Protective Effect of 3-Bromo-4,5-Dihydroxybenzaldehyde from *Polysiphonia morrowii* Harvey against Hydrogen Peroxide-Induced Oxidative Stress In Vitro and In Vivo

Su-Hyeon Cho^{1,2†}, Soo-Jin Heo^{3†}, Hye-Won Yang², Eun-Yi Ko⁴, Myeong Seon Jung¹, Seon-Heui Cha⁵, Ginnae Ahn⁶, You-Jin Jeon^{2*}, and Kil-Nam Kim^{1*}

¹Chuncheon Center, Korea Basic Science Institute (KBSI), Chuncheon 24341, Republic of Korea

²Department of Marine Life Science, Jeju National University, Jeju 63243, Republic of Korea

³Jeju International Marine Science Center for Research & Education, Korea Institute of Ocean Science and Technology, Jeju 63349, Republic of Korea

⁴Bio Research Center, Dermapro, Jeju 63309, Republic of Korea

⁵Department of Marine Biomedical Science, Hanseo University, Chungcheongnamdo 31962, Republic of Korea

⁶Department of Marine Bio-food Science, College of Fisheries and Ocean Sciences, Chonnam National University, 59626, Republic of Korea

Received: April 29, 2019

Revised: June 25, 2019

Accepted: July 2, 2019

First published online
July 9, 2019

*Corresponding authors

K.N.K.

Phone: +82-33-815-4607,

E-mail: knkim@kbsi.re.kr

Y.J.J.

Phone: +82-64-754-3475,

E-mail: youjinj@jejunu.ac.kr

pISSN 1017-7825, eISSN 1738-8872

Copyright© 2019 by
The Korean Society for Microbiology
and Biotechnology

We investigated the protective effects of 3-bromo-4,5-dihydroxybenzaldehyde (BDB) from *Polysiphonia morrowii* Harvey against hydrogen peroxide (H₂O₂)-induced apoptosis in Vero cells. BDB exhibited scavenging activity for DPPH, hydroxyl, and alkyl radicals. BDB also inhibited H₂O₂-induced lipid peroxidation, cell death, and apoptosis in Vero cells by inhibiting the production of ROS. To evaluate the molecular mechanisms of apoptosis inhibition, the expression of Bax/Bcl-xL and NF-κB was assessed by western blot assay. BDB significantly suppressed the cleavage of caspase-9 and PARP and reduced Bax levels in H₂O₂-induced Vero cells. Besides, BDB suppressed the phosphorylation of NF-κB and the translocation of p65 in H₂O₂-induced cells. Furthermore, we evaluated the effect of BDB on ROS production, cell death, and lipid peroxidation in an H₂O₂-stimulated zebrafish embryo model. Taken together, these results indicated that ROS generation and cell death were significantly inhibited by BDB in zebrafish embryos, thereby proving that BDB exerts excellent antioxidant activity in vitro and in vivo.

Keywords: *Polysiphonia morrowii* Harvey, 3-bromo-4,5-dihydroxybenzaldehyde, oxidative stress, reactive oxygen species, antioxidant, zebrafish embryos

Introduction

The modern society is exposed to various types of oxidative stresses that originate from the environment, diet, or lifestyle. Highly reactive molecules such as reactive oxygen species (ROS) and free radicals such as superoxide anion radicals (O₂[•]), hydrogen peroxide (H₂O₂), hydroxyl radicals (HO[•]), and singlet oxygen (¹O₂) are generated by oxidative stress in the body. ROS produced in the body are eliminated by the antioxidant defense mechanisms. However, when these defense mechanisms do not function,

ROS can structurally damage the cell components [1–3]. The resulting oxidative stress damages DNA, lipids, proteins, and other molecules, leading to harmful consequences such as Parkinson's disease, Alzheimer's disease, diabetes, liver injury, cancer, inflammation, and aging [4–7]. Antioxidants have long been studied, and currently, several chemically synthesized antioxidants such as BHA (butylated hydroxyanisole) and BHT (butylated hydroxytoluene) are in use. However, it is necessary to develop safer and active natural antioxidants because the synthesized compounds act as mutagens and tumor

promoters at high dosages, and there have been a number of recent studies on antioxidants derived from natural products [8–10].

Marine algae have traditionally been used as food and therapeutic agents for various diseases. Such algae generally produce diverse compounds, some of which have biological activity of therapeutic value [11, 12]. Identifying biologically active compounds in marine algae has been a focus of research, and currently, interest in natural physiological materials from natural organisms continues to increase. Moreover, studies on biologically active substances from marine algae are being carried out to determine not only their nutritious aspects but also their possible use for treating diseases and maintaining health [13]. Marine algae-derived compounds have been shown to have many functions such as antiviral, antioxidant, anti-inflammatory, and anticoagulant activities [14–17]. Previous studies have reported that products derived from *Polysiphonia* sp. have various functions such as antioxidant, antiviral, and antimycobacterial activities [18, 19]. Further, Hyun *et al.* and Piao *et al.* demonstrated the protective effect of 3-bromo-4,5-dihydroxybenzaldehyde (BDB) from red algae against ultraviolet (UVB)-induced oxidative stress in human keratinocytes [20, 21]. However, the protective effects of BDB via the apoptosis pathway and NF- κ B signaling pathway against oxidative stress-induced apoptosis in vitro have not been determined. In addition, studies using zebrafish models are lacking. Therefore, the purpose of the present study was to examine the antioxidant activity of BDB from *P. morrowii* Harvey in H₂O₂-induced oxidative stress in cells and zebrafish.

