A THESIS

FOR THE DEGREE OF MASTER OF PHYLOSOPHY

Antioxidant activity of 5-bromoprotocatechualdehyde from *Polysiphonia morrowii* Harvey

Su-Hyeon Cho

Department of marine life science

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

2018. 02

Antioxidant activity of 5-bomoprotocatechualdehyde from *Polysiphonia morrowii* Harbvey

Su-Hyeon Cho

(Supervised by Professor You-Jin Jeon)

A thesis submitted in partial fulfillment of the requirement for the degree of Master of Science

This thesis has been examined and approved by

Thesis director, Seungheon Lee, Professor of Marine Life Science

Kil-Nam Kim, Senior researcher of Korea Basic Science Institute

You-Jin Jeon, Professor of Marine Life Science

2018. 2

Department of marine life science

GRADUATE SCHOOL

JEJU NATIONAL UNIVERSITY

CONTENTS

국문초록

현대 사회는 환경, 식품, 또는 생활 습관에 의한 다양한 산화스트레스에 노출되어 있다. 대부분의 생물은 정상적인 호흡 과정에서 산소를 체내로 받아들이게 된다. 체내로 들어온 산소는 물질대사과정에서 활성산소를 생성하는데, 신체 내에 병원균이 침투했을 때 백혈구가 작용하게 되는데, 이 때 적당한 양의 활성산소는 이에 도움을 주어 병원균을 제거하여 신체를 보호하는 기능을 한다. 하지만 과도하게 축적이 되었을 경우, 세포에 손상을 줌으로써 여러 가지 질병을 일으키는 원인이 되기도 한다. 이 때 우리 몸에서 생성되는 superoxide dismutase, catalase, 그리고 glutathione peroxidase (GPX)와 같은 항산화 효소에 의해서 대사과정 중에 생성되는 활성산소를 제거가 됨으로써 신체 내에서 ROS가 일정한 양으로 유지된다. 하지만 자외선, 대기오염, 흡연 등과 같은 환경적인 요인에 의해서 과도하게 발생된 활성산소는 항산화 효소 기전 만으로 완전히 조절을 할 수 없기 때문에 결국 다양한 질병을 유발한다. 그렇기 때문에 이를 억제할 수 있는 효과적인 항산화제가 필요한 실정이다. 항산화제는 합성항산화제와 천연항산화제로 나뉘어 있는데, 합성항산화제는 변이성과 암을 유발할 수 있는 독성이 있기 때문에, 보다 안전한 천연물 유래의 항산화제에 대한 연구가 많이 이루어지고 있다. 최근 해조류 유래의 소재들을 이용하여 기능성 소재로서의 후보물질을 탐색하는 연구가 많이 이루어지고 있다. 본 연구에서 선택한 해조류는 홍조류인 *Polysiphonia mnorrowii* Harvey이며 part 1에서는 in vitro와 in vivo 상에서 *Polysiphonia* sp. 에서 분리한 단일물질인 5-bromoprotocatechualdehyde (BPCA)의 항산화 활성을 평가하였다. 그 결과 in

vitro 상에서 항산화 효소의 발현 증가, Bax와 NF-κB 기전 억제를 통해 산화스트레스에 의한 apoptosis를 억제하였으며, in vivo 모델인 zebrafish에서 산화스트레스에 의한 ROS, cell death, 그리고 지질과산화 발생을 억제함으로써, in vitro와 in vivo 상에서 BPCA가 항산화 활성이 뛰어남을 확인할 수 있었다.

최근 전 세계적으로 인구 고령화 현상이 빠르게 진행되고 소득 수준 높아지면서 삶의 질에 대한 관심이 증가하면서 의약시장과 항노화 산업이 부상하고 있으며, 국가 사회적 중요성도 높아지고 있다. 노화는 시간의 흐름에 따라 신체의 구조와 기능이 저하되고 질병과 사망에 대한 감수성이 증가하여 쇠약해지는 과정이다. 노화는 크게 내재적 노화와 외재적 노화로 나눌 수 있다. 내재적 노화는 유전적인 요인이 원인인 반면, 외재적 노화는 환경적인 요인이 원인이다. 이러한 환경적인 요인에 의해 발생한 ROS가 노화를 일으키는 중요한 인자이다. 과산화수소에 의해 발생한 산화스트레스는 세포 노화를 일으키는 요인으로 잘 알려져 있기 때문에, 노화연구에 과산화수소가 많이 사용되고 있다. 하지만 노화가 일어나는 기전이 아직 명확히 밝혀지지 않은 실정이다. Part 2에서는 in vitro와 in vivo 상에서 BPCA의 항노화 활성을 평가하였다. 그 결과, in vitro 상에서 BPCA가 노화의 중요한 표지로 알려진 senescence-associated β-galactosidase의 활성을 억제하였다. 또한 노화와 cell cycle arrest의 관련성을 확인한 결과, 과산화수소를 처리하였을 때 G2기가 arrest 되는 것을 확인하였다. 하지만 BPCA를 같이 처리하였을 때 arrest를 억제하지 못함으로써 본 실험의 노화억제와 관련이 없음을 확인하였다. ROS 조절에 있어서 중요한 역할을 하는 항산화 효소의 작용을 확인한 결과 과산화수소를 처리하였을 때 GPX가 감소하는 것을 확인하였으나 BPCA를 같이 처리하였을 때 증가하는 것을 확인하였다. 이와 같은

결과는 BPCA가 항산화 효소 발현을 증가시킴으로써 산화스트레스를 줄여 노화 억제에 관여하는 것으로 예측되었다. In vivo 모델인 zebrafish에서도 senescence-associated β-galactosidase의 활성이 억제됨으로써 BPCA가 zebrafish에서도 노화를 억제할 수 있다는 것을 확인할 수 있었다.

그러므로 BPCA는 항산화 및 항염증 활성을 가진 기능성 소재로서 이용이 가능할 것으로 보여진다.

LIST OF FIGURE

Introduction

Modern society is exposed to various types of oxidative stress by environment, diet, or lifestyle. Highly reactive molecules such as reactive oxygen species (ROS) and free radicals such as hydrogen peroxide (H_2O_2), superoxide anion radicals (O_{2-}), hydroxyl radicals ($HO^{\cdot}$), and singlet oxygen ($^1O_2$) are generated by oxidative stress in body. ROS produced in body is eliminated by the antioxidant defense mechanisms. However, when these defense mechanisms do not work properly, ROS causes damage to the constituent parts of the cell (Alfadda et al., 2012; da Silva et al., 2010; Baek et al, 2015). It causes oxidative stress resulting in damage of DNA, lipid, protein and other molecules that lead to harmful effects such as Parkinson's disease, Alzheimer's disease, diabetes, liver injury, cancer, inflammation, and aging (Barbara S. Berlett and Earl R. Stadtman, 1997; Hong et al., 2011; Kang et al., 2012; Heo et al., 2006). The study of antioxidants has been done from long ago and currently, there are a number of used chemical synthesis antioxidants such as BHA (butylated hydroxyanisol) and BHT (butylated hydroxytoluene). It requires development of more secure and active natural antioxidants due to function as mutagens and tumor promoters at high dosages (Kasuga et al., 1988; Branen, 1975; Song et al., 2000). Therefore, recently there is a lot of researches about antioxidants derived from natural products. (Kasuga et al., 1988; Kim et al., 2005; Choi and Lee, 2014).

Recent changes in the standard of living and the development of medical technology have been accompanied by an aging population as the lifespan has been extended. The entry of an aging society, it is growing interest in improving the quality of life and health, not simply extending life expectancy. Accordingly anti-aging markets are rapidly increasing and anti-aging research is progressed actively (Manosroi et al., 2009; Satoh et al., 2005). Aging refers to the process of increasing the risk of death due to change in the body according to the

passage of time. When aging happens, there is the reduction on adaptive capability about stress such as decreasing physiological functions and increasing susceptibility to diseases (Harman, 2006; Marieke et al., 2007; Cutler and Mattson, 2006; Harman, 2001; Vijg and Wei, 1995; Kwon, 2007). The theory of aging is divided into two categories. Intrinsic aging is the theory of genetic etiology that there is the information determining the lifespan preserved in the gene and according to the information, aging is progressed. On the other hands, extrinsic aging is the theory of environmental etiology, which is progressed by various barriers or aging materials. In particular, the ROS produced by UV and respiration is considered to be the most important factors of the aging (Farage et al., 2007; Hong, 2009). Among ROS, H_2O_2 is widely used to aging research because it is known to lead to aging of cells (Choi et al., 2012; Suo et al., 2013; Kim et al., 2011). When the cells were treated H_2O_2, it is known that cellular aging is induced after cell cycle is leaded (Zhou et al., 2005; Duan et al., 2005; Choo et al., 2014). Therefore, in this study, we identified effect of anti-aging using the H_2O_2.

For the past, marine algae traditionally have been used as food and therapeutic agent for various diseases. Marine algae are broadly well-known that produce a various compounds and some of them have biological activity of therapeutic value. (Fujihara and Nagumo, 1993; Konig et al., 1994; Farvine and Jacobsen, 2013) Research at marine algae is focused according to finding biologically active compounds. Recently, interest in natural physiological materials of natural organisms is continuously increasing. And the studies of biological active substances from marine algae are being carried out by many researchers, not only nutritious aspects but also treatment of diseases and health maintenance (Cha et al., 2006). Marine algae-derived compounds have been proven to many functions such as antiviral, antioxidant, anti-inflammatory, and anticoagulant activities (Radonic et al., 2011; Spitz et al., 2005; Matsui et al., 2003; Jung et al., 2001). In previous studies, various function of *Polysiphonia* sp. have been reported such as antioxidant, antviral, antimycobacterial

activity (Kim et al., 2011; Saravanakumar et al., 2008). However, There is a lack of research about anti-aging activity of *Polysiphonia* sp.. Expecially, studies on antioxidant and anti-aging activity of 5-bromoprotocatechualdehyde (BPCA) from *P. morrowii* Harvey has not been conducted.

Therefore, the purpose of the present study was to examine the antioxidant activity and anti-aging of BPCA from *P. morrowii* Harvey in hydrogen peroxide (H_2O_2)-stimulated cell and zebrafish.

