Anti-platelet activity of mineral-balanced deep sea water is mediated via the regulation of Akt and ERK pathway crosstalk

GI SUK NAM, KYU-SHIK LEE and KYUNG-SOO NAM

Department of Pharmacology and Intractable Disease Research Center, School of Medicine, Dongguk University, Gyeongju, Gyeongsangbuk-do 38066, Republic of Korea

Received July 9, 2019; Accepted November 14, 2019

DOI: 10.3892/ijmm.2019.4424

Abstract. Mineral-balanced deep sea water (MBDSW), an unlimited natural sea source, has been demonstrated to minimize the risk of developing cardiovascular diseases, such as obesity, hypertension, inflammation and hyperlipidemia. This study investigated the effects of MBDSW [magnesium (Mg):calcium (Ca) ratio, 3:1] on platelet activation. MBDSW significantly inhibited the collagen- and thrombin-induced platelet aggregation of human platelets. In collagen-induced platelets, MBDSW inhibited intracellular calcium mobilization, granule secretion [serotonin, adenosine triphosphate (ATP) and P-selectin expression] and thromboxane A_2 (TXA_2) production. Moreover, MBDSW markedly inhibited Akt and extracellular signal-regulated kinase (ERK) phosphorylation, but not that of c-Jun N-terminal kinase (JNK) and p38. Moreover, MBDSW phosphorylated inositol 1,4,5-triphosphate receptor (IP, R) and vasodilator-stimulated phosphoprotein (VASP), and it increased the cyclic adenosine monophosphate (cAMP) level in collagen-induced human platelets. Dipyridamole, a phosphodiesterase (PDE) inhibitor, significantly increased the cAMP level and regulated the Akt, ERK and VASP (Ser^{157}) levels in a manner similar to that of MBDSW. In addition, LY294002, an Akt inhibitor, inhibited the phosphorylation of ERK, and U0126, an ERK inhibitor, inhibited the phosphorylation of Akt. Taken together, the results of the present investigation suggest that the inhibitory effects of MBDSW on platelet aggregation may be associated with the cross-inhibition of Akt and ERK phosphorylation. These results strongly indicate that MBDSW may have preventive or therapeutic potential for platelet aggregation-mediated diseases, such as thrombosis, atherosclerosis and myocardial infarction.

Introduction

Platelets are non-nucleated cells that are produced in the cytoplasm of intramedullary megakaryocytes (1). These platelets circulate throughout the bloodstream, performing hemostasis and preventing bleeding in conjunction with coagulation factors. However, these platelet functions produce blood clots that potentially block arterial, microvascular and capillary blood flows, causing cardiovascular diseases (CVDs), including angina pectoris, myocardial infarction and atherosclerosis (2).

Collagen is a protein abundantly present in the extracellular matrix of the blood vessel wall, providing flexibility to withstand the outward forces exerted by arterial pressure (3). Under normal blood flow situations, collagen is not exposed to the lumen. However, when the collagen leaks into the lumen, due to damage or sclerosis of the blood vessel walls by CVD risk factors, glycoprotein (GP)-VI and GP-VI-V complex receptors on the surface of platelets bind to the collagen (4). Fc receptor-γ chain and Src family kinase associated with GP-VI lead to the phosphorylation and activation of phospholipase C_2, catalyzing the hydrolysis of phosphatidylinositol 4,5-bisphosphate to diacylglycerol and inositol trisphosphate (IP_3). In such instances, agonist-binding receptors induce granule release, integrin activation, thromboxane A_2 (TXA_2) production, and as a consequence, platelet aggregation (5,6).

Deep sea water (DSW) is an abundant sea resource located at depths as deep as 200 m below sea level. It contains high concentrations of minerals, such as magnesium (Mg), calcium (Ca), potassium (K) and sodium (Na), as well as several trace minerals. Currently, DSW is being developed in Korea, Japan and Taiwan for use in various applications in the aquaculture, agriculture and food industries (7,8). Although various industries are trying to adopt and use DSW, such as the food industry (e.g., mineral water, deep sea water salt, confectioneries, etc.), there is a lack of evidence that DSW has biologically beneficial functions. The biological effects of DSW on obesity, hypertension, inflammation, atopic eczema/dermatitis syndrome and cancer have been reported in several studies (7,9-12). Moreover, DSW has been shown to exert suppressive effects on hyperlipidemia and atherosclerosis in animals fed a high cholesterol diet (13-15). Previously, it was demonstrated that DSW inhibited doxorubicin-induced epithelial-mesenchymal transition by inhibiting the extracellular signal-activated protein kinase (ERK1/2), p38 and PI3K/Akt signaling.
pathways (15). Mitogen-activated protein kinases (MAPKs), such as the ERK, e-Jun N-terminal kinase (JNK) and p38, and Akt signaling pathways, play primary roles in the amplification of platelet aggregation, granule secretion and integrin αIIbβ3 activation induced by agonists (16-18). However, to the best of our knowledge, there is no study available to date describing a role of DSW in the inhibition of platelet activation, which is another cause of CVIs, including angina pectoris, myocardial infarction and atherosclerosis.