Materials and Methods

Extraction and Isolation of BDB from *P. morrowii* Harvey

The red alga *P. morrowii* Harvey was collected along the coast of Jeju Island, Korea. The preparation, extraction and isolation methods of the sample were described in previous study [22]. The purified compound ($\geq 99\%$) was identified by comparing its ¹H and ¹³C NMR data with those in the literature [23]. The chemical structure of the purified compound is indicated in Fig. 1. The compound was dissolved in 100% ethanol (Sigma-Aldrich, USA) and employed in experiments in which the final concentration of 100% ethanol in the culture medium was adjusted to $<0.01\%$.

Cell Culture

Vero cells, kidney cells from African green monkeys, were purchased from the Korean Cell Line Bank (KCLB, Korea). The cells were grown at 37°C in a 5% CO₂ humidified atmosphere incubator using RPMI-1640 medium (Gibco/BRL, Canada) added

Fig. 1. (A) Isolation scheme for BDB from the alga *Polysiphonia morrowii* Harvey, (B) NMR data of 3-bromo-4,5-dihydroxybenzaldehyde (BDB), and (C) chemical structure of BDB.

with 10% fetal bovine serum (FBS; Welgene, Korea) and 1% antibiotics (Gibco/BRL).

DPPH, Hydroxyl, and Alkyl Radical Scavenging Activity

1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl, and alkyl radical scavenging activity were analyzed by altering the method described by Ahn *et al.* [24].

Measurement of Cell Viability

The cells were seeded in a 96-well plate at a concentration of 1.0×10^5 cells/ml. After 24 h, the cells were pre-treated with 12.5, 25, and 50 μ M BDB and 1 M *N*-acetyl-L-cysteine (NAC; Sigma-Aldrich) for 2 h at 37°C. Then, 500 μ M H₂O₂ was added for 24 h at 37°C. Cell viability was measured using the method described by Ko *et al.* [25]. The absorbance was read at 540 nm using a spectrophotometer (Molecular Devices, USA).

Measurement of Intracellular ROS Production

The cells were seeded in a 96-well plate at a concentration of 1.0×10^5 cells/ml. After 24 h, the cells were pre-treated with 12.5, 25, and 50 μM BDB for 2 h at 37°C. Then, 500 μM H_2O_2 was added followed by incubation for 30 min at 37°C. ROS production was measured using the method described by Yang *et al.* [26]. Detection of DCHF-DA was analyzed at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a spectrophotometer (Molecular Devices).

Lipid Peroxidation Inhibitory Activity

Lipid peroxidation was measured using an MDA assay kit (Northwest Life Science Specialties, LLC) following the manufacturer's instructions. Briefly, 2 μl of BHT reagent was added into a microtube, and then a calibrator or lysates, acid reagent, and TBA reagent were progressively added. Then, the mixture was mixed vigorously and incubated for 1 h at 60°C, after which it was centrifuged at $10,000 \times g$ for 3 min. The generation of MDA, the substrate of TBA, was read at 514 nm using a spectrophotometer (Molecular Devices).

Nuclear Staining with Hoechst 33342

The nuclear morphology was studied using the cell-permeable DNA dye Hoechst 33342 (Sigma-Aldrich). The cells were seeded in a 24-well plate at a concentration of 1.0×10^5 cells/ml. After 24 h, the cells were pre-treated with 12.5, 25, and 50 μM BDB and 1 M NAC for 2 h at 37°C. Then, 500 μM H_2O_2 was added followed by incubation for 6 h at 37°C. Nuclear staining was performed by modifying the method described by Yang *et al.* [26]. The cells were observed using a fluorescence microscope (Zeiss, Germany).

Cell Cycle Analysis

Cell cycle analysis was carried out to determine the rate of apoptotic sub- G_1 cells. The cells were seeded in a 60-mm dish at a concentration of 2.0×10^5 cells/ml. After 24 h, the cells were pre-treated with 50 μM BDB for 2 h at 37°C. Then, 500 μM H_2O_2 was added followed by incubation for 6 h at 37°C. Apoptotic sub- G_1 of cell cycle was measured using the method described by Yang *et al.* [26]. Flow cytometry was conducted using a Beckman Coulter CytoFLEX Flow Cytometer (Beckman Coulter, USA).

Western Blot Analysis

The protein extraction and western blot analysis were conducted by altering the method described by Ko *et al.* [25]. Primary antibodies used were the following: anti-bax, anti-bcl-xL, anti-PARP, anti-cleaved caspase-9 anti-phospho-NF- κB p105, anti-phospho-NF- κB p65 (Cell Signaling Technologies, USA,) and β -actin (Santa Cruz Biotechnology, USA). Secondary antibodies used were the following: anti-rabbit IgG, HRP-linked antibody and anti-mouse IgG, HRP-linked antibody (Cell Signaling Technologies).