Part 1

Evaluation of antioxidant activity of 5-bromoprotocatechualdehyde from *Polysiphonia morrowii* Harvey

1. Abstract

We investigated the protective effects of 5-bromoprotocatechualdehyde (BPCA) from *Polysiphonia morrowii* Harvey against hydrogen peroxide (H_2O_2)-induced apoptosis in Vero cells. BPCA exhibited scavenging activity for 1,1-diphenyl-2-picrylhydrazyl (DPPH), hydroxyl, and alkyl radicals. BPCA also inhibited H_2O_2-induced cell death and apoptosis in Vero cells by inhibiting the production of ROS and lipid peroxidation. Regarding the antioxidant enzymes in the cells, there were no significant changes in superoxide dismutase and catalase levels but a marked decrease in glutathione peroxidase (GPx) following treatment with H_2O_2. Pretreatment with BPCA prevented the reduction in GPx. To investigate the molecular mechanisms of apoptosis inhibition, the expression of Bax/Bcl-xL and NF-κB was assessed by western blot assay. H_2O_2 induced the cleavage of caspase-9 and poly(ADP-ribose) polymerase (PARP) and increased Bax levels. However, BPCA significantly inhibited the cleavage of caspase-9 and PARP and reduced Bax levels. In addition, BPCA suppressed the activation of the phosphorylation of NF-κB and the translocation of p65 in H_2O_2-induced cells. Furthermore, we evaluated the effect of BPCA on ROS production, cell death, and lipid peroxidation generation in a H_2O_2-stimulated zebrafish embryo model. The results indicated that cell death and ROS production were significantly inhibited by BPCA in zebrafish embryos. Taken together, BPCA has excellent antioxidant activity *in vitro* and *in vivo*.

2. MATERIAL AND METHOD

2. 1. Materials

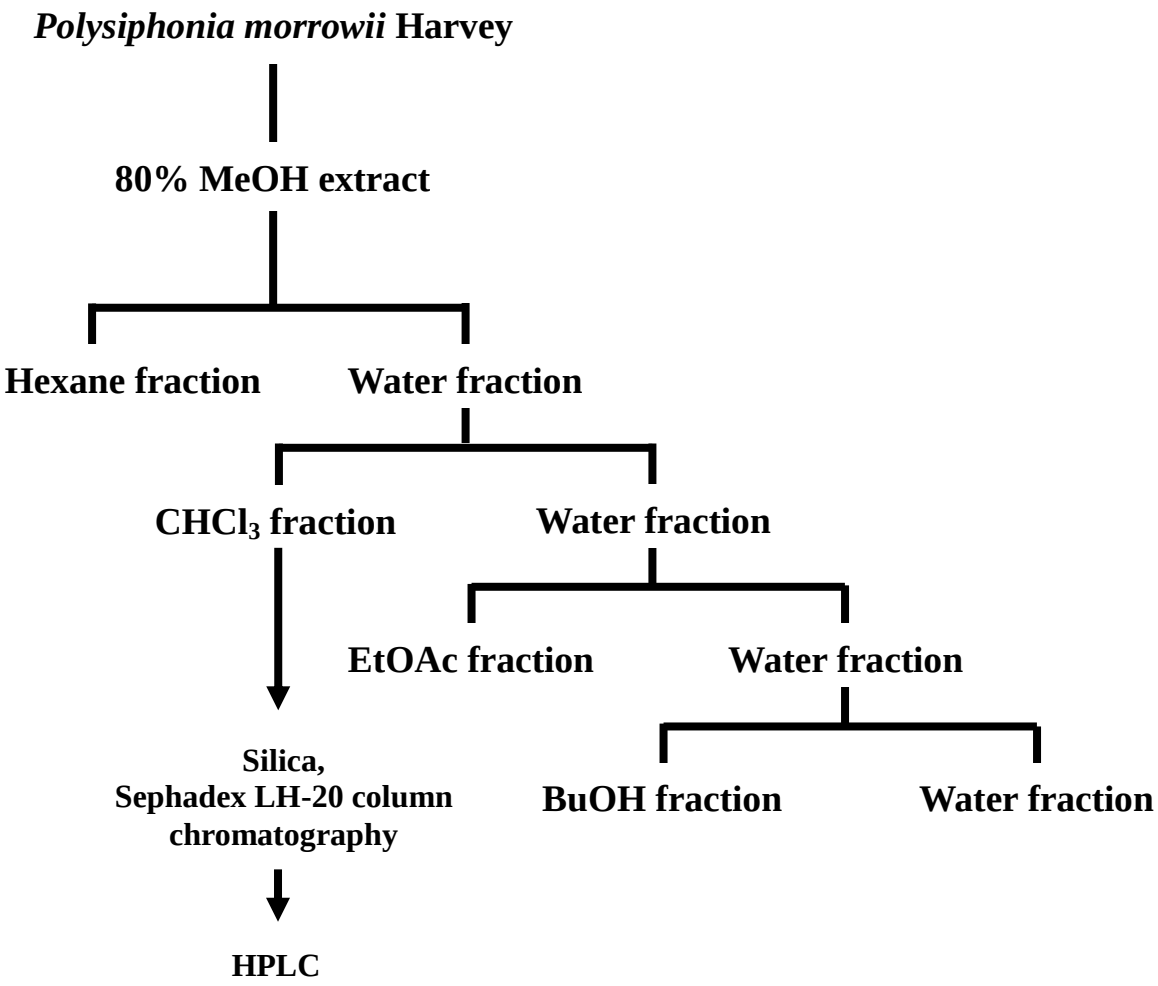
Roswell Park Memorial Institute (RPMI)-1640, phosphate buffered saline (PBS), antibiotics, and trypsin-EDTA were purchased from Gibco/BRL (Burlington, Ont, Cananda). Fetal bovine serum (FBS) was purchased from Welgene (Daegu, Korea). Dimethyl sulfoxide, 1,1-diphenyl-2-picrylhydrazyl (DPPH), 2′,7′-dichlorodihydrofluorescein diacetate (DCHF-DA), diphenyl-1-pyrenylphosphine (DPPP), acridine orange, *N*-acetyl-*L*-cysteine (NAC), Hoechst 33342, propidium iodide (PI), and RIPA buffer were purchased from Sigma (St. Louis, MO, USA). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Amresco (Solon, OH, USA). RNase A was purchased from Roche (Canada). NUPAGE 4~12% Bis-Tris gel and iBlot® Transfer Stack, PVDF Regular were purchased from Invitrogen (Carlsbad, CA). Primary antibodies against Bax, Bcl-xL, cleaved caspase-9, PARP, phospho-NF-κB p65, phospho-NF-κB p105, NF-κB p65, superoxide dismutase 1 (SOD1), SOD2, catalase, and glutathione peroxidase 1 (GPX1) and secondary antibodies against anti-rabbit IgG, HRP-linked antibody and anti-mouse IgG, HRP-linked antibody were purchased from Cell signaling Technologies (Bedford, MA, USA). Primary antibodies against β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). SuperSignal West Femto Trial kits were purchased from Thermo Fisher Scientific (Waltham, Massachusetts, USA). Vectashield mounting solution was purchased from Vector (Burlingame, CA).

2. 2. Extraction and isolation of BPCA from *P. morrowii* Harvey

The red alga *P. morrowii* Harvey was collected along the coast of Jeju Island, Korea.

The sample was washed thrice with tap water to remove salt, sand, and epiphytes attached to its surface, followed by careful rinsing with fresh water and freezing in a medical refrigerator at -20℃. Thereafter, the frozen sample was lyophilized and homogenized with a grinder prior to extraction. All chemicals and reagents used were of analytical grade and sourced from trusted commercial sources. The dried *P. japonica* powder was extracted thrice with 80% aqueous methanol at room temperature. The liquid layer was obtained via filtration, and the filtrate was concentrated using an evaporator under reduced pressure. The extract was suspended in water, and the aqueous layer was partitioned with chloroform. Then, the chloroform fraction was fractionated by silica column chromatography with stepwise elution with a chloroform-methanol mixture (30:1→1:1) to separate the active fractions in the chloroform extract. A combined active fraction was further subjected to a Sephadex LH-20 column saturated with 100% methanol and then purified by reversed phase high-performance liquid chromatography (HPLC) using a Waters HPLC system (Alliance 2690; Waters Corp., Milford, MA, USA) equipped with a Waters 996 photodiode array detector and C18 column (J'sphere ODS-H80, 250 × 4.6 mm, 4 μm; YMC Co., Kyoto, Japan) by stepwise elution with a methanol-water gradient (UV range, 290 nm; flow rate, 1 ml/min). Finally, the purified compound was identified by comparing their ^{1}H and ^{13}C NMR data with those in the literature (Mikami et al., 2013). The chemical structure of the purified compound is indicated in Fig. 1-1. The compound was dissolved in 100 % ethanol and employed in experiments in which the final concentration of 100 % ethanol in the culture medium was adjusted to <0.01 %.

(A)



(B)

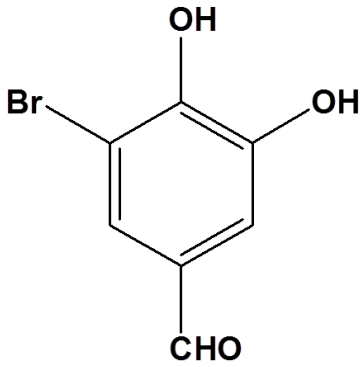


Fig. 1-1. (A) Isolation scheme for BPCA from the algae *Polysiphonia morrowii* Harvey and (B) chemical structure of BPCA

2. 3. Cell culture

Vero cells, kidney cells from African green monkeys, were purchased from the Korean Cell Line Bank (KCLB, Seoul, Korea). The cells were grown at 37 ℃ in a 5 % CO_2 humidified atmosphere incubator using RPMI-1640 medium supplemented with 10 % FBS and 1 % antibiotics.

2. 4. DPPH radical scavenging activity

First, 60 μl of BPCA (or ethanol as a control) was added to 60 μl of 60 μM 1,1-diphenyl-2-picrylhydrazyl (DPPH). The mixture was vortexed and incubated for 2 h and then transferred to a 100 μl capillary tube, which was fitted into the cavity of an ESR spectrometer (JES-FA machine, JOEL, Tokyo, Japan). The measurement conditions were as follows: power, 1 mW; amplitude, 5 × 100; modulation width, 0.8 mT; sweep width, 10 mT; sweep time, 30 s; and time constant, 0.03 s.

2. 5. Hydroxyl radical scavenging activity

First, 20 μl of BPCA (or ethanol as a control) was added to 20 μl of 10 mM H_2O_2, 10 mM $FeSO_4$, and 0.3 M DMPO. The mixture was vortexed and incubated for 3 min and then transferred to a 100 μl capillary tube, which was fitted into the cavity of an ESR spectrometer (JES-FA machine, JOEL, Tokyo, Japan). The measurement conditions were as follows: power, 1 mW; amplitude, 1 × 1000; modulation width, 0.1 mT; sweep width, 10 mT; sweep time, 30 s; and time constant, 0.03 s.

2. 6. Alkyl radical scavenging activity

First, 20 μl of BPCA (or ethanol as a control) was added to 20 μl of 40 mM AAPH and 40 mM POBN. The mixture was vortexed and incubated at 37℃ for 30 min in a water bath and then transferred to a 100 μl capillary tube, which was fitted into the cavity of an ESR spectrometer (JES-FA machine, JOEL, Tokyo, Japan). The measurement conditions were as follows: power, 7~8 mW; amplitude, 5 × 100; modulation width, 0.2 mT; sweep width, 10 mT; sweep time, 30 s; and time constant, 0.03 s.