In this study, since the original DSW is high in Na and requires hardness control, experiments were performed using a mineral-balanced DSW (MBDSW). The anti-platelet activity of MBDSW was investigated using collagen- and thrombin-induced platelet aggregation. Furthermore, whether MBDSW inhibits granule secretion, platelet-activating molecules, integrin and the MAPK and Akt signaling pathways was also evaluated.

Materials and methods

Materials and reagents. Collagen and thrombin were purchased from the Chrono-Log Co. The thromboxane B2 (TXB2) ELISA kit, Cyclic AMP ELISA kit and dipyridamole were purchased from Cayman Chemical Co. The CytoTox 96® Non-Radioactive Cytotoxicity assay (cat. no. G1780) was purchased from Promega. The serotonin ELISA kit was purchased from Labor Diagnostika Nord GmbH & Corp. Fura-2/acetoxymethyl ester (AM) and Alexa Fluor 488-conjugated fibrinogen were purchased from Molecular Probes. APC anti-human CD62P (P-selectin, cat. no. 304910) antibody was purchased from BioLegend Inc. The adenosine triphosphate (ATP) assay kit was purchased from Biomek Laboratories. Protease inhibitor cocktail and phosphatase inhibitor cocktail were purchased from GenDEPOT, and antibodies against ERK1/2 (cat. no. 4695), phospho-ERK1/2 (Thr202/Tyr204; cat. no. 4370), p38 (cat. no. 8690), phospho-p38 (cat. no. 4511), JNK (cat. no. 9258), phospho-JNK (cat. no. 4668), Akt (cat. no. 4691), phospho-Akt (Ser473; cat. no. 9271), phospho-Akt (Thr308; cat. no. 2965), vasodilator-stimulated phosphoprotein (VASP; cat. no. 3112), phospho-VASP (Ser57; cat. no. 3111), phospho-inositol 1,4,5-trisphosphate receptor (IP3R; cat. no. 8548) and glyceraldehyde 3-phosphate dehydrogenase (GAPDH; cat. no. 5174) were purchased from Cell Signaling Technology. β-actin antibody (cat. no. sc-69879) was purchased from Santa Cruz Biotechnology. LY294002 and U0126 were purchased from GendEPOT, and antibodies against phospho-ERK1/2 (Thr202/Tyr204), phospho-p38 (Thr180/Tyr182), phospho-IP3R, phospho-ERK1/2, phospho-p38, phospho-JNK, phospho-Akt, β-actin, GAPDH, IP3R and phospho-IP3R were purchased from Cell Signaling Technology. Polyvinylidene fluoride (PVDF) membrane was purchased from PallLife Sciences.

Preparation of MBDSW. MBDSW was obtained from the Seawater Utilization Plant Research Center in the Research Institute of Ships and Ocean Engineering. DSW was pumped from a depth of 500 m in the East Sea of Korea. To remove salts and minerals, it underwent reverse osmosis filtration (sample LDH release×maximum LDH release).