Immunofluorescence Staining

The cells were seeded on a confocal slide at a concentration of

1.0×10^5 cells/ml. After 24 h, the cells were pre-treated with 50 μM BDB for 2 h at 37°C. Then, 500 μM H_2O_2 was added followed by incubation for 45 min at 37°C. Immunofluorescence staining was conducted using the method described by Ko *et al.* [27]. The cells were photographed using an LSM 780 Zeiss Confocal Laser Microscope.

Maintenance of Zebrafish

Zebrafish were kept under the following conditions: $28.5 \pm 0.5^\circ\text{C}$ with a 14/10 h light/dark cycle. The zebrafish were fed two times a day, 6 days a week. Zebrafish were selected for mating at a female-to-male ratio of 1:2. Embryos were obtained via mating and spawning. After spawning, the embryos were moved to a Petri dish containing 1 mg/ml methylene blue solution. After disinfection for 1.5 h, the methylene blue solution was changed to fresh embryo media (600 mg/l red sea salt in distilled water). All animal experiments were approved by the Jeju National University Animal Care and Use Committee (2016-0052).

Effect of BDB on H_2O_2 -Induced ROS Generation, Cell Death, and Lipid Peroxidation in Zebrafish

After fertilization, 7–9 h post-fertilization (hpf) zebrafish embryos were moved to a 12-well plate. The embryos were pre-treated with various concentrations of BDB (12.5, 25, and 50 μM) for 2 h and then treated with 5 mM H_2O_2 for 3 days post-fertilization (dpf). The zebrafish larvae were transferred to 24-well plates and stained with specific fluorescent dye to examine ROS production (DCFH-DA, 1 h incubation in the dark at 37°C), cell death (acridine orange, 30 min incubation in the dark at 28.5°C; Sigma-Aldrich), and lipid peroxidation (DPPP, 30 min incubation in the dark at 28.5°C; Dojindo Inc., Kumamoto, Japan). Zebrafish larvae were photographed under a microscope equipped with a CoolSNAP-Pro color digital camera (Olympus, Japan) after the anesthetization using 0.03% ethyl 3-aminobenzoate methane-sulfonate (MS-222, Sigma-Aldrich). The fluorescence intensity was quantified using ImageJ software.

Statistical Analysis

The level of significance was defined as $p < 0.05$ and the value was given as mean $\pm$ standard deviation (SD) using the unpaired Student's *t*-test in Microsoft Excel 2010 (Microsoft, USA).

Results

Free Radical Scavenging Activities of BDB

We measured the inhibition activity of BDB against DPPH, hydroxyl, and alkyl radicals (Fig. 2). BDB exhibited profound scavenging activities for DPPH, hydroxyl, and alkyl radicals in a dose-dependent manner. The half maximal inhibitory concentration (IC_{50}) values for DPPH, hydroxyl, and alkyl radical scavenging were 13.17 ± 0.13 ,

Fig. 2. (A) DPPH, (B) hydroxyl, and (C) alkyl radical scavenging activities of BDB were measured by ESR spectrum. All results were expressed as the mean $\pm$ standard deviation (SD) from more than three individual experiments; * $p < 0.05$ compared with the H_2O_2 -stimulated group.

60.12 $\pm$ 3.31, and 7.58 $\pm$ 0.37 μ M, respectively. These data indicate that BDB has excellent inhibition activity against DPPH and alkyl radicals.

Effect of BDB on H_2O_2 -Induced Cytotoxicity in Vero Cells

We measured the effect of BDB on cell viability using the principle that MTT reacts with the mitochondria in living cells and produces formazan (Fig. 3A). Vero cells were pre-treated with BDB and NAC for 2 h and then treated with 500 μ M H_2O_2 for 24 h. After the cells were treated with H_2O_2 , cell viability decreased to 30.15% compared with that of the control. However, when the cells were treated with 50 μ M BDB and 1 mM NAC, cell viability increased up to 78.8% and 60.8%, respectively.

Fig. 3. Effect of BDB on cell viability and ROS production in H_2O_2 -stimulated Vero cells.

The cells were pre-treated with BDB (12.5, 25, 50 μ M) and NAC (1 mM) and treated with H_2O_2 (500 μ M) for 24 h or 30 min. (A) Cell viability was assessed with the MTT assay. (B) ROS production was measured by DCFH-DA methods. All results were expressed as the mean $\pm$ standard deviation (SD) from more than three individual experiments; * $p < 0.05$ compared with the H_2O_2 -stimulated group.

Effect of BDB on ROS Production in H_2O_2 -Stimulated Vero Cells

We measured the intracellular ROS scavenging activity of BDB and NAC using the principle that DCFH-DA reacts with reactive oxygen in cells and is oxidized to a fluorescent substance, DCF (Fig. 3B). Vero cells were pre-treated with BDB and NAC for 2 h and then treated with 500 μ M H_2O_2 for 30 min. After 30 min, ROS scavenging activity was approximately 54% for 12.5 and 25 μ M BDB and approximately 59% for 50 μ M BDB. These results indicated that BDB protects against H_2O_2 -induced cytotoxicity by scavenging radicals and ROS.