2. 7. 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 μM BPCA and 1 M *N*-acetyl-*L*-cysteine (NAC) for 2 h at 37 ℃. Then, 500 μM H_2O_2 was added for 24 at 37 ℃. The cells were incubated with 3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) dissolved in PBS for 3 h. After the supernatants were removed, formazan was dissolved in dimethyl sulfoxide (DMSO). The absorbance was read at 540 nm using a spectrophotometer (Molecular Devices, USA).

2. 8. Measurement of intracellular reactive oxygen species (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 BPCA for 2 h at 37 ℃. Then, 500 μM H_2O_2 was added followed by incubation for 30 min at 37 ℃. Then, the cells were treated with DCHF-DA (20 μM) for 30 min at 37 ℃ in the dark. DCHF-DA was detected at an excitation wavelength of 485 nm and an emission wavelength of 535 nm using a

spectrophotometer (Molecular Devices, USA).

2. 9. Nuclear staining with Hoechst 33342

The nuclear morphology of cells was studied using the cell-permeable DNA dye Hoechst 33342. 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 BPCA and 1 M NAC for 2 h at 37 ℃. Then, 500 μM H_2O_2 was added followed by incubation for 6 h at 37 ℃. Then, 2 μl of Hoechst 33342 (10 mg/ml) was added to cells followed by incubation for 10 min at 37 ℃. The cells were observed using a fluorescence microscope (Zeiss, Iena, Germany).

2. 10. 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 BPCA for 2 h at 37 ℃. Then, 500 μM H_2O_2 was added followed by incubation for 6 h at 37 ℃. After the media were removed, the cells were washed with PBS. The cells were collected using trypsin-EDTA and washed twice with PBS. The cells were then fixed using cold 70 % ethanol at 4 ℃ before use. The fixed cells were washed with PBS and incubated in PBS-2 mM EDTA containing 100 μg/ml PI and 100 μg/ml RNase for 30 min at 37 ℃ in the dark. Flow cytometry was performed using a Beckman Coulter CytoFLEX flow cytometer (Beckman Coulter, Miami, FL).

2. 11. Western blot analysis

After the media were removed, the cells were washed two times using PBS. RIPA buffer (Sigma Aldrich) was added followed by incubation for 10 min at 4 ℃. Then, cell lysates were collected, vortexed 6 times for 1 h, and centrifuged at 13,300 rpm at 4 ℃ for 25 min. After the protein concentration was determined using Protein Assay Dye Reagent Concentrate (BIO-RAD), the protein samples were electrophoresed and transferred to PVDF membranes (Invitrogen). The membranes were blocked for 2 h at room temperature and incubated with the primary antibody (anti-bax, anti-bcl-xL, anti-PARP, and anti-cleaved caspase 9, anti-GPX1, anti-SOD1, anti-SOD2, anti-catalase, anti-phospho-NF-κB p65, anti-phospho-NF-κB p105, and anti-β-actin) overnight at 4 ℃. The membranes were washed using TTBS (20 mM Tris, 137 mM NaCl, 0.1 % tween 20) and incubated with the secondary antibody (anti-rabbit IgG, HRP-linked antibody and anti-mouse IgG, HRP-linked antibody) at room temperature. After 2 h, bands were detected using a SuperSignal West Femto Trial kit (Thermo Fisher Scientific).

2. 12. 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 BPCA for 2 h at 37℃. Then, 500 μM H_2O_2 was added followed by incubation for 45 min at 37℃. The cells were fixed using 4 % formaldehyde for 10 min at room temperature and permeabilized with 100 % methanol for 10 min at -20℃. After the cells were rinsed three times using PBS, the cells were blocked with 1 % BSA in PBS at room temperature for 1 h. After the cells were rinsed two times using PBS, the cells were incubated with 4 % Triton X-100 at room temperature for 30 min. The cells were rinsed three times using PBS and incubated with the primary antibody p65

(1:200, Cell signaling) overnight at -20 ℃. Next, the cells were rinsed three times using PBS and incubated with Alexa Fluor488-labeled Rabbit IgG (1:500, Invitrogen) for 1 h at room temperature. The cells were rinsed three times with PBS and then stained with DAPI using Vectashield mounting solution. Fluorescence was analyzed using an LSM 780 Zeiss confocal laser microscope (Zeiss, Iena, Germany).

2. 13. Origin and maintenance of zebrafish

Zebrafish were kept under the following conditions: 28.5±0.5℃ 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 transferred 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).

2. 14. Effect of BPCA on H_2O_2-induced ROS generation in zebrafish

ROS production in zebrafish was analyzed using the oxidation sensitive fluorescent dye 2,7-dichlorodihydrofluorescin diacetate (DCFH-DA). After fertilization, 7-9 hours post-fertilization (hpf) zebrafish embryos were transferred to a 12 well plate. The embryos were pre-treated with various concentrations of BPCA (6.25, 12.5, 25, and 50 μM) for 2 h and then treated with 5 mM H_2O_2 for 3 dpf. The zebrafish larvae were transferred to a 24 well plate, and 4 μg/ml DCFH-DA solution was followed by incubation for 1 h in the dark at 37℃. After incubation, the zebrafish larvae were rinsed twice using fresh embryo media and anesthetized using 0.03 % ethyl 3-aminobenzoate methanesulfonate before observation. The

zebrafish larvae were photographed under a microscope equipped with a CoolSNAP-pro color digital camera (Olympus, Japan). The fluorescence intensity of zebrafish larvae was quantified using ImageJ software.

2. 15. Effect of BPCA on H_2O_2-induced cell death in zebrafish

Cell death in zebrafish was measured using a nucleic acid-selective metachromatic dye, acridine orange, which interacts with DNA and RNA. After fertilization, 7-9 hpf zebrafish embryos were transferred to a 12 well plate. The embryos were pre-treated with various concentrations of BPCA (6.25, 12.5, 25, and 50 μM) for 2 h and then treated with 5 mM H_2O_2 for 3 day post-fertilization (dpf). The zebrafish larvae were transferred to a 24 well plate, and 7 μg/ml acridine orange solution was added followed by incubation for 30 min in the dark at 28.5℃. After incubation, the zebrafish larvae were rinsed twice using fresh embryo media and anesthetized using 0.03 % ethyl 3-aminobenzoate methanesulfonate before observation. The zebrafish larvae were photographed under a microscope equipped with a CoolSNAP-pro color digital camera (Olympus, Japan). The fluorescence intensity of zebrafish larvae was quantified using ImageJ software.

2. 16. Effect of BPCA on H_2O_2-induced lipid peroxidation in zebrafish

Lipid peroxidation in zebrafish was detected using the fluorescent dye DPPP to detect cell membrane lipid peroxidation. After fertilization, 7-9 hpf zebrafish embryos were transferred to a 12 well plate. The embryos were pre-treated with various concentrations of BPCA (6.25, 12.5, 25, and 50 μM) for 2 h and then treated with 5 mM H_2O_2 for 3 dpf. The zebrafish larvae were transferred to a 24 well plate, and 25 μg/ml DPPP solution was added

followed by incubation for 30 min in the dark at 28.5℃. After incubation, the zebrafish larvae were rinsed twice using fresh embryo media and anesthetized using 0.03 % ethyl 3-aminobenzoate methanesulfonate before observation. The zebrafish larvae were photographed under a microscope equipped with a CoolSNAP-pro color digital camera (Olympus, Japan). The fluorescence intensity of zebrafish larvae was quantified using ImageJ software.

2. 17. Statistical analysis

All data are expressed as means ± S.D. Significant differences among groups were determined using the unpaired Student's t-test. A value of $p < .05$ was considered to be statistically significant.

3. RESULTS

3. 1. Free radical scavenging activities of BPCA

We measured the protective effects of BPCA against DPPH, hydroxyl, and alkyl radicals (Fig. 1-2). BPCA exhibited profound scavenging activities for DPPH, hydroxyl, and alkyl radicals in a dose-dependent manner. The IC_{50} values for DPPH, hydroxyl, and alkyl radical scavenging were 13.17±0.13, 60.12±3.31, and 7.58±0.37 μM, respectively. These results indicate that BPCA has excellent protective effect against DPPH and alkyl radicals.

3. 2. Effect of BPCA on H_2O_2-induced cytotoxicity in Vero cells

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

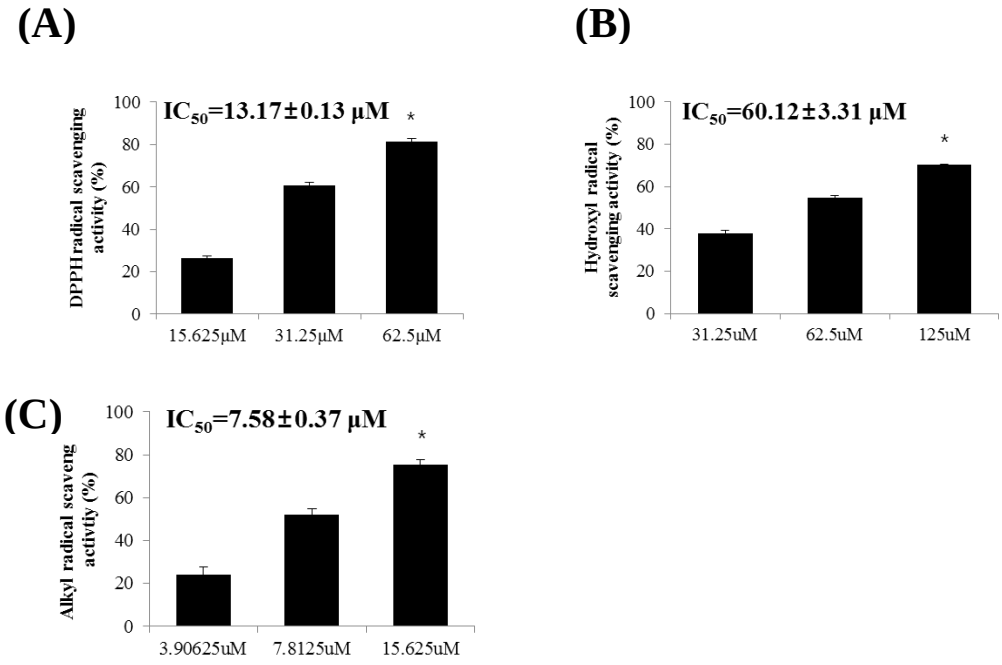


Fig. 1-2 (A) DPPH, (B) hydroxyl, and (C) alkyl radical scavenging activities of BPCA were measured by ESR spectrum. All results are expressed as the mean ± S.E. from more than three individual experiments; *P < 0.05.