Preparation of washed human platelets and measurement of platelet aggregation. Once the platelet plug has been formed by the activated platelets, the coagulation factors presented in plasma are activated in a sequence of events known as the ‘coagulation cascade’, which leads to the formation of fibrin from fibrinogen plasma protein (19). Therefore, this study evaded all response to any secondary hemostasis effects using the washed platelet aggregation method. This study used blood samples obtained from healthy volunteers (17 to 59 years of age). This study was approved by the Dongguk University Gyeongju Institutional Review Board (DUG IRB 20180004-04). Volunteer written consent was waived in this study. A total of 13 human platelet-rich plasma (PRP) with an acid-citrate-dextrose solution (0.8% citric acid, 2.2% sodium citrate and 2.45% glucose) was collected and obtained from the Korean Red Cross Blood Center (Daegu, Korea) from July, 2018 to December, 2018. Human PRP was centrifuged for 10 min at 125 x g at 25°C to remove other cells and then centrifuged for 10 min at 1,300 x g at 25°C to obtain platelet pellets. The platelet pellets were washed twice with washing buffer (138 mM NaCl, 2.7 mM KCl, 12 mM NaHCO3, 5.5 mM glucose and 1 mM Na2EDTA, pH 6.5) and then resuspended in a suspension buffer (138 mM NaCl, 2.7 mM KCl, 12 mM NaHCO3, 0.36 mM NaH2PO4, 0.49 mM MgCl2, 5.5 mM glucose and 0.25% gelatin, pH 6.9) to a final concentration of 1x10⁶ cells/ml, as previously described (20). All of the above-mentioned procedures were performed at 25°C to avoid any effects (i.e., platelet activation) related to temperature.

Platelet aggregation was measured using an aggregometer (Chrono-Log, Corp.) at a constant stirring speed of 50 °C. In the experiment, platelets were pre-incubated with MBDSW at 37°C in the presence of 2 mM CaCl2 prior to the addition of collagen (3 µg/ml) or thrombin (0.05 U/ml). Each aggregation rate (%) was recorded for 5 min.

Measurement of lactate dehydrogenase. Platelet cytotoxicity was determined by the leakage of lactate dehydrogenase (LDH) from the cytosol. Washed human platelets (10⁶ cells/ml) were incubated with various concentrations of MBDSW (H250 to H2,000) for 30 min at 37°C, and then centrifuged with 2,000 x g for 5 min at room temperature. The supernatant (50 µl) incubated with cytotoxicity assay reagent mix (50 µl) for 30 min at room temperature was measured using a SpectraMax M2e (Molecular Devices). A maximal value of cytotoxicity was recorded in 0.05% triton X-100. Percentage cytotoxicity was calculated as follows: Cytotoxicity (%)=100×(sample LDH release/maximum LDH release).

Measurement of cytosolic calcium ion concentration. Human PRP was incubated with Fura-2/AM (5 µM) at 37°C for 1 h in the dark. Washed, Fura-2/AM-loaded human platelets
Measurement of P-selectin expression and fibrinogen binding to integrin α_{IIb}β_{3}. Platelets, pre-incubated with MBDSW (H250 to H2,000) or dipyridamole (40 µM) for 2 min at 37°C, were stimulated with collagen (3 µg/ml) at 37°C in the presence of 2 mM CaCl₂. Platelet aggregation was terminated after 5 min of stimulation by the addition of 5 mM ice-cold EDTA including 0.2 mM indomethacin followed by centrifuging the samples (5,000 x g, 3 min, at 4°C). Supernatants were diluted 1:500 and used in assays. Samples were reacted with TXB₂, antiserum and TXB₂ tracers in a mouse anti-rabbit IgG-coated plate overnight at 4°C. The level of TXB₂ in the supernatant was determined using a SpectraMax M2e (Molecular Devices) microplate reader.

**Western blot analysis.** Platelets, pre-incubated with MBDSW (H250 to H2,000), dipyridamole (40 µM), LY294002 (2 and 20 µM) for 2 min at 37°C, were stimulated with collagen (3 µg/ml) for 5 min at 37°C in the presence of 2 mM CaCl₂. Platelet aggregation was terminated after 5 min of stimulation by the addition of 5 mM ice-cold EDTA including 0.2 mM indomethacin followed by centrifuging the samples (5,000 x g, 3 min, at 4°C). Supernatants were diluted 1:500 and used in assays. Samples were reacted with TXB₂, antiserum and TXB₂ tracers in a mouse anti-rabbit IgG-coated plate overnight at 4°C. The level of TXB₂ in the supernatant was determined using a SpectraMax M2e (Molecular Devices) microplate reader.

**Statistical analysis.** The experimental results are presented as the means ± standard deviation (SD) values accompanied by the number of observations. Statistical significance was determined by analyzing variance. Significant differences among groups in experiments were assessed by undertaking analysis.
of variance (ANOVA). When the ANOVA results indicated significant differences among group means, the groups were compared by the Newman-Keuls method. A value of P<0.05 was considered to indicate a statistically significant difference.