Effect of BDB on H_2O_2 -Induced Lipid Peroxidation in Vero Cells

We measured the intracellular ROS scavenging effect of BDB as well as the effect of BDB on lipid peroxidation in

Fig. 4. Effect of BDB on H₂O₂-induced lipid peroxidation in Vero cells.

The cells were pre-treated with BDB (50 μM) and treated with H₂O₂ (500 μM) for 6 h. The generation of MDA was measured by MDA assay kit. All results were expressed as the mean ± standard deviation (SD) from more than three individual experiments; **p* < 0.05.

H₂O₂-stimulated Vero cells (Fig. 4). The cells were pre-treated with BPCA and then treated with 500 μM H₂O₂ for 6 h. H₂O₂ increased the generation of MDA compared to the control, but 50 μM of BDB significantly reduced MDA to levels similar to those in the control. This result shows that BPCA protects against H₂O₂-induced lipid peroxidation in Vero cells.

Effect of BDB on H₂O₂-Induced Apoptosis in Vero Cells

We assessed the protective effect of BDB on H₂O₂-induced apoptosis through nuclear staining with Hoechst 33342 (Fig. 5A). When the cells were treated with H₂O₂, we observed nuclei shrinkage, membrane blebbing, and apoptotic bodies. However, when the cells were treated with 50 μM BDB, we observed a significant reduction in apoptotic bodies. Moreover, to study the role of BDB in cell cycle progression under oxidative stress, we performed cell cycle analysis using flow cytometry (Fig. 5B). The sub-G₁ DNA content of the control cells was 14.5%. On the other hand, the sub-G₁ DNA content of cells treated with 500 μM H₂O₂ was 25.99%, indicating the induction of apoptosis. But 50 μM BDB treatment suppressed the sub-G₁ DNA content decreased to 21.89%. These results suggest that BDB prevents cytotoxicity by inhibiting apoptosis in H₂O₂-induced Vero cells.

Effect of BDB on the Reduction of Bax and Cleaved Caspase-9 and PARP Cleavage Activation in H₂O₂-Stimulated Vero Cells

We evaluated the expression of Bax, cleaved caspase-9,

Fig. 5. Effect of BDB on H₂O₂-induced apoptosis in Vero cells.

The cells were pre-treated with BDB (50 μM) and treated with H₂O₂ (500 μM) for 6 h. (A) Apoptotic bodies were observed under a fluorescent microscope after Hoechst 33342 staining. (B) Apoptotic sub-G₁ phase cells were assessed using flow cytometry after PI staining.

and PARP in Vero cells exposed to 500 μM of H₂O₂ in the presence or absence of BDB (Fig. 6). The expression levels of Bax and cleaved caspase-9 were increased by H₂O₂ treatment. However, when cells were treated with 50 μM BDB and H₂O₂, the expression levels decreased. The expression level of PARP was reduced by H₂O₂, but after treatment with 50 μM BDB and H₂O₂, protein expression increased. The results demonstrated that BDB inhibits apoptosis by reducing the expression of Bax and cleaved caspase-9 and increasing the expression of PARP.

Fig. 6. Effect of BDB on the reduction of Bax and cleaved caspase-9 and PARP cleavage activation in H₂O₂-stimulated Vero cells. The cells were pre-treated with BDB (50 μM) and treated with H₂O₂ (500 μM) for 6 h. Bax, Bcl-xL, cleaved caspase-9, and PARP protein levels were determined using western blot analysis. All results were expressed as the mean ± standard deviation (SD) from more than three individual experiments; **p* < 0.05 compared with the H₂O₂-stimulated group.

Effect of BDB on the Activation of NF-κB in H₂O₂-Stimulated Vero Cells

We evaluated the activation of NF-κB in Vero cells

exposed to 500 μM of H₂O₂ in the presence or absence of BDB (Fig. 7). When cells were treated with 500 μM H₂O₂ for 45 min, NF-κB was activated. However, the activation of

Fig. 7. Effect of BDB on the activation of NF-κB in H₂O₂-stimulated Vero cells. (A) The cells were pre-treated with BDB (50 μM) and treated with H₂O₂ (500 μM) for 6 h. The expression levels of p-p105 and p-p65 were analyzed by western blot analysis. (B) The nuclear translocation of p65 in Vero cells was observed with an anti-p65 and Alexa Fluor 488 goat anti-rabbit antibody by confocal laser microscopy.

Fig. 8. Effect of BDB on survival rate and H₂O₂-induced ROS production, cell death, and lipid peroxidation in zebrafish.

(A) Survival rate of zebrafish embryos was measured for 7 dpf after treatment with BDB (12.5, 25, and 50 µM). Zebrafish embryos were pre-treated with BDB (12.5, 25, and 50 µM) and then treated with H₂O₂ (5 mM). Imaging of (B) ROS production, (C) cell death, and (D) lipid peroxidation was performed using fluorescence microscopy. The fluorescence intensity of zebrafish was quantified using ImageJ software. All results were expressed as the mean ± standard deviation (SD) from more than three individual experiments; **p* < 0.05 compared with the H₂O₂-stimulated group.