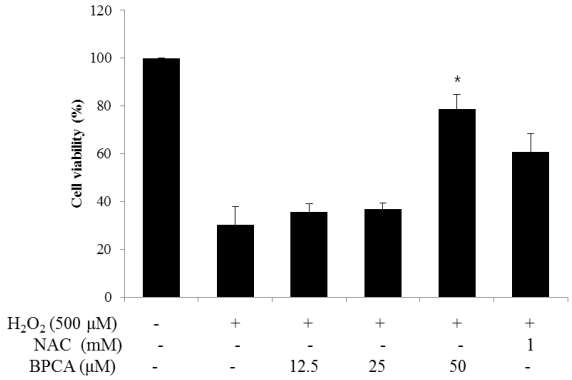


Fig. 1-3. Effect of BPCA on cell viability in H_2O_2-stimulated Vero cells. The cells were pre-treated with BPCA (12.5, 25, 50 μM) and NAC (1 mM) and treated with H_2O_2 (500 μM) for 24 h. Cell viability was assessed with the MTT assay. All results are expressed as the mean ± S.E. from more than three individual experiments; *P < 0.05.

3. 3. Effect of BPCA on ROS production in H_2O_2-stimulated Vero cells

We measured the intracellular ROS scavenging activity of BPCA and NAC using the principle that DCFH-DA reacts with the reactive oxygen in cells and is oxidized to a fluorescent substance, DCF (Fig. 1-4). Vero cells were pre-treated with BPCA and NAC for 2 h and then treated with 500 μM H_2O_2 for 30 min. After 30 min, ROS scavenging activity was approximately 54% for 12.5 and 25 μM BPCA and approximately 59% for 50 μM BPCA. These results indicated that BPCA protects against H_2O_2-induced cytotoxicity by scavenging radicals and ROS.

3. 4. Effect of BPCA on H_2O_2-induced apoptosis in Vero cells

We assessed the protective effect of BPCA on H_2O_2-induced apoptosis via nuclear staining with Hoechst 33342 (Fig. 1-5 (A)). When the cells were treated with H_2O_2, we observed nuclei shrinkage, membrane blebbing, and apoptotic bodies. However, when the cells were treated with 50 μM BPCA, we observed a significant reduction in apoptotic bodies. Moreover, to study the role of BPCA in cell cycle progression under oxidative stress, we performed cell cycle analysis using flow cytometry (Fig. 1-5 (B)). The sub-G_1 DNA content of the control cells was 14.5 %. On the other hand, the sub-G_1 DNA content of cells treated with 500 μM H_2O_2 was 27.17 %, indicating the induction of apoptosis. However, when te cells were treated with 50 μM BPCA, the sub-G_1 DNA content decreased to 21.89 %. Therefore, these results indicate that BPCA prevents cytotoxicity by inhibiting apoptosis in H_2O_2-induced Vero cells.

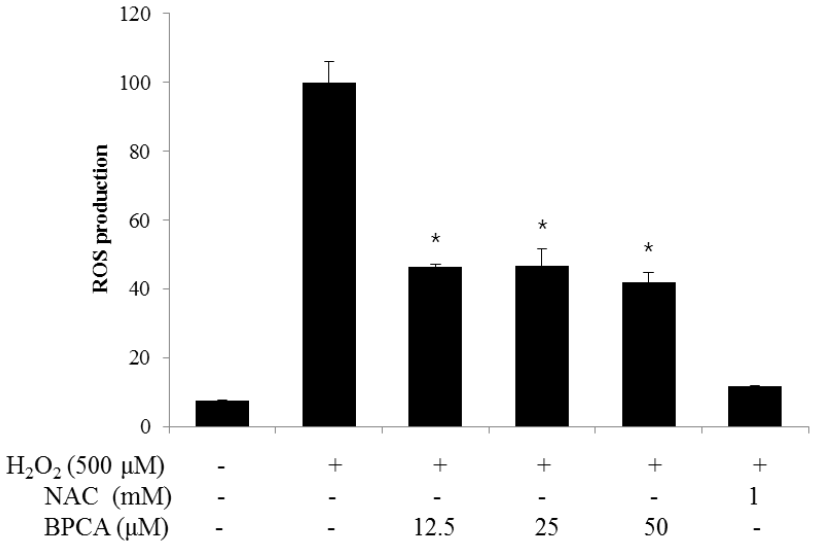


Fig. 1-4. Effect of BPCA on ROS production in H_2O_2-stimulated Vero cells. The cells were pre-treated with BPCA (12.5, 25, 50 μM) and NAC (1 mM) and treated with H_2O_2 (500 μM) for 30 min. ROS production was measured by DCFH-DA methods. All results are expressed as the mean ± S.E. from more than three individual experiments; *P < 0.05.

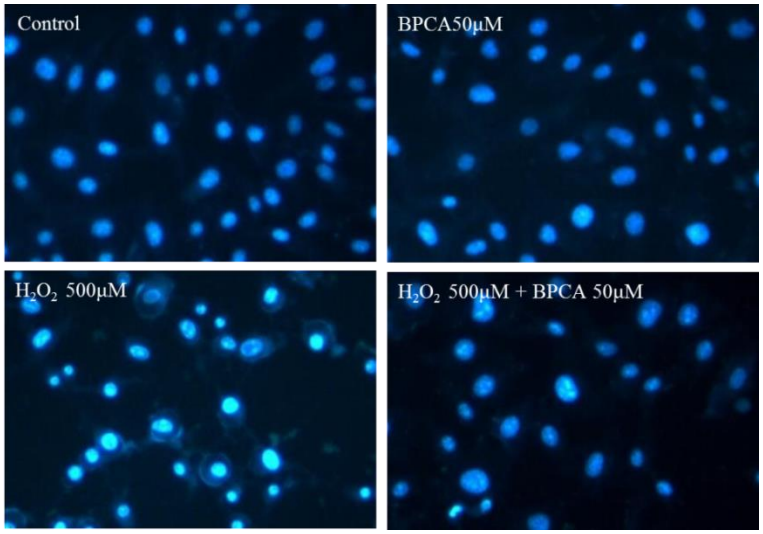
3. 5. Effect of BPCA on antioxidant enzymes in H_2O_2-stimulated Vero cells

Because we found that H_2O_2 treatment induced ROS in Vero cells, we confirmed the expression levels of antioxidant enzymes in Vero cells exposed to 500 μM H_2O_2 in presence or absence of BPCA (Fig. 1-6). When cells were treated with 500 μM H_2O_2 for 6 h, the expression of GPX1 was inhibited compared to the control, whereas there was little change in the expression levels of catalase, SOD1, and SOD2. However, the 50 μM BPCA treatment with H_2O_2 increased the expression of GPX1. This result indicates that BPCA has an antioxidant effect by increasing the levels of GPX1 in H_2O_2-induced Vero cells.

3. 6. Effect of BPCA on reduction of Bax and cleaved caspase-9, and PARP cleavage activation in H_2O_2-stimulated Vero cells

We evaluated the expression of Bax, cleaved caspase-9, and PARK in Vero cells exposed to 500 μM H_2O_2 in presence or absence of BPCA (Fig. 1-7). The expression levels of Bax and cleaved caspase-3 were increased by H_2O_2 treatment. However, when cells were treated with 50 μM BPCA and H_2O_2, the expression levels decreased. The expression level of PARP was decreased by H_2O_2, but after treatment with 50 μM BPCA and H_2O_2, protein expression increased. The results demonstrated that BPCA inhibits apoptosis by decreasing the expression of Bax and cleaved caspase-3 and increasing the expression of PARP.

(A)



(B)

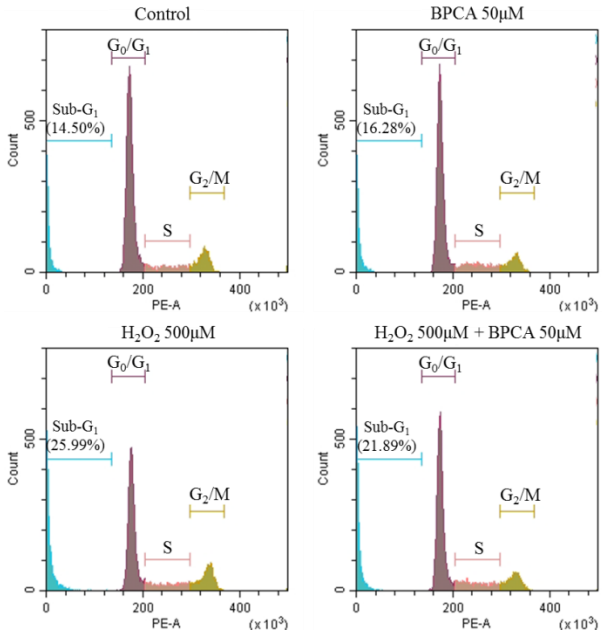


Fig. 1-5. Effect of BPCA on H_2O_2-induced apoptosis in Vero cells. The cells were pre-treated with BPCA (50 μM) and treated with H_2O_2 (500 μM) for 6 h. (A) Apoptotic bodies were observed under a fluorescent microscope after Hoechst 33342 staining. (B) Apoptotic sub-G_1 phase cells were assessed using flow cytometry after PI staining.

3. 7. Effect of BPCA on activation of NF-κB in H_2O_2-stimulated Vero cells

We evaluated the activation of NF-κB in Vero cells exposed to 500 μM H_2O_2 in presence or absence of BPCA (Fig. 1-8). When cells were treated with 500 μM H_2O_2 for 45 min, NF-κB was activated. However, the activation of NF-κB was reduced after treatment with 50 μM BPCA and H_2O_2 (Fig. 1-8 (A)). The nuclear translocation of p65 increased when cells were treated with H_2O_2, but BPCA inhibited the nuclear translocation of p65. (Fig. 1-8 (B)). These results demonstrate that BPCA inhibits the activation and translocation of NF-κB in H_2O_2-stimulated Vero cells.

3. 8. Effect of BPCA on H_2O_2-induced oxidative stress in zebrafish

To confirm the protective effect of BPCA on H_2O_2-induced oxidative stress in zebrafish, we analyzed survival rates, ROS production, cell death, and lipid peroxidation (Fig. 1-9). The survival rate was more than 80 % for all concentrations compared to the control, thus indicating that all tested concentrations of BPCA were non-toxic in zebrafish (Fig. 1-9 (A)). H_2O_2-induced ROS production in zebrafish was 214 % compared to the control group. However, treatment with 6.25, 12.5, 25, and 50 μM BPCA 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 treatment of zebrafish embryos with BPCA markedly suppressed H_2O_2-induced ROS generation (Fig. 1-9 (B)). H_2O_2-induced cell death in zebrafish increased more than double compared to the control group. However, treatment with 6.25, 12.5, 25, and 50 μM BPCA and H_2O_2 significantly reduced H_2O_2-induced cell death in a concentration-dependent manner. Thus, these results indicate that BPCA significantly inhibits H_2O_2-induced cell death in zebrafish embryos via inhibition of ROS production (Fig. 1-9 (C)). H_2O_2-induced lipid peroxidation in zebrafish was 115.5 % compared to the control group.

Lipid peroxidation was dose-dependently reduced by BPCA, but there was no significant difference, and treatment with 50 μM BPCA resulted in a similar value as in the control group (Fig. 1-9 (D)). Taken together, these results demonstrate that BPCA has a protective effect on H_2O_2-induced oxidative stress in zebrafish.