Results

Effects of MBDSW on collagen- and thrombin-induced human platelet aggregation, and cytotoxicity. Platelet aggregation was determined using a light transmission aggregometer. The concentration of collagen used was 3 µg/ml, which was the threshold concentration required to induce maximal aggregation (Fig. S1). As the threshold concentration of thrombin (0.2 U/ml) is a very high concentration, the concentration of thrombin used was 0.05 U/ml. The effects of various hardness levels of MBDSW on platelet aggregation induced by collagen and thrombin were examined and it was observed that MBDSW inhibited platelet aggregation induced by collagen (3 µg/ml) and thrombin (0.05 U/ml) in a hardness-dependent manner (Fig. 1A and B). Subsequently, it was confirmed that the presence of calcium in MBDSW did not automatically affect platelet aggregation (Fig. 1C). Furthermore, Ca plus Mg, equivalent to MBDSW (H250 and H1,000), inhibited platelet aggregation induced by collagen (3 µg/ml) (Fig. S2). In addition, LDH assay revealed that MBDSW (H250 to H2,000) incubated with the platelets for 30 min did not increase LDH leakage in the platelets (Fig. 1D). These results suggested that MBDSW inhibited platelet aggregation without affecting cytotoxicity.

MBDSW reduces collagen-induced [Ca^{2+}], mobilization and increases IP_{3}R phosphorylation. Intracellular calcium ([Ca^{2+}]) is considered an important molecule for platelet activation, such as the inside-out signaling activation of integrin α_{IIb}β_{3} and thrombus formation (22). Thus, the inhibitory effects of MBDSW on the collagen-induced increase in [Ca^{2+}] levels were investigated. Pre-treatment of the platelets with various hardness levels of MBDSW significantly prevented the collagen-induced increase in [Ca^{2+}] levels (Fig. 2A). To reveal the inhibitory mechanisms of MBDSW associated with [Ca^{2+}], mobilization, the association between IP_{3}R and [Ca^{2+}], mobilization was evaluated. As shown in Fig. 2B, MBDSW increased IP_{3}R phosphorylation in a hardness-dependent manner.

MBDSW reduces granule secretion. When platelets are activated via agonist stimulation, platelets release granules. Therefore, it was investigated whether MBDSW affects α-granule and dense granule secretion in collagen-induced platelet aggregation. Collagen (3 µg/ml) alone markedly increased ATP and serotonin secretion from dense granules. However, the ATP and serotonin secretions were significantly decreased by MBDSW in a hardness-dependent manner (Fig. 3A and B). To determine whether MBDSW inhibits α-granule secretion, P-selectin expression was examined by flow cytometry. The results revealed that MBDSW significantly inhibited P-selectin expression in a hardness-dependent manner (Fig. 3C and D).

MBDSW inhibits activation of integrin α_{IIb}β_{3}. Integrin α_{IIb}β_{3} undergoes a conformational transition upon platelet activation and binds to fibrinogen (23). To determine whether MBDSW inhibits the activation of integrin α_{IIb}β_{3}, the binding of fibrinogen to the activated integrin α_{IIb}β_{3} was examined by flow cytometry. The binding of fibrinogen to the activated integrin α_{IIb}β_{3} was significantly inhibited in MBDSW-treated platelets (Fig. 4A and B). Furthermore, we examined the effect of MBDSW on VASP phosphorylation as a regulator of integrin α_{IIb}β_{3} activation (24). In collagen-activated platelets, VASP (ser157) was shown to be directly phosphorylated (Fig. 4C), an action that involves a collagen-related feedback inhibitory signaling pathway (25). However, MBDSW markedly increased the phosphorylation of VASP (Ser157) to a level greater than that observed with collagen only (Fig. 4C).

MBDSW alters the cAMP level. Since it has been well established that cAMP inhibits the activation of platelets and stimulates VASP phosphorylation in activated platelets, the effects of MBDSW on the cAMP level in collagen-activated platelets were investigated. As shown in Fig. 5A, MBDSW (H2,000) increased the cAMP level. In addition, dipyridamole (40 µM), a PDE inhibitor, significantly increased the cAMP level and VASP (ser157) phosphorylation. (Figs. 5A and S3).