NF- κ B was reduced after treatment with 50 μ M BDB and H_2O_2 (Fig. 7A). The nuclear translocation of p65 increased when cells were treated with H_2O_2 , but BDB inhibited the nuclear translocation of p65 (Fig. 7B). These results demonstrate that BDB suppresses the phosphorylation and nuclear translocation of NF- κ B in H_2O_2 -induced oxidative stress in Vero cells.

Effect of BDB on H_2O_2 -Caused Oxidative Stress in Zebrafish Larvae

To confirm the protective effect of BDB on H_2O_2 -induced oxidative stress in zebrafish larvae, we analyzed survival rates, ROS production, cell death, and lipid peroxidation (Fig. 8). The survival rate was more than 80% for all concentrations compared to the control, thus indicating that all tested levels of BDB were non-toxic in zebrafish larvae (Fig. 8A). H_2O_2 -caused ROS production in zebrafish larvae was 214% compared with that of the control group. However, treatment with 12.5, 25, and 50 μ M BDB and H_2O_2 considerably reduced H_2O_2 -induced ROS production to 98.6%, 95.4%, 66.3%, and 67.8%, respectively. These results indicated that the treatment of zebrafish larvae with BDB markedly suppressed H_2O_2 -induced ROS generation (Fig. 8B). H_2O_2 -induced cell death in zebrafish larvae increased more than double that in the control group. However, treatment with 12.5, 25, and 50 μ M BDB and H_2O_2 significantly reduced H_2O_2 -induced cell death in a concentration-dependent manner. Thus, these results indicate that BDB significantly inhibits H_2O_2 -induced cell death in zebrafish larvae via inhibition of ROS production (Fig. 8C). H_2O_2 -caused lipid peroxidation in zebrafish was 115.5% compared with that of the control group. Lipid peroxidation was dose-dependently reduced by BDB, but there was no significant difference, and treatment with 50 μ M BDB resulted in a similar value as in the control group (Fig. 8D). Taken together, these results demonstrate that BDB has a protective effect on H_2O_2 -stimulated oxidative stress in zebrafish larvae.

Discussion

ROS cause cellular and tissue injuries by inducing oxidative damage in DNA, RNA, proteins, and lipids [28, 29]. The overexpression of ROS promotes apoptosis via various mechanisms [29, 30]. Therefore, suppressing free radicals such as DPPH, hydroxyl, and alkyl radicals is vital to prevent apoptosis. In this study, BDB was shown to protect against DPPH and alkyl radicals significantly and to suppress intracellular ROS production considerably. The

results indicated that BDB has a vigorous scavenging activity for free radicals and ROS and protects against oxidative damage in cells.

The peroxidation of cell membrane lipids is one of the major processes for oxidative damage. Lipid peroxidation induces MDA, one of the essential by-products and a marker for oxidative stress [31]. Hence, the inhibition of lipid peroxidation is crucial for protecting against oxidative stress in H_2O_2 -stimulated cells. Therefore, we evaluated the protective effect of BDB on H_2O_2 -induced lipid peroxidation in Vero cells. Treatment with 500 μ M H_2O_2 increased lipid peroxidation compared with that of the control. However, H_2O_2 -induced lipid peroxidation was inhibited by BDB in Vero cells. The result indicates that BDB can suppress oxidative stress-induced lipid peroxidation in cells.

Apoptosis is called programmed cell death. It is a generally occurring cell death process that is vital for the healthy development and homeostasis of multicellular organisms [32, 33]. The morphologic pattern of cell death is characterized by the shrinking of the cell, membrane blebbing, production of apoptotic bodies, and chromatin condensation, concluding in cell fragmentation [34]. The phenomenon can be induced by various factors, including receptor-mediated signals such as those from ROS, metals, and pathophysiologic conditions. Regarding these factors, recent studies have proved that ROS and oxidative stress play a critical role in apoptosis. In this study, Vero cells exposed to 500 μ M H_2O_2 exhibited distinct features of apoptosis, including the presence of apoptotic bodies and increased sub-G₁ DNA content. However, BDB considerably inhibited the formation of apoptotic bodies and decreased the sub-G₁ DNA content, resulting in a lower percentage of apoptotic cells. BDB also protected against H_2O_2 -induced cell death. Thus, the results show that BDB protects against cell death by inhibiting H_2O_2 -induced apoptosis.

The Bcl-2 family of proteins plays major roles in apoptosis, and Bax, a member of the Bcl-2 family, is a pro-apoptotic factor [35, 36]. Bax promotes the release of mitochondrial cytochrome *c* and causes caspase activation. Bcl-2 and Bcl-xL prevent Bax-regulated cytochrome *c* release and cell death [36, 37]. Caspases, a family of cysteine proteases, including caspase-3 and caspase-9, play an essential role in the execution stage of apoptosis and are involved in many changes associated with apoptosis [38–40]. Cleaved caspase-9 processes other caspase members, including caspase-3 and caspase-9, to begin the caspase cascade, which leads to apoptosis [39, 40]. These caspases cleave intracellular substrates, such as poly (ADP-ribose) polymerase (PARP) and lamins, during the execution stage

of apoptosis [39]. PARP plays a critical role in the maintenance of cell viability. However, the cleavage of PARP promotes cellular degradation and is widely used as a marker for apoptotic cells [40, 41]. In this study, we performed western blot analysis to reveal the mechanisms of apoptosis in H₂O₂-stimulated Vero cells. When the cells were treated with H₂O₂, the protein levels of Bax, cleaved caspase-9, and PARP increased; however, BDB inhibited the expression of Bax, cleaved caspase-9, and PARP, demonstrating that BDB significantly affects the mechanism of apoptosis by regulating Bax.