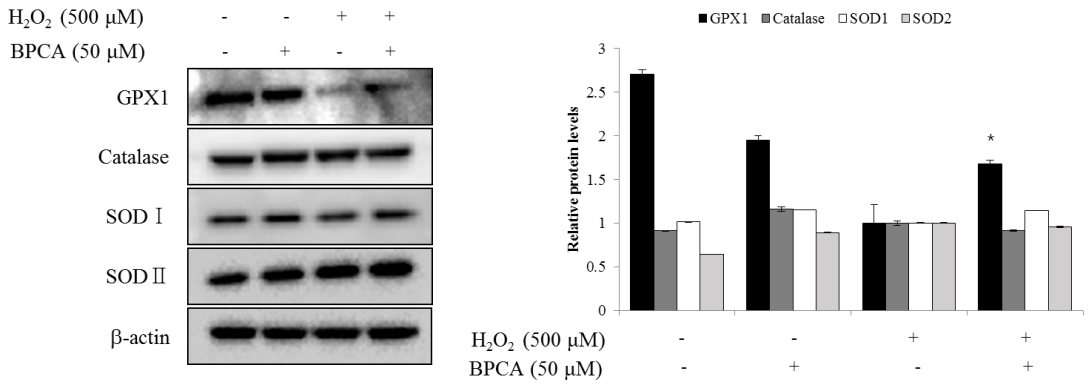


Fig. 1-6. Effect of BPCA on antioxidant enzymes in H_2O_2-stimulated Vero cells. The cells were pre-treated with BPCA (50 μM) and treated with H_2O_2 (500 μM) for 6 h. GPX1, catalase, SOD1, and SOD2 protein levels were determined using western blot analysis. All results are expressed as the mean ± S.E. from more than three individual experiments; *P < 0.05.

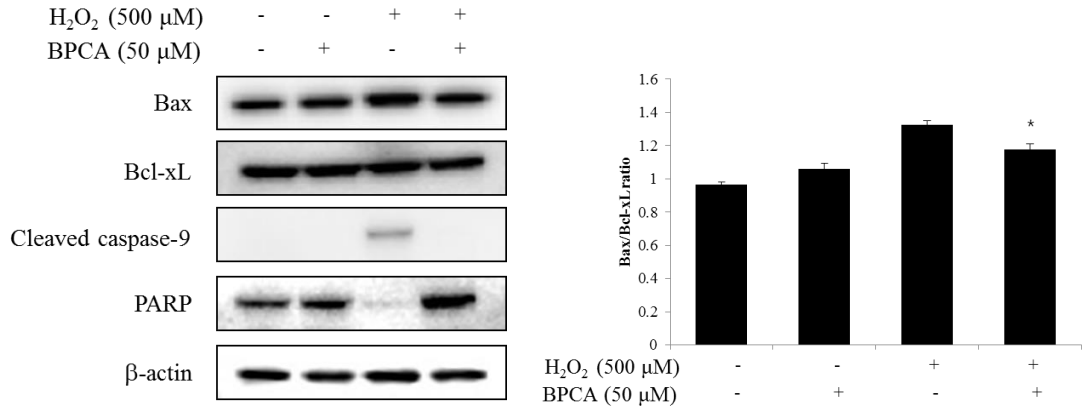


Fig. 1-7. Effect of BPCA on the reduction of Bax and cleaved caspase-9 and PARP cleavage activation in H_2O_2-stimulated Vero cells. The cells were pre-treated with BPCA (50 μM) and treated with H_2O_2 (500 μM) for 6 h. Bax, Bcl-xL, cleaved caspase-9, and PARP protein levels were determined using western blot analysis. All results are expressed as the mean ± S.E. from more than three individual experiments; *P < 0.05.

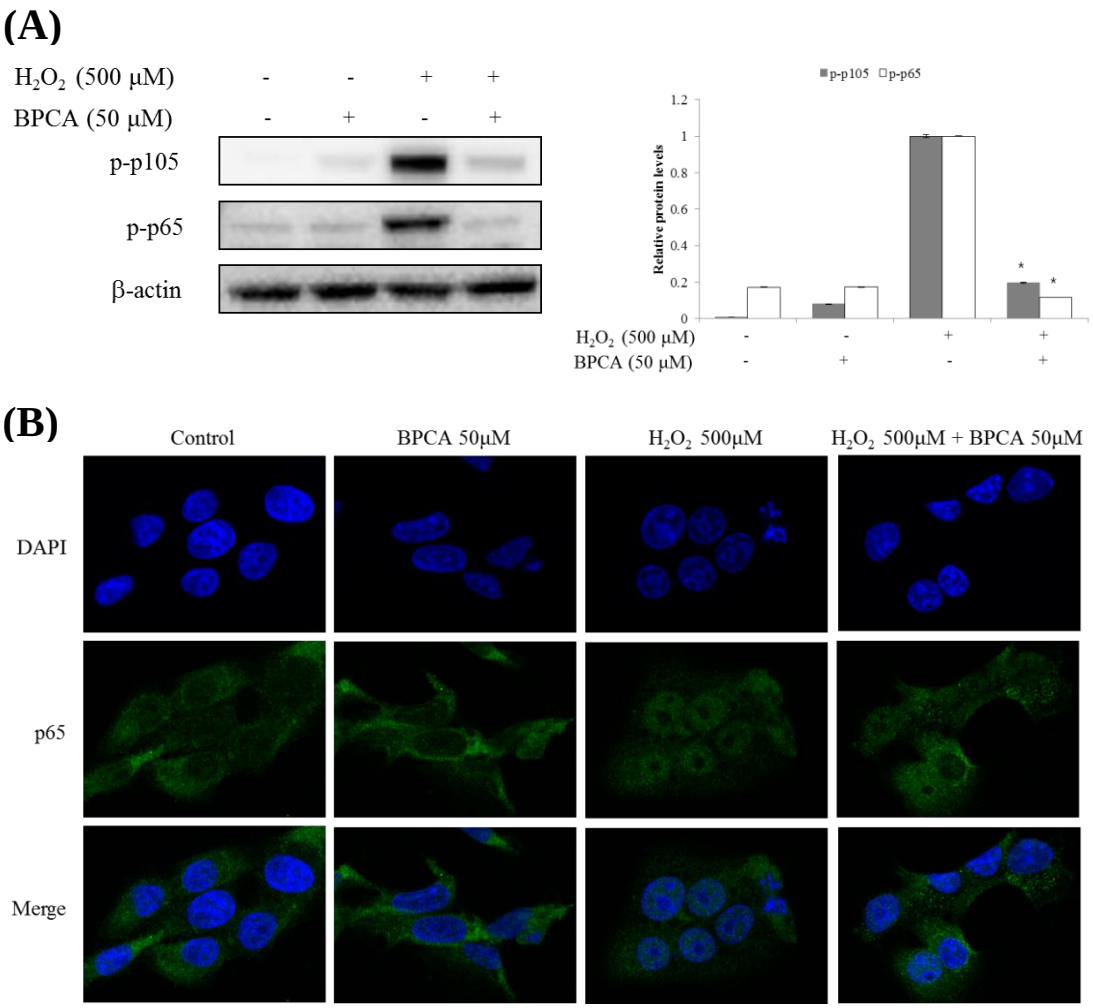
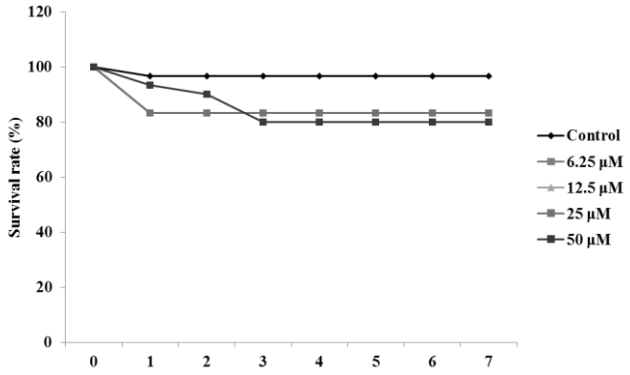
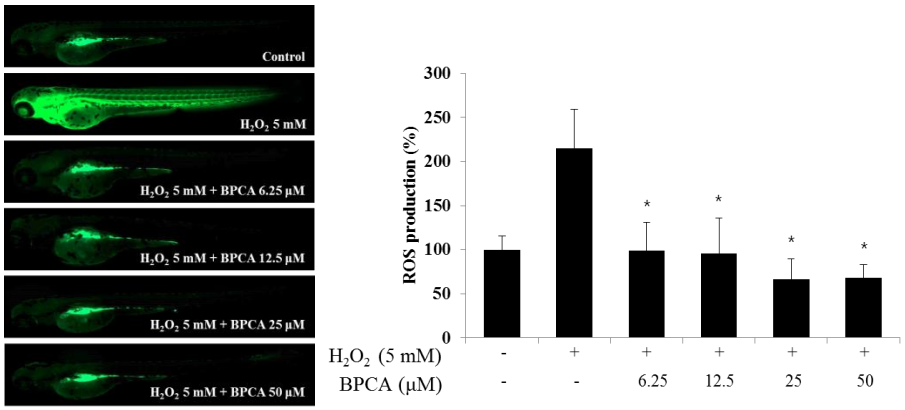


Fig. 1-8. Effect of BPCA on the activation of NF-κB in H_2O_2-stimulated Vero cells. (A) The cells were pre-treated with BPCA (50 μM) and treated with H_2O_2 (500 μM) for 6 h. The expression levels of p-p105 and p-p65 were analyzed by western blot analysis. (B) The nuclear translocation of p65 in Vero cells was observed with an anti-p65 and Alexa Fluor 488 got anti-rabbit antibody by confocal laser microscope (LSM 780).

