyalinocytes had phagocytosis capacities against latex microbeads. Dang et al. (2013) also reported that the granulocytes and hyalinocytes of *A. trapezia* exhibit phagocytic activities, while the erythrocytes did not show phagocytosis capacity. In this study, the granulocytes of the three blood cockle species demonstrated 2 to 4 times higher phagocytosis than the

hyalinocytes. Phagocytosis capacity in the hemocytes of *A. kagoshimensis* (Zhihong et al., 2003, 2005) and *T. granosa* (Liu et al., 2016; Shi et al., 2017, 2018; Su et al., 2017; Zhu et al., 2011) were also confirmed by microscopy using yeast or zymosan as the phagocytosis inducers (Table 3). Zhihong et al. (2003, 2005), also reported phagocytosis capacity of *A. kagoshimensis* as 62%, which is significantly higher than the capacity determined in this study as 7.3 % (Table 3). According to the Table 3, the phagocytosis capacity of *T. granosa* ranges from 0.5% (Liu et al., 2016; Su et al., 2017) to 38% (Shi et al., 2018). In the present study, we determined the phagocytosis capacity of whole hemocytes of *T. granosa* as 2.1%. The phacocytosis capacities measured in this study is somewhat lower than the values reported from elsewhere, and the difference is believed to be derived from the substrate used in the induction of phagocytosis and subsequent method (i.e., microscopy and/or flow cytometry) to determine the capacity.

Numerous studies have reported the oxidative capacities of granulocytes and hyalinocytes in marine bivalves including oysters (Bachère et al., 1991; Donaghy et al., 2009b; Goedken and DeGuise, 2004; Hégaret et al., 2003b; Labreuche et al., 2006; Lambert et al., 2003; Larson et al., 1989;), mussels (Carballal et al., 1997; Ordás et al., 2000; Pipe, 1992), clams (da Silva et al., 2008; Delaporte et al., 2003; Hégaret et al., 2007; Hong et al., 2014), and scallops (Chen et al., 2007; Le Gall et al., 1991). In contrast, studies in the reactive oxygen species production in hemocyte blood cells are rare, as Dang et al. (2013) first reported the oxidative capacities of the erythrocytes and the leukocytes in *A. trapezia* in Australian water. In the present study, we detected PMA-stimulated oxidative activity in three blood cockle species, and erythrocytes-II and granulocytes were the main hemocytes engaged in the oxidative activity. The erythrocytes-II of *A. kagoshimensis* and *T. granosa* were more active in the production of reactive oxygen species than the granulocytes, whereas, the oxidative capacity of erythrocytes-II of *A. broughtonii* was similar to that of the granulocytes. Dang et al. (2013) also reported no significant difference in the oxidative capacity of *A. trapezia* of the erythrocytes and the leucocytes. Compared to *A. broughtonii* and *A. kagoshimensis* hemocytes,

T. granosa hemocytes produced a significantly higher (up to 52-fold) amount of reactive oxygen species and reactive nitrogen. Such differences in the oxidative capacity among different blood cockle species are believed to be species-specific, which could be controlled by a different pathway.

5. Conclusion

Using flow cytometry and microscopy, we identified five different types of hemocytes from three common blood cockles *A. broughtonii*, *A. kagoshimensis*, and *T. granosa*, as erythrocytes-I, erythrocytes-II, granulocytes, hyalinocytes, and blast-like cells. The erythrocytes were the most abundant (79.5-89.2% of the total circulating hemocytes) and the biggest hemocyte containing hemoglobin and numerous granules in the cytoplasm. The flow cytometry and microscopy indicated that there are two types of erythrocytes, which have different cell size and immunological activities. The granulocytes were characterized by numerous granules in the cytoplasm and many long pseudopodia on the cell surface. The hyalinocytes exhibited no granules in the cytoplasm, and they were comparatively smaller than the erythrocytes and the granulocytes. The blast-like cells were characterized by the smallest size and very thin cytoplasm. The granulocytes of the three species showed active cellular defensive activities, including the highest lysosome content, phagocytosis, and oxidative activities. The flow cytometry also indicated that the erythrocytes-II of the three species generate more ROS and RNS than those of the granulocytes. The blast-like cells did not show any phagocytosis and oxidative capacity, suggesting that this population is not directly involved in the cell-mediated immune activities.