MBDSW decreases TXA_{2} production. Upon exposure to collagen, the platelet membrane is catabolized from a phospholipid to TXA_{2}. The produced TXA_{2} acts as a positive feedback mediator in the activation of nearby platelets (26). Therefore, in this study, whether MBDSW decreases the production of TXA_{2} under collagen exposure was then examined. In this experiment, TXA_{2} was detected using TXB_{2}, a stable metabolite of TXA_{2}. The results revealed that treatment with collagen (3 µg/ml) markedly increased TXB_{2} production; however, the platelets pre-treated with MBDSW exhibited significantly decreased TXB_{2} levels, with the reduction occurring in a hardness-dependent manner (Fig. 5B).

MBDSW inhibits Akt and ERK phosphorylation. Previous studies have demonstrated that Akt plays a role in the amplification of platelet aggregation, granule secretion and integrin α_{IIb}β_{3} activation induced by agonists (16,17); therefore, this study investigated whether MBDSW inhibits the collagen-stimulated phosphorylation of Akt; the results revealed that the inhibition of Akt phosphorylation occurred in a hardness-dependent manner (Fig. 6A).

MAPKs, including ERK, JNK and p38, are activated and phosphorylated in platelets following treatment with various agonists (18). Thus, this study whether MBDSW downregulates MAPKs. The results revealed that MBDSW inhibits the phosphorylation of ERK, in a hardness-dependent manner; however, it did not inhibit the phosphorylation of JNK or p38 (Fig. 6B).

LY294002 and U0126 inhibit Akt and ERK phosphorylation. In addition, it was investigated whether LY294002, an Akt inhibitor, or U0126, an ERK inhibitor, can cross-inhibit the phosphorylation of Akt and ERK. Collagen (3 µg/ml) was shown to extensively increase phosphorylation of Akt and ERK. By contrast, both LY294002 and U0126 markedly inhibited the phosphorylation of Akt and ERK (Fig. 7A and B). In addition, dipyridamole inhibited the phosphorylation of Akt and ERK (Fig. S3).
Discussion

CVDs are the most common underlying cause of mortality in developed nations and are associated with various risk factors, such as abnormal cholesterol levels, high blood pressure, diabetes, smoking and obesity (27,28). In addition, the activation of platelets, which is caused by a series of processes, can increase the risk of CVDs, when combined with risk factors (29). Previous studies have demonstrated that MBDSW can regulate various risk factors for CVDs (7,9,11,30‑32); however, there is incomplete information on its effects on platelet activation. Therefore, the present study evaluated the inhibitory activity of MBDSW on agonist‑induced human platelet aggregation, and cytotoxicity. (A) Washed human platelets (1x10⁸ cells/ml) were pre‑incubated with MBDSW at various levels of hardness (H250, 500, 1,000 and 2,000) and subsequently treated with collagen (3 µg/ml) to induce platelet aggregation. (B) Washed human platelets (1x10⁸ cells/ml) were pre‑incubated with MBDSW at various levels of hardness (H250, 500, 1,000 and 2,000) and subsequently treated with thrombin (0.05 U/ml) to induce platelet aggregation. (C) Washed human platelets (1x10⁸ cells/ml) were pre‑incubated with MBDSW (H1,000) or the vehicle in the presence or absence of 2 mM CaCl₂ and subsequently treated with collagen (3 µg/ml). (D) Effects of MBDSW on cytotoxicity. MBDSW (H) was at the ratio of Mg:Ca, 3:1, and hardness. All data are presented as the means ± SD (n=3). Vehicle is collagen only. NS, not significant. *P<0.05 and **P<0.001 vs. the vehicle. H, hardness; MBDSW, mineral‑balanced deep sea water.
**Figure 2.** Inhibitory effects of MBDSW on collagen-induced cytosolic calcium ion concentration and IP3R phosphorylation. (A) Washed platelets were loaded with Fura-2/AM at 37˚C for 1 h. Fura-2/AM-loaded platelets were pretreated with MBDSW at 37˚C for 2 min and stimulated with collagen (3 µg/ml). Fluorescence was recorded with a spectrofluorometer (SFM 25). (B) Washed platelets were pre-incubated with MBDSW at 37˚C for 2 min and stimulated with collagen (3 µg/ml). Platelet aggregation was terminated by the addition of protease and phosphatase inhibitor-contained RIPA lysis buffer; the phosphorylation of IP3R was examined by western blot analysis. All data are presented as the means ± SD (n=3). NSB, non-specific band. †P<0.05 and ††P<0.001 vs. intact; *P<0.05 and **P<0.001 vs. collagen-activated control. H, hardness; MBDSW, mineral-balanced deep sea water; IP3R, inositol 1,4,5-triphosphate receptor.