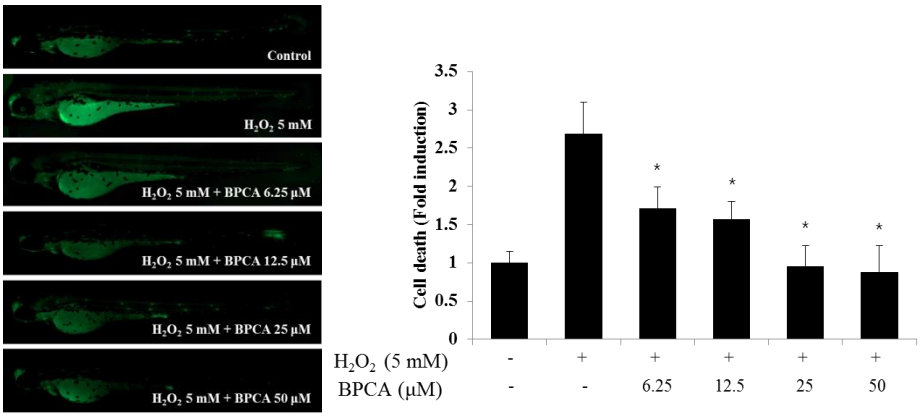
(A)



(B)



(C)



(D)

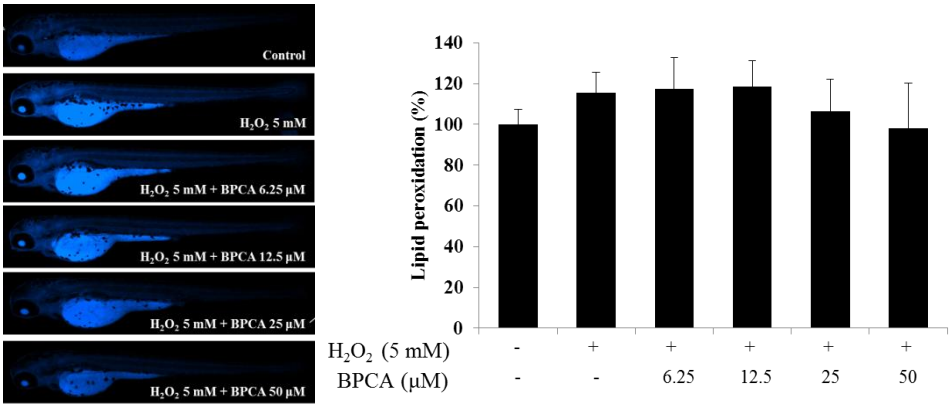


Fig. 1-9. Effect of BPCA on survival rate and H_2O_2-induced ROS production, cell death, and lipid peroxidation in zebrafish. (A) Survival rate of zebrafish embryos was measured for 7 dpf after treatment with BPCA (6.25, 12.5, 25, and 50 μM). Zebrafish embryos were pre-treated with BPCA (6.25, 12.5, 25, and 50 μM) and then treated with H_2O_2 (5 mM). Imaging of (B) ROS production, (C) cell death, and (D) lipid peroxidation generation was performed using fluorescent microscopy. The fluorescence intensity of zebrafish was quantified using ImageJ software. All results are expressed as the mean ± S.E. from more than three individual experiments; *P < 0.05.

4. Discussion

ROS cause cellular and tissue injuries by inducing oxidative damage in DNA, RNA, proteins, and lipids (Aybek et al., 2008; Li et al., 2013). The overexpression of ROS promotes apoptosis via various mechanisms (Tang et al., 2008; Li et al., 2013). Therefore, suppressing free radicals such as DPPH, hydroxyl, and alkyl radicals is important to prevent apoptosis. In this study, BPCA was shown to significantly protect against DPPH and alkyl radicals and to considerably suppress intracellular ROS production. The results indicated that BPCA has a strong scavenging activity for free radicals and ROS that protects against oxidative damage in cells.

Apoptosis, or programmed cell death (PCD), is a naturally occurring cell death process that is vital for the normal development and homeostasis of multicellular organisms (Schwartzman and Cidlowski, 1993; Kannan and Jain, 2000). 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 (Kerr et al., 1972; Hockenbery et al., 1993). 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 demonstrated that ROS and oxidative stress play a critical role in apoptosis. In this study, Vero cells exposed to 500 μM H_2O_2 exhibited distinct features of apoptosis, including the presence of apoptotic bodies and an increased sub-G_1 DNA content. However, BPCA considerably inhibited the formation of apoptotic bodies and decreased the sub-G_1 DNA content, resulting in a lower percentage of apoptotic cells. BPCA also protected against H_2O_2-induced cell death. Thus, the results show that BPCA 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 (Adams and Cory, 1998; Jones et al., 2000). Bax induces the release of mitochondrial cytochrome *c* and causes caspase activation. Bcl-2 and Bcl-xL prevent Bax-regulated cytochrome *c* release and cell death (Finucane et al., 1999; Jones et al., 2000). Caspases, a family of cysteine proteases, including caspase-3 and caspase-9, play an important role in the execution stage of apoptosis and are involved in many changes associated with apoptosis (Cohen, 1997; Sun et al., 1999, Ham et al., 2012). These caspases cleave intracellular substrates, such as poly(ADP-ribose) polymerase (PARP) and lamins, during the execution stage of apoptosis (Sun et al., 1999). Cleaved caspase-9 processes other caspase members, including caspase-3 and caspase-9, to begin the caspase cascade, which leads to apoptosis (Sun et al., 1999; Ham et al., 2012). 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 (Oliver et al., 1998; Ham et al., 2012). In this study, we performed western blot analysis to reveal the mechanisms of apoptosis in H_2O_2-stimulated Vero cells. When the cells were treated with H_2O_2, the protein levels of Bax, cleaved caspase-9, and PARP increased; however, BPCA inhibited the expression of Bax, cleaved caspase-9, and PARP, demonstrating that BPCA significantly affects the mechanism of apoptosis by regulating Bax.

NF-κB is a transcription factor thought to play an important role in the onset of cell apoptosis (). The transcription factor NF-κB exists as heterodimer of p50 and p65 and is bound in the cytoplasm by IκB (Baeuerle, 1998; Stark et al., 2007). In previous studies, it was shown that H_2O_2 induced the activation of NF-κB in several types of cells (Schreck et al., 1991; Meyer et al., 1993), and ROS were found to stimulate the NF-κB signaling pathway directly (Chen et al., 2011; Morgan and Liu, 2011). Moreover, the activation of NF-κB is inhibited by the antioxidants (Schenk et al., 1994). In this study, we confirmed the effect of BPCA on the activation of NF-κB in H_2O_2-exposed Vero cells. H_2O_2 induced the

activation of NF-κB in Vero cells; however, BPCA markedly inhibited this activation. In addition, when Vero cells were exposed to H_2O_2, the nuclear translocation of p65 increased, but when cells were pre-treated with BPCA, the translocation of p65 was considerably suppressed. These results suggest that BPCA inhibits H_2O_2-induced apoptosis by regulating the NF-κB pathway.

The enzymatic antioxidant defense system includes SOD, catalase, and GPX (Chen et al., 2009; Melegari et al., 2012). SODs are the most important factors in antioxidant defense system against ROS and superoxide anion radicals (Zelko et al., 2002), and they convert superoxide anions to H_2O_2 (Wijeratne et al., 2005; Fridovich, 1997). There are two types of SOD, Cu/Zn-SOD (SOD1) exists mainly in the cytoplasm and mitochondria, and Zn-SOD (SOD2) exists mainly in mitochondria (Zelko et al., 2002; Jeon et al., 2007; Wijeratne et al., 2005). In previous studies, SOD1 and SOD2 were shown to play a protective role against ROS in cells (Fridovich., 1995; Bannister J. V., 1987). Catalase catalyzes the degradation of H_2O_2 to water and oxygen (Weydert and Cullen, 2010; Chelikani et al., 2004). Catalase activity is mainly located in the subcellular organelles peroxisomes (Weydert and Cullen, 2010). It is important factor in protecting cells from oxidative stress induced by ROS, and one catalase molecule can convert millions of H_2O_2 molecules to water and oxygen (Weydert and Cullen, 2010; Chelikani et al., 2004). GPXs are a family of enzymes that reduce of H_2O_2 to water or reduce lipid hydroperoxides to the corresponding alcohols using glutathione (Chen et al., 2009, Brigelius-Flohe, 2006). Among the GPXs, GPX1 exists in the cytoplasm of many mammalian tissues and is considered the critical enzyme in the removal of H_2O_2 (Weydert and Cullen, 2010). Moreover, antioxidant enzymes control ROS to ensure that metabolic processes can function in organisms. Therefore, in this study, we evaluated the effect of BPCA on the protein expression levels of antioxidant enzymes such as SOD1, SOD2, catalase, and GPX1 in H_2O_2-stimulated Vero cells. When cells were treated with

H_2O_2, the expression levels of SOD1, SOD2, and catalase did not change compared to those of the control. However, the expression level of GPX1 was suppressed by H_2O_2, and BPCA treatment increased GPX1 protein expression. Thus, BPCA suppresses oxidative stress damage by restoring the antioxidant enzyme defense system, particularly GPX1, in H_2O_2-induced Vero cells.

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 (Kim et al., 2015; Lan et al., 2013; Zou et al., 2017). In particular, researchers used zebrafish as animal models in drug discovery studies investigating oxidative stress (Kim et al., 2015; Du et al., 2017; Kobayashi et al., 2009). Therefore, we evaluated the effect of BPCA on survival rate, ROS generation, cell death, and lipid peroxidation using H_2O_2-induced oxidative stress in the zebrafish model. All concentrations of BPCA were non-toxic in zebrafish. H_2O_2 induced ROS generation, cell death, and lipid peroxidation in zebrafish embryos. However, treatment of zebrafish embryos exposed to H_2O_2 with BPCA significantly reduced ROS generation and cell death. BPCA reduced lipid peroxidation, although the difference was not significant. Therefore, these results demonstrated that BPCA can protect against H_2O_2-induced oxidative stress in the zebrafish model.

In conclusion, we demonstrated that BPCA decreased H_2O_2-induced oxidative stress in Vero cells by decreasing ROS levels and restoring endogenous antioxidant enzymatic activities, such as GPX. Furthermore, BPCA blocked H_2O_2-induced apoptosis via the regulation of Bax and NF-kB. Moreover, BPCA significantly suppressed ROS production and cell death in response to H_2O_2-induced oxidative stress in a zebrafish model. Taken together, BPCA protects against oxidative stress *in vitro* and *in vivo*, demonstrating that BPCA may be an alternative to more toxic synthetic antioxidants for use as an additive in food and pharmaceutical preparations.

Part 2

Evaluation of anti-aging activity of 5-bromoprotocatechualdehyde from *Polysiphonia morrowii* Harvey

1. Abstract

We investigated anti-aging activity of 5-bromoprotocatechualdehyde (BPCA) from *P. morrowii* Harvey in human dermal fibroblasts (HDF). HDF cells were subjected to treatment of BPCA and then treated with H_2O_2 to induce premature senescence. . Treatment of HDF with H_2O_2 increased senescence-associated β-galactosidase (SA-β-gal) activity but BPCA resulted in reduction of SA-β-gal activity. Also, BPCA significantly reduced H_2O_2-induced intracellular reactive oxygen species (ROS) production. To investigate the relation of aging and cell cycle, we performed the cell cycle analysis. Treatment of H_2O_2 is arrested the G_2/M phase, but BPCA was not suppressed the G_2/M arrest on H_2O_2. Therefore, the aging and cell cycle arrest was not related each other. Also, we demonstrated the effect of BPCA on antioxidant enzymes against oxidative H_2O_2-stimulated HDF. Among the antioxidant enzymes, when the 400 μM of H_2O_2 were treated in the cells for 6 h, the expression level of GPX1 inhibited. However, 50 μM of BPCA increased considerably. Furthermore, we evaluated the effect of BPCA on SA-β-gal activity in H_2O_2-stimulated zebrafish model. Treatment of 500 μM of H_2O_2 significantly induced the SA-β-gal activity than 600 μM of H_2O_2. The 500 μM of H_2O_2 increased the SA-β-gal activity and treatment of NAC suppressed the SA-β-gal activity in zebrafish. Also, BPCA decreased the SA-β-gal activity in zebrafish. Taken together, BPCA has the excellent anti-aging activity in *in vitro* and *in vivo*.