Table. 3. Total hemocyte count (THC) and hemocyte types of blood cockles observed in the present and previous studies

Species	THC (cells mL^{-1})	Hemocyte types and proportion						Methods	References
		Erythrocytes	%	Granulocytes	%	Hyalinocytes	%		
A. broughtonii	2.8 x 10^8	Erythrocytes (2 types)	91	Granulocytes	4	Hyalinocytes	2	Light microscopy, SEM, Flow cytometry	Present study
A. kagoshimensis	1.9 x 10^7	Erythrocytes (2 types)	81	Granulocytes	8	Hyalinocytes	5	Light microscopy, SEM, Flow cytometry	Present study
T. granosa	2.3 x 10^8	Red granulocytes	91	Basophil granulocytes	8	Hyalinocytes	1	Light microscopy, SEM, Flow cytometry	Present study
A. broughtonii	-	Red Cells		White Cells		-		Light microscopy, Flow cytometry	Zhou et al., 2017
A. kagoshimensis	-	Erythrocytes		Granulocytes		Hyaline leucocytes		Light microscopy	Zhihong et al., 2003
A. kagoshimensis		Erythrocytes		Granulocytes		Hyalinocytes		Light microscopy	Zhihong et al., 2005
A. kagoshimensis		Erythrocytes		Amoebocytes		Intermediate type cells		Light microscopy, Flow cytometry	Kladchenko et al., 2020
T. granosa	3.29 x 10^6	Red granulocytes	89.67	Basophil granulocytes	7.05	Hyalinocytes	8.14	Light microscopy, SEM, TEM, Flow cytometry	Zhu et al., 2011
T. granosa	7.61 x 10^6	Red granulocytes	86	Basophil granulocytes	9	Hyalinocytes	1	Light microscopy	Liu et al., 2016
T. granosa	8.7 x 10^7	Red granulocytes	84	Basophil granulocytes	12	Hyalinocytes	3	Light microscopy	Su et al., 2017
T. granosa	1.3 x 10^8	Red granulocytes	84	Basophil granulocytes	12	Hyalinocytes	3	Light microscopy	Shi et al., 2017
T. granosa	1.2 x 10^8	Red granulocytes	80	Basophil granulocytes	15	Hyalinocytes	5	Light microscopy	Shi et al., 2018
A. ovalis	-	Erythrocytes	95.1	Granulocytes	1.6	Agranulocytes	3.3	Light microscopy, SEM, TEM	Rodrick and Ulrich, 1984
A. inaequivalvis	-	Red blood cells		Granulocytes		Agranulocytes		Light microscopy, TEM	Holden et al., 1994
A. inaequivalvis	2.1 x 10^7	Erythrocytes	50	Granular amoebocytes	25	Nongranular amoebocytes	25	Light microscopy	Kolyuchkina and Ismailov, 2007
A. inequivalvis	3.54 x 10^6	Red blood cells		Granulocytes (Neutrophils, eosinophil, basophil)		Agranulocytes (lymphocytes and monocytes)		Light microscopy	Suganthi et al., 2009
A. trapezia	5 x 10^5	Erythrocytes	79	Amebocytes	21	-		Light microscopy, Flow cytometry	Dang et al., 2013
A. antiquata	-	Red blood cell		Granulocytes		Agranulocytes		Light microscopy	Hameed et al., 2018

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감사의 글

주변의 많은 분들의 도움이 있었기에 석사학위과정을 마칠 수 있었습니다. 이 논문을 빌어 감사 드립니다.

우선 연구주제를 제공해 주시고, 이 논문이 완성되기까지 많은 관심과 도움을 주신 최광식 교수님께 진심으로 감사를 드립니다. 그리고 바쁜 시간을 내어 주셔서 제 졸업논문을 심사해 주신 박경일 교수님과 김기영 교수님께 감사를 드립니다. 또한 학위과정 동안 수업과 세미나를 통해 조언과 가르침을 주신 이제희 교수님, 이경준 교수님, 김기영 교수님께도 감사를 드립니다.

지난 2 년간 실험실 생활을 처음 해보면서 많은 것을 배우고, 매우 소중한 사람들을 얻었습니다. 저의 실험 처음 시작부터 글을 쓰는 것까지 모두 친절히 가르쳐 주시고, 하나하나 신경써주신 홍현기 박사님, 언제나 저를 많이 생각해 주시며, 항상 자신의 일처럼 걱정해 주시는 혜미언니, 많이 도와주시고, 걱정해 주시고, 챙겨 주시는 영관오빠, 보이는 곳에서나 안 보이는 곳에서나 항상 챙겨주는 종섭오빠, 제 장난을 다 받아주던 Subramaniam Thachaneshkanth, 실험 방법도 알려주고, 많은 도움을 준 Nainanayaka Pathirannehelage Harshani, 말없이 조용히 어느 곳에서나 도움을 주시는 진수오빠, 저에게 많이 맞추어 주시고 도움을 주시는 경태오빠, 자신의 일처럼 도와주는 Mai Nguyen Anh Thu 에게 깊은 감사를 드립니다.

저를 위해 타지로 이사하여 많은 지원과 응원을 아끼지 않는 가족들에게도 깊은 감사를 드립니다.

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