**Figure 3.** Effects of MBDSW on collagen-induced α-granule and dense granule secretions. (A) Effect of MBDSW on ATP release. (B) Effect of MBDSW on serotonin release. (C) Flow cytometric histograms of collagen-induced P-selectin expression. (D) Effect of MBDSW on P-selectin expression. Washed platelets were incubated with MBDSW at 37˚C for 2 min, and the platelets were then stimulated with collagen (3 µg/ml) for 5 min. ATP and serotonin release was determined using an ELISA assay kit. P-selectin expression was assessed using a FACSCalibur II flow cytometer. All data are presented as the means ± SD (n=3). ††P<0.001 vs. intact; **P<0.001 vs. collagen-activated control. H, hardness; MBDSW, mineral-balanced deep sea water.
inhibitory effects of MBdSW on platelet activity by analyzing granule release, integrin α\textsubscript{IIb}β\textsubscript{3} activation, calcium mobilization, TXA\textsubscript{2} production, cAMP level, and the functioning of the Akt and MAPK pathways.

When the extracellular matrix of the blood vessel wall is damaged, the released collagen induces platelet aggregation; thrombin, a coagulation protein produced during the coagulation process, also causes platelet aggregation (4,33). Although these mechanisms differ, they ultimately produce the same result: platelet aggregation and activation. Therefore, this study initially examined whether MBdSW can inhibit both collagen- and thrombin-induced platelet aggregation. As shown in Fig. 1A and B, MBdSW markedly inhibited collagen- and thrombin-induced platelet aggregation in a hardness-dependent manner. In addition, the presence of calcium in MBdSW did not automatically affect platelet aggregation (Fig. 1C). This indicates that calcium, which is essential for platelet activation, is abundant in MBdSW but does not affect for platelet aggregation. In addition, MBdSW contains Mg, K, Na and Sr. Mg and K are known to inhibit platelet aggregation and Na intake tends to increase platelet aggregation (34,35). However, the suspension buffer contains much higher Na and K concentrations than MBdSW. Therefore, Na and K in MBdSW are not affected [suspension buffer, 138 mM NaCl, 2.7 mM KCl and MBdSW (H2,000): 1 mM Na, 0.25 mM K]. Sr is an antagonist of calcium and is known to act similar to calcium in the platelet aggregation (36). However, MBdSW contains a very small amount of strontium and will not be affected. In this study Fig. S2 shows Ca and Mg information for platelet aggregation. However, more detailed research is required and thus further studies need to be carried out on this matter.

To elucidate the underlying inhibitory mechanisms of MBdSW, platelet activation markers and downstream molecules were investigated. To determine whether MBdSW
regulates the intracellular calcium levels, a calcium assay was carried out using a fluorescence spectrophotometer. The data revealed that MBDSW significantly decreased the collagen-induced intracellular calcium levels in a hardness-dependent manner (Fig. 2A). The IP$_3$ generated by phospholipase C is able to release [Ca$^{2+}$] from the endoplasmic reticulum by directly activating IP$_3$R channels. In platelets, the regulation of this receptor is considered to occur through receptor phosphorylation. Previous studies have reported the inhibition of IP$_3$R function following phosphorylation (37,38). This study demonstrated that MBDSW markedly increased the phosphorylation of IP$_3$R (Fig. 2B).
functions including degranulation, adhesion and thrombus formation are activated by an elevation in the intracellular calcium level (22). Hence, these results suggest that MBDSW containing calcium inhibits collagen-induced calcium mobilization via the phosphorylation of IP3R. Moreover, it is also suggested that MBDSW can prevent calcium-dependent signaling in platelets. A previous study suggested that the calcium/calmodulin-induced phosphorylation of the myosin light chain contributes to platelet granule secretion (39).

Platelet α-granules contain protein molecules, such as coagulation factors (e.g., fibrinogen, factor V), growth factors (e.g., platelet-derived growth factor, epidermal growth factor) and adhesion molecules (P-selectin). Dense granules contain small non-protein molecules, such as adenosine diphosphate, ATP and serotonin that amplify the activation of platelets (40).

We found that MBDSW significantly inhibited P-selectin expression and dense granule secretion (Fig. 3). These results indicate that MBDSW, by suppressing P-selectin expression, inhibits platelet-mediated inflammation and leukocyte migration during thrombus formation and prevents platelet aggregation.