2. MATERIAL AND METHOD

2. 1. Materials

F12 media (Ham's F-12 Nutrient mix), phosphate buffered saline (PBS), antibiotic, and trypsin-EDTA were purchased from Gibco/BRL (Burlington, Ont, Cananda). Fetal bovine serum (FBS) was purchased from Welgene (Daegu, Korea). Dulbecco's Modified Eagle Medium (DMEM) was purchased from Hyclone (Logan, Utah, USA). Dimethyl sulfoxide, 2',7'-dichlorodihydrofluorescein diacetate (DCHF-DA), 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (X-gal), potassium hexacyanoferrate (Ⅱ) trihydrate, potassium hexacyanoferrate (Ⅲ), propidium iodide (PI), and RIPA buffer were purchased from Sigma (St. Louis, MO, USA). Magnesium chloride was purchased from Biosesang (Seongnam, Korea). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) was purchased from Amresco (Solon, OH, USA). RNase A was purchased from Roche (Canada). NUPAGE 4~12% Bis-Tris gel and iBlot® Transfer Stack, PVDF Regular were purchased from Invitrogen (Carlsbad, CA). Primary antibodies against phospho-p53, p27, p21, phospho-Rb, Rb, CDK4, cyclin B1, cyclin D1, SOD1, SOD2, catalase, and GPX1 and secondary antibodies against anti-rabbit IgG, HRP-linked antibody and anti-mouse IgG, HRP-linked antibody were purchased from Cell signaling Technologies (Bedford, MA, USA). Primary antibodies against β-actin was Santa Cruz Biotechnology (Santa Cruz, CA). SuperSignal West Femto Trial kit was purchased from Thremo Fisher Scienticfic (Waltham, Massachusetts, USA).

2. 2. Cell culture

Human dermal fibroblasts (HDF) were purchased by Korean Cell Line Bank (KCLB,

Seoul, Korea). The cells were grown at 37 ℃ in 5% CO_2 humidified atmosphere incubator using F12 media (Ham's F-12 Nutrient mix) and Dulbecco's Modified Eagle Medium (DMEM) mixture (1:3) included 10 % fetal bovine serum (FBS), 1 % antibiotic.

2. 3. Measurement of cell viability

The cells were seeded in 96 well plate at a concentration of 1.5×10^4 cells/ml. After 24h, 125, 50, and 100 μM of BPCA were pre-treated for 2 h at 37 ℃. Then 400 μM of H_2O_2 was added for 72 h at 37 ℃. The cells were incubated with 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) dissolved in PBS for 3 h. After the supernatant were removed, formazan were dissolved in Dimethyl sulfoxide (DMSO). The absorbance was read at 540 nm.

2. 4. Senescence-associated β-galactosidase (SA-β-gal) staining

The cells were washed once with PBS and fixed using 4 % formaldehyde for 15 min. After the cells were washed twice, the cells were incubated SA-β-gal staining solution containing 1 mg/ml X-gal, 5 mM potassium hexacyanoferrate (Ⅱ) trihydrate, 5 mM potassium hexacyanoferrate (Ⅲ), and 2 mM $MgCl_2$ in 40mM citric acid/sodium phosphate buffer (pH 6.0) for 24 h at 37 ℃ in a CO_2 free atmosphere in the dark. The SA-β-gal stained cells were observed using inverted microscope (Olympus, Japan).

2.5. Measurement of intracellular reactive oxygen species (ROS) production

The cells were seeded in 96 well plate at a concentration of 8×10^4 cells/ml. After 24 h, 25 and 50 μM of BPCA were pre-treated for 2 h at 37 ℃. And 400 μM of H_2O_2 was added for 20 min at 37 ℃. Then the cells were treated 2',7'-dichlorodihydrofluorescein diacetate (DCHF-DA, 20 μM) for 30 min at 37 ℃ in dark. DCHF-DA was detected at an excitation wavelength of 485 nm and an emission wavelength of 535 nm, using a spectrophotometer (Molecular Devices, USA).

2. 6. Cell cycle analysis

Cell cycle analysis was carried out to determine the rate of apoptotic sub-G_1 cells. The cells were seeded in 60 mm dish at a concentration of 2.0×10^5 cells/ml. After 24 h, 50 μM of BPCA were pre-treated for 2 h at 37 ℃. And 500 μM of H_2O_2 were treated for 6 h at 37 ℃. After the media were removed, the cells were washed phosphate buffered saline (PBS). The cells were collected using trypsin-EDTA and washed twice with PBS. And the cells were fixed using cold-70 % ethanol at 4 ℃ before using it. The fixed cells were washed with PBS and incubated in PBS-2 mM EDTA containing 100 μg/ml PI and 100 μg/ml RNase for 30 min at 37 ℃ in dark. Flow cytometry was performed using Beckman Coulter CytoFLEX flow cytometer (Beckman Coulter, Miami, FL).

2. 7. Western blot analysis

The cells were seeded in 60mm dish at a concentration of 1.0×10^5 cells/ml. After 24 h, 50 μM of BPCA were pre-treated for 2 h at 37 ℃. And 400 μM of H_2O_2 were treated for 6 h

at 37 ℃. After the media were removed, the cells were washed two times using phosphate buffered saline (PBS). RIPA buffer (Sigma Aldrich) was added and incubated for 10 min at 4 ℃. Then cell lysates were collected, vortexed 6 times for 1 h, and centrifuged at 13,300 rpm at 4 ℃ for 25 min. After protein concentration was determined by Protein Assay Dye Reagent Concentrate (BIO-RAD), protein samples were electrophoresised and transferred to PVDF membrane (Invitrogen). The membranes were blocked for 2 h at room temperature and incubated with primary antibody (anti-GPX1, anti-SOD1, anti-SOD2, anti-catalase, and anti-β-actin) for overnight at 4 ℃. The membranes were washed using TTBS (20 mM Tris, 137 mM NaCl, 0.1 % tween 20) and incubated with secondary antibody (anti-rabbit IgG, HRP-linked antibody and anti-mouse IgG, HRP-linked antibody) at room temperature. After 2 h, bands were detected using SuperSignal West Femto Trial kit (Thermo Fisher Scientific).

2. 8. Origin and maintenance of zebrafish

Zebrafish was kept under following conditions: 28.5±0.5 ℃, with 14/10 h light/dark cycle. The zebrafish were fed two times a day, 6 days a week. Zebrafish was selected for mating in the female-to-male ratio of 1:2. Embryos were obtained via mating and spawning. After spawning, the embryos were transferred into the petri dish containing 1 mg/ml of methylene blue solution. After disinfection for 1.5 h, methylene blue solution was changed to fresh embryo media (600 mg/L red sea salt in distilled water).

2. 9. Effect of BPCA on SA-β-gal activity in H_2O_2-stimulated zebrafish

After 3 hdf, zebrafish larvae were rinsed once time using fresh embryo media and

anesthetized using 0.03 % of ethyl 3-aminobenzoate methanesulfonate. After anesthetization, they were rinsed two times using PBS and fixed using 4 % formaldehyde at 4 ℃ for overnight. The fixed zebrafish larvae were rinsed three times using PBS (pH 6.0) for 1 h and they were stained using SA-β-gal staining solution containing 0.75 mg/ml X-gal, 5 mM potassium hexacyanoferrate (Ⅱ) trihydrate, 5 mM potassium hexacyanoferrate (Ⅲ), and 2 mM $MgCl_2$ in PBS (pH 6.0) for 24 h at 37 ℃ in a CO_2 free atmosphere in the dark. The SA-β-gal stained cells were observed using inverted microscope (Olympus, Japan).

2. 10. Statistical analysis

All data are expressed as means ± S.D. Significant differences among groups were determined using the unpaired Student's t-test. A value of $p < .05$ was considered to be statistically significant.

3. Results

3. 1. Effect of BPCA on cell viability in HDF

We measured the cell viability of BPCA using the principle that MTT reacts to the mitochondria in living cell and produces formazan (Fig. 2-1). HDF cell was pre-treated BPCA for 2 h and treated 400 μM of H_2O_2 for 72 h. 25 and 50 μM of BPCA was non-cytotoxic but 100 μM were a little toxic compared to control. When the cells were treated H_2O_2, cell viability was decreased to 79.52 %. However, when the cells were treated 25 and 50 μM of BPCA, cell viability was increased until 87.5 % and 88.76 % each other. The results are indicated that BPCA has protective effect against H_2O_2 in HDF.

3. 2. Effect of BPCA on H_2O_2-induced aging in HDF

We observed the SA-β-gal expression in HDF exposed to H_2O_2 in presence or absence of BPCA (Fig. 2-2). HDF cells were pre-treated BPCA for 2 h and treated 400 μM of H_2O_2 for 72 h. When 400 μM of H_2O_2 treated, SA-β-gal activity increased to 52.87 % and induced morphological enlargement. However, SA-β-gal activity reduced to 30.08 % and 26.19 % at 25 and 50 μM of BPCA each other. The result is show that BPCA inhibits the cellular aging H_2O_2-stimulated HDF.

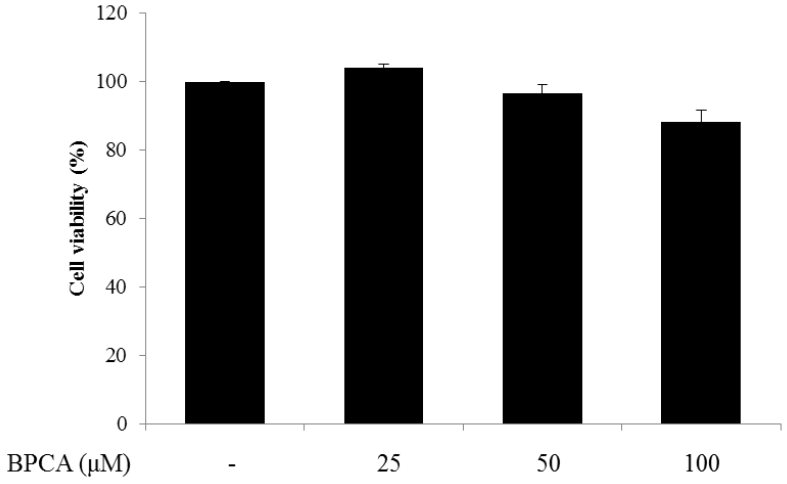


Fig. 2-1. Effect of BPCA on cell viability in HDF. The cells were pre-treated with BPCA (25, 50, and 100 μM) and for 72 h. Cell viability was indicated by MTT assay. All the results were expressed as means ± S.E. of over three individual experiments; *P < 0.05.