NF-κB is a transcription factor thought to play a crucial role in the onset of cell apoptosis. The transcription factor NF-κB exists as a heterodimer of p50 and p65 and is bound in the cytoplasm by IκB [42]. In previous studies, it was indicated that H₂O₂ induced the activation of NF-κB in several types of cells [43], and ROS were found to stimulate the NF-κB signaling pathway directly [44, 45]. Moreover, the activation of NF-κB is inhibited by antioxidants [46]. In the present study, we confirmed the effect of BDB on the activation of NF-κB in H₂O₂-exposed Vero cells. H₂O₂ induced the activation of NF-κB in Vero cells; however, BDB markedly inhibited this activation. Besides, when Vero cells were exposed to H₂O₂, the nuclear translocation of p65 increased, but when cells were pre-treated with BDB, the translocation of p65 was considerably suppressed. These results suggest that BDB suppresses H₂O₂-induced apoptosis by regulating the NF-κB pathway.

The zebrafish model has been used in a variety of research fields, and it has many benefits such as a small size, rapid development, optical transparency, and easy handling in experimental settings [47–49]. In particular, researchers have used zebrafish as animal models in drug discovery studies investigating oxidative stress [47, 50]. Therefore, we evaluated the effect of BDB on survival rate, generation of ROS, cell death, and lipid peroxidation using H₂O₂-induced oxidative stress in the zebrafish model. All concentrations of BDB were non-toxic in zebrafish. H₂O₂ induced ROS generation, cell death, and lipid peroxidation in zebrafish larvae. However, treatment of zebrafish larvae exposed to H₂O₂ with BDB significantly reduced generation of ROS and induction of cell death. BDB reduced lipid peroxidation, although the difference was not significant. Therefore, these results demonstrated that BDB could protect against H₂O₂-caused oxidative stress in the zebrafish model.

In conclusion, we demonstrated that BDB decreased H₂O₂-induced oxidative stress in Vero cells by reducing ROS levels and lipid peroxidation. Furthermore, BDB

blocked H₂O₂-induced apoptosis via the regulation of Bax and NF-κB. Moreover, BDB inhibited oxidative stress, including ROS and NO generation in zebrafish larvae. Taken together, BDB protects against oxidative stress in vitro and in vivo, demonstrating that BDB may be an effective antioxidant for use as an additive in functional foods.

Acknowledgments

This research was supported by Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (grant number: NRF-2016R1D1A1B03933092) and supported by a grant of the Korea Health Technology R&D Project through the Korea Health Industry Development Institute (KHIDI), funded by the Ministry of Health & Welfare, Republic of Korea (grant number: HI15C1540).

Conflict of Interest

The authors have no financial conflicts of interest to declare.

References

1. Alfadda A-A, Sallam R-M. 2012. Reactive oxygen species in health and disease. *J. Biomed. Biotechnol.* **2012**: 1-14.
2. Oh Baek S, Kim H, Jeong H, Ju E, Kong CS, Seo Y. 2015. Antioxidant activity of the halophyte *ligustrum japonicum*. *KSBB J.* **30**: 275-282.
3. Silva F, Marques A, Chaveiro A. 2010. Reactive oxygen species: a double-edged sword in reproduction. *Open Vet. Sci. J.* **4**: 127-133.
4. Hong CO, Hong ST, Lee YH, Lee KW. 2012. Protective effects of *Plantago asiatica* L. extract against ferric-nitrosyltriacetate (Fe-NTA) induced liver oxidative stress in Wistar rats. *FASEB J.* **26**: 107-113.
5. Kang MC, Kim EA, Kang SM, Wijesinghe WJ, Yang X, Kang NL, et al. 2012. Thermostability of a marine polyphenolic antioxidant dieckol, derived from the brown seaweed *Ecklonia cava*. *Algae*. **27**: 205-213.
6. Berlett B-S, Stadtman E-R. 1997. Protein oxidation in aging, disease, and oxidative stress. *J. Biol. Chem.* **272**: 20313-20316.
7. Heo SJ, Cha SH, Lee KW, Jeon YJ. 2006. Antioxidant activities of red algae from Jeju Island. *Algae* **21**: 149-156.
8. Kasuga A, Aoyagi Y, Sugahara T. 1988. Antioxidant activities of edible plants. *Nippon Shokuhin Kogyo Gakkaishi*. **35**: 828-834.
9. Kim JJ, Kim SJ, Kim SH, Park HR, Lee SC. 2005. Antioxidant and anticancer activities of extracts from *styela plicata*. *J. Korean Soc. Food. Sci. Nutr.* **34**: 937-941.