In platelets, abundant integrin αIIbβ3 plays an essential role in both inside-out and outside-in signaling. Integrin αIIbβ3-mediated signaling transduction is achieved when it binds to fibrinogen, which leads to platelet aggregation (41). In this study, MBDSW markedly inhibited its binding to fibrinogen, blocking inside-out signaling (Fig. 4A and B).

VASP links the extracellular matrix through integrin to the actin cytoskeleton and is involved in the inactivation of integrin, and Akt is required for full activation of integrin and granule secretion (16,17). In the present study, it was demonstrated that MBDSW activated VASP (Ser157) and inactivated the phosphorylation of Akt (Figs. 4C and 6A). This result indicates that MBDSW inhibits the activation of integrin αIIbβ3 by regulating both VASP and Akt. In addition, the activation of integrin αIIbβ3 and granule secretion are known to be inhibited by the cAMP pathway (42,43). As the cAMP pathway inhibits the activation of integrin αIIbβ3 and granule secretion through VASP and Akt proteins, this study examined whether MBDSW increases the cAMP level. MBDSW (H250-H1,000) restored the cAMP level to that of unstimulated platelets, although this change was not significant. However, MBDSW did increase the cAMP level at H2,000 (Fig. 5A).

Further investigation of the molecular mechanisms revealed that MBDSW may target ERK (Fig. 6B). Indeed, human platelets contain the MAPK family and Akt proteins, and these proteins are involved in the platelet activation, adhesion, and granule secretion (16-18). However, this study demonstrated that p38 and JNK are not the targets of MBDSW, based on the observation that MBDSW failed to inhibit the phosphorylation of p38 and JNK (Fig. 6B). Since MBDSW selectively suppressed the phosphorylation of Akt and ERK, whether the cross-inhibition of Akt and ERK was possible was investigated. The phosphorylation of Akt and ERK was assessed by using LY294002, an
Platelet aggregation was markedly inhibited by MBdSW that contains abundant Mg. Therefore, the results suggest that Mg is a key component in determining the anti-platelet factors (7,9,11,13,14). The present study demonstrated that MBdSW more effectively inhibits the production of TXA2 through the inhibition of ERK phosphorylation. The released TXA2, serotonin, and ATP act as positive feedback mediators in the activation of more platelets to participated in platelet aggregation (31,45,46). Therefore, the inhibition of secretion of these molecules by MBdSW is a source of effective inhibition of platelet aggregation.

Platelet aggregation influences the development of CVDs. Of note, the majority of risk factors of CVDs, including hypercholesterolemia, hypertension, smoking and diabetes are able to increase platelet activation (47-50). In other study, DSW has been shown to be effective in reducing CVD risk factors (7,9,11,13,14). The present study demonstrated that platelet aggregation was markedly inhibited by MBdSW that contains abundant Mg. Therefore, the results suggest that Mg in MBdSW is a key component in determining the anti-platelet aggregation activity of MBdSW.

In conclusion, this study demonstrated that the inhibitory effect of MBdSW on collagen- and thrombin-induced platelet aggregation occurred in a hardness-dependent manner. In addition, MBdSW markedly suppressed intracellular downstream signal transduction; that is, MBdSW significantly inhibited collagen-induced [Ca2+]i mobilization, granule secretion and TXA2 production. Furthermore, it was found that MBdSW regulated the cAMP level and the phosphorylation of ERK, Akt and VASP (Ser157). Finally, the activity of integrin αIIbβ3 was inhibited by MBdSW. Anti-platelet drugs used for the prevention and treatment of thrombosis target TXA2 production, integrin inhibition and increases in cAMP levels (51); therefore, MBdSW can be regarded as a potential prophylactic agent for application in the prevention of CVDs.

Acknowledgements

Not applicable.

Funding

This study was financially supported by the National R&D project ‘Development of new application technology for deep seawater industry’ supported by the Ministry of Oceans and Fisheries of the Republic of Korea.

Availability of data and materials

All data generated or analyzed during this study are included in this published article or are available from the corresponding author on reasonable request.

Authors’ contributions

KSN and GSN designed the experiments. KSN, GSN and KSL analyzed and interpreted the data. GSN performed the experiments and wrote the manuscript. All authors have read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by Dongguk University Gyeongju Institutional Review Board (DUG IRB 20180014-04).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References