10. Choi W-Y, Lee H. 2014. Enhancement of antioxidant activities of curcuma longa leaves by ultra high pressure extraction. *Korean J. Med. Crop. Sci.* **22**: 121-126.
11. Fujihara M, Nagumo T. 1993. An influence of the structure of alginate on the chemotactic activity of macrophages and the antitumor activity. *Carbohydr. Res.* **243**: 211-216.
12. M König G, Wright A-D, Sticher O, Angerhofer C, M Pezzuto J. 1995. Biological activities of selected marine natural products. *Planta. Med.* **60**: 532-537.
13. Cha SH, Ahn GN, Heo SJ, Kim KN, Lee KW, Song CB, et al. 2006. Screening of extracts from marine green and brown algae in Jeju for potential marine angiotensin-I converting enzyme (ACE) inhibitory activity. *J. Korean Soc. Food. Sci. Nutr.* **35**: 307-314.
14. Aleksandar R, Stefanie T, Achenbach J, Andreas K, Anna V, Tanja K, et al. 2010. Anionic polysaccharides from phototrophic microorganisms exhibit antiviral activities to vaccinia virus. *J. Antivir. Antiretrovir.* **2**: 51-55.
15. Tannin-Spitz T, Bergman M, van-Moppes D, Grossman S, Arad S. 2005. Antioxidant activity of the polysaccharide of the red microalga *Porphyridium* sp. *J. Appl. Phycol.* **17**: 215-222.
16. Matsui M, Muizzuddin N, Arad S, Marenus K. 2003. Sulfated polysaccharides from red microalgae have anti-inflammatory properties in vitro and in vivo. *Appl. Biochem. Biotechnol.* **104**: 13-22.
17. Jung WK, Je JY, Kim HJ, Kim SJ. 2002. A novel anticoagulant protein from scapharca broughtonii. *J. Biochem. Mol. Biol.* **35**: 199-205.
18. Kim SY, Ryel Kim S, Oh MJ, Jung SJ, Kang S. 2011. In vitro antiviral activity of red alga, polysiphonia morrowii extract and its bromophenols against fish pathogenic infectious hematopoietic necrosis virus and infectious pancreatic necrosis virus. *J. Microbiol.* **49**: 102-106.
19. Saravanakumar D, Folb P, Campbell B, Smith P. 2008. Antimycobacterial activity of the red alga polysiphonia virgata. *Pharm. Biol.* **46**: 254-260.
20. Jae Hyun Y, Jing Piao M, Zhang R, Choi Y, Chae S, Won Hyun J. 2012. Photo-protection by 3-bromo-4, 5-dihydroxybenzaldehyde against ultraviolet B-induced oxidative stress in human keratinocytes. **83**: 71-78.
21. Piao M-J, Kang K-A, Ryu Y-S, Shilnikova K, Park J-E, Hyun Y-J, et al. 2017. The red algae compound 3-bromo-4,5-dihydroxybenzaldehyde protects human keratinocytes on oxidative stress-related molecules and pathways activated by UVB irradiation. *Marine Drugs* **15**: 268.
22. Choi K-Y, Kim J, Lee M-K, Choi YJ, Ye BR, Kim MS, et al. 2017. Tuberatolide B suppresses cancer progression by promoting ROS-mediated inhibition of STAT3 signaling. *Marine Drugs* **15**(3). pii: E55. doi: 10.3390/md15030055.
23. Mikami D, Kurihara H, Kim S-M, Takahashi K. 2013. Red algal bromophenols as glucose 6-phosphate dehydrogenase inhibitors. *Mar. Drugs* **11**: 4050-4057.
24. Ahn GN, Kim KN, Cha SH, Song CB, Lee J, Heo MS, et al. 2007. Antioxidant activities of phlorotannins purified from Ecklonia cava on free radical scavenging using ESR and H2O2-mediated DNA damage. *Eur. Food Res. Technol.* **226**: 71-79.
25. Ko YJ, Ahn G, Ham YM, Song SM, Ko EY, Cho SH, et al. 2017. Anti-inflammatory effect and mechanism of action of Lindera erythrocarpa essential oil in lipopolysaccharide-stimulated RAW264.7 cells. *EXCLI J.* **16**: 1103-1113.
26. Yang HM, Ham YM, Yoon WJ, Roh S-W, Jeon YJ, Oda T, et al. 2012. Quercitrin protects against ultraviolet B-induced cell death in vitro and in an in vivo zebrafish model. *J. Photochem. Photobiol. B.* **114**: 126-131.
27. Ko EY, Cho SH, Kang K, Kim G, Lee JH, Jeon YJ, et al. 2017. Anti-inflammatory activity of hydrosols from Tetragonia tetragonoides in LPS-induced RAW 264.7 cells. *EXCLI J.* **16**: 521-530.
28. Aybek H, Aybek Z, Rota S, Şen N, Akbulut M. 2008. The effects of diabetes mellitus, age, and vitamin E on testicular oxidative stress. *Fertil. Steril.* **90**: 755-760.
29. Li M, Liu Z, Zhuan L, Wang T, Guo S, Wang S, et al. 2012. Effects of apocynin on oxidative stress and expression of apoptosis-related genes in testes of diabetic rats. *Mol. Med. Rep.* **7**: 47-52.
30. Tang XY, Zhang q, Dai DZ, Ying HJ, Wang QJ, Dai Y. 2008. Effects of strontium fructose 1,6-diphosphate on expression of apoptosis-related genes and oxidative stress in testes of diabetic rats. *Int. J. Urol.* **15**: 251-256.
31. Niki E. 2008. Lipid peroxidation products as oxidative stress biomarkers. *BioFactors* **34**: 171-180.
32. Cidlowski J-A, Schwartzman R-A. 1993. Apoptosis: the biochemistry and molecular biology of programmed cell death. *Endocr. Rev.* **14**: 133-151.
33. Kannan K, Jain S-K. 2000. Oxidative stress and apoptosis. *Pathophysiology* **7**: 153-163.
34. Kerr J-F, Wyllie A-H, Currie A-R. 1972. Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics. *Br. J. Cancer* **26**: 239-257.
35. M, Adams J, Cory S. 1998. The Bcl-2 protein family: arbiters of cell survival. *Science* **281**: 1322-1326.
36. L. Jones N, Islur A, Haq R, Mascarenhas M, Karmali M, Perdue M, et al. 2000. Escherichia coli Shiga toxins induce apoptosis in epithelial cells that is regulated by the BCL-2 family. *Am. J. Physiol. Gastrointest. Liver Physiol.* **278**: G811-819.
37. Finucane D-M, Bossy-Wetzel E, Waterhouse N-J, Cotter T-G, Green D-R. 1999. Bax-induced caspase activation and apoptosis via cytochrome c release from mitochondria is inhibitable by Bcl-xL. *J. Biol. Chem.* **274**: 2225-2233.
38. Cohen G-M. 1997. Caspases: the executioners of apoptosis. *Biochem. J.* **326**: 1-16.
39. Sun XM, MacFarlane M, Zhuang J, Wolf B-B, Green D-R, Cohen G-M. 1999. Distinct caspase cascades are initiated in

- receptor-mediated and chemical-induced apoptosis. *J. Biol. Chem.* **274**: 5053-5060.
40. Ham YM, Yoon WJ, Park SY, Song GP, Jung YH, Jeon YJ, *et al.* 2012. Quercitrin protects against oxidative stress-induced injury in lung fibroblast cells via up-regulation of Bcl-xL. *J. Funct. Foods* **4**: 253-262.
 41. Oliver F-J, de la Rubia G, Rolli V, Ruiz-Ruiz M-C, de Murcia G, Ménissier-de Murcia J. 1998. Importance of poly (ADP-ribose) polymerase and its cleavage in apoptosis Lesson from an uncleavable mutant. *J. Biol. Chem.* **273**: 33533-33539.
 42. Baeuerle P-A. 1998. Pro-inflammatory signaling: last pieces in the NF- κ B puzzle? *Curr. Biol.* **8**: R19-R22.
 43. Schreck R, Rieber P, Baeuerle P-A. 1991. Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-kappa B transcription factor and HIV-1. *EMBO J.* **10**: 2247-2258.
 44. CH Chen A, Arany P, Huang YY, M Tomkinson E, Sharma S, B Kharkwal G, *et al.* 2011. Low-level laser therapy activates NF- κ B via generation of reactive oxygen species in mouse embryonic fibroblasts. *PLoS One* **6**: e22453.
 45. Morgan M-J, Liu Zg. 2011. Crosstalk of reactive oxygen species and NF- κ B signaling. *Cell Res.* **21**: 103-115.
 46. Schenk H, Klein M, Erdbrugger W, Droge W, Schulze-Osthoff K. 1994. Distinct effects of thioredoxin and antioxidants on the activation of transcription factors NF-kappa B and AP-1. *Proc. Natl. Acad. Sci. USA* **91**: 1672-1676.
 47. Kim EA, Kang MC, Lee JH, Kang N, Lee W, Oh JY, *et al.* 2015. Protective effect of marine brown algal polyphenols against oxidative stressed zebrafish with high glucose. *RSC Adv.* **5**: 25738-25746.
 48. Lan CC, Tang R, San Leong I-U, Love D-R. 2009. Quantitative real-time RT-PCR (qRT-PCR) of zebrafish transcripts: optimization of RNA extraction, quality control considerations, and data analysis. *Cold Spring Harb. Protoc.* **2009(10)**: pdb. prot5314.
 49. Zou Y, Zhang Y, Han L, He Q, Hou H, Han J, *et al.* 2017. Oxidative stress-mediated developmental toxicity induced by isoniazide in zebrafish embryos and larvae. *J. Appl. Toxicol.* **37**: 842-852.
 50. Kobayashi M, Li L, Iwamoto N, Nakajima-Takagi Y, Kaneko H, Nakayama Y, *et al.* 2009. The antioxidant defense system Keap1-Nrf2 comprises a multiple sensing mechanism for responding to a wide range of chemical compounds. *Mol. Cell. Biol.* **29**: 493-502.