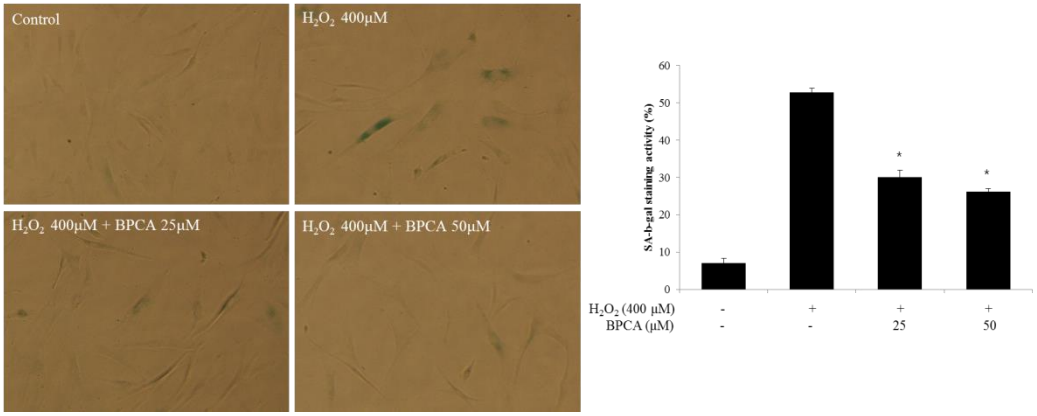


Fig. 2-2. Effect of BPCA on SA-β-gal activity against H_2O_2-induced aging in HDF. The cells were pre-treated with BPCA (25, 50, and 100 μM) and treated H_2O_2 (400 μM) for 72 h. The cells were then stained with X-gal to observe SA-β-gal activity and photographed under the inverted microscope. All the results were expressed as means ± S.E. of over three individual experiments; *P < 0.05.

3. 3. Effect of BPCA on H_2O_2-induced ROS production in HDF

We measured the intracellular ROS scavenging activity of BPCA using the principle that the DCFH-DA reacts to the reactive oxygen in the cells and is oxidize to fluorescent substance DCF (Fig. 2-3). HDF cells were pre-treated BPCA for 2 h and treated 400 μM of H_2O_2 for 30 min. After 30 min, ROS production was reduced to 34.16 % and 30.06 % at 25 and 50 μM of BPCA. The result shows that BPCA has intracellular ROS scavenging activity against H_2O_2 in HDF.

3. 4. Effect of BPCA on H_2O_2-induced cell cycle arrest in HDF

To study the role of BPCA on cell cycle arrest in oxidative stress, we performed cell cycle analysis using flow cytometer (Fig. 2-4). HDF cells were pre-treated 50 μM of BPCA for 2 h and treated 400 μM of H_2O_2 for 24 h. When 400 μM of H_2O_2 treated, G_2/M DNA content increased to 45.65%. Nevertheless, the cells were treated with 50 μM of BPCA, G_2/M DNA content almost not reduced. The results indicate that BPCA can't inhibit G_2/M cell cycle arrest in H_2O_2-stimulated HDF.

3. 5. Effect of BPCA on antioxidant enzymes in H_2O_2-stimulated HDF

Because we found that H_2O_2 treatment was induced ROS in HDF, we evaluated the expression levels of antioxidant enzymes in HDF exposed to 400 μM of H_2O_2 in presence or absence of BPCA (Fig. 2-5). When 400 μM of H_2O_2 were treated for 6 and 12 h, expression of GPX1 was inhibited compared to control. However 50 μM of BPCA treatment with H_2O_2 increased expression of GPX1. The expression levels of Catalase, SOD1, and SOD2 except to GPX1 was not much difference. Therefore, the result indicates that BPCA induces expression levels of GPX1 inhibited by H_2O_2 among the antioxidant enzymes in HDF.

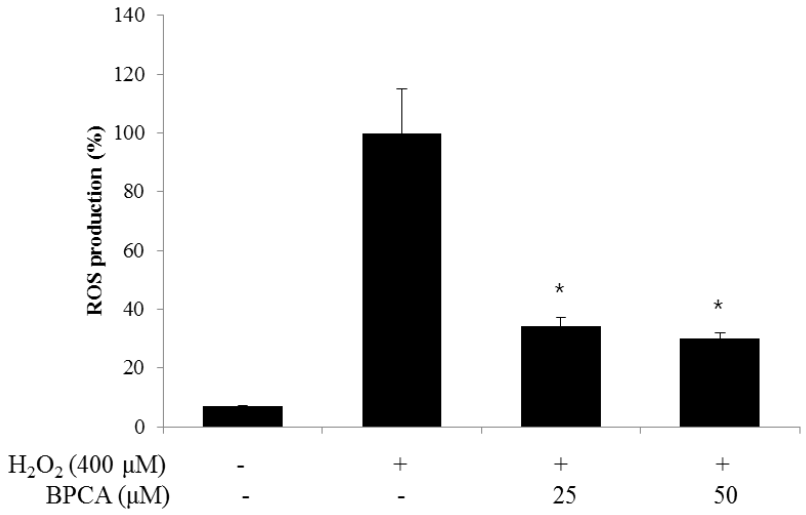


Fig 2-3. Effect of BPCA on intracellular ROS production in H_2O_2-stimulated HDF. The cells were pre-treated with BPCA (25, 50, and 100 μM) and treated H_2O_2 (400 μM) for 30 min. ROS production is measured using 20 μg/ml of DCFH-DA. All the results were expressed as means ± S.E. of over three individual experiments; *P < 0.05.

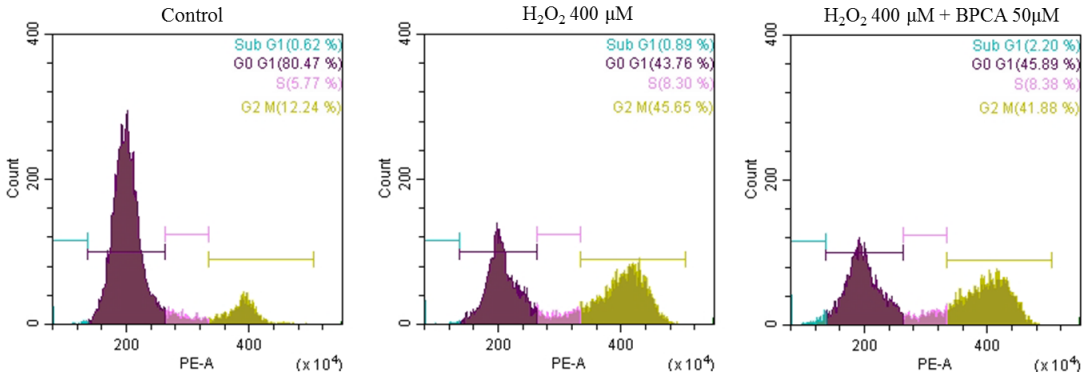


Fig. 2-4. Effect of BPCA on H_2O_2-induced cell cycle arrest in HDF. The cells were pre-treated with BPCA (50 μM) and treated H_2O_2 (400 μM) for 24 h. Cell cycle arrest was measured using flow cytometry analysis.

3. 6. Effect of BPCA on SA-β-gal activity in H_2O_2-stimulated zebrafish

We observed the expression of SA-β-gal in zebrafish exposed to H_2O_2 and effect of BPCA and NAC on expression of SA-β-gal in zebrafish exposed to H_2O_2 (Fig. 2-6). When 500 and 600 μM of H_2O_2 were treated, SA-β-gal activity significantly increased compared to control group. Treatment group of 500 μM of H_2O_2 was more increased than treatment group of 600 μM. The result show that 500 μM of H_2O_2 more induces the aging in zebrafish than 600 μM. Therefore, we determined the H_2O_2 concentration of aging induction at 500 μM (Fig. 2-6 (A)). Next, to confirm the relationship between aging according to oxidative stress and antioxidant NAC in zebrafish, we treated the H_2O_2 and NAC and carried out the SA-β-gal staining in zebrafish. As previous results, 500 μM of H_2O_2 induced the aging in zebrafish. However, treatment of 100 μM of NAC preserved against the oxidative stress-induced aging. Therefore, aging and oxidative stress are related and antioxidant NAC can suppress the H_2O_2-induced aging in zebrafish. (Fig. 2-6 (B)). Also, we observed the effects of BPCA on oxidative stress-induced aging in zebrafish. When 500 μM of H_2O_2 were treated, SA-β-gal activity considerably increased compared to control group. However, 50 μM of BPCA suppressed the SA-β-gal activity. Therefore, it is demonstrated that BPCA can inhibit the oxidative stress-induced aging in zebrafish (Fig. 2-6 (C)). Taken together, these results identify that H_2O_2 induces the aging in zebrafish, oxidative stress-induced aging can inhibit using NAC, and BPCA can protect against oxidative stress-induced aging in zebrafish.

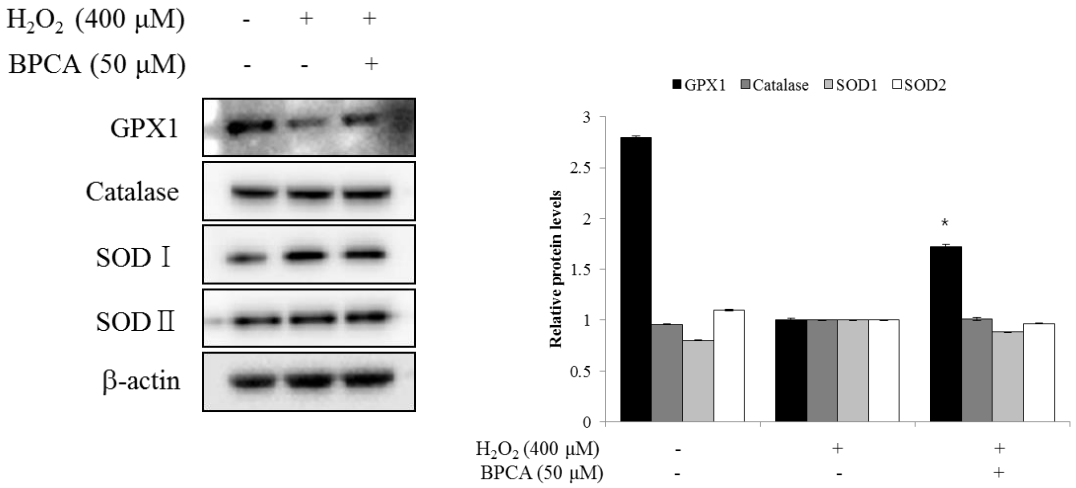


Fig. 2-5. Effect of BPCA on antioxidant enzymes in H_2O_2 -stimulated HDF. The cells were pre-treated with BPCA (50 μM) and treated with H_2O_2 (400 μM) for 6 and 12 h. GPX1, catalase, SOD1, and SOD2 protein level were determined using Western blot analysis. All the results were expressed as means ± S.E. of over three individual experiments; *P < 0.05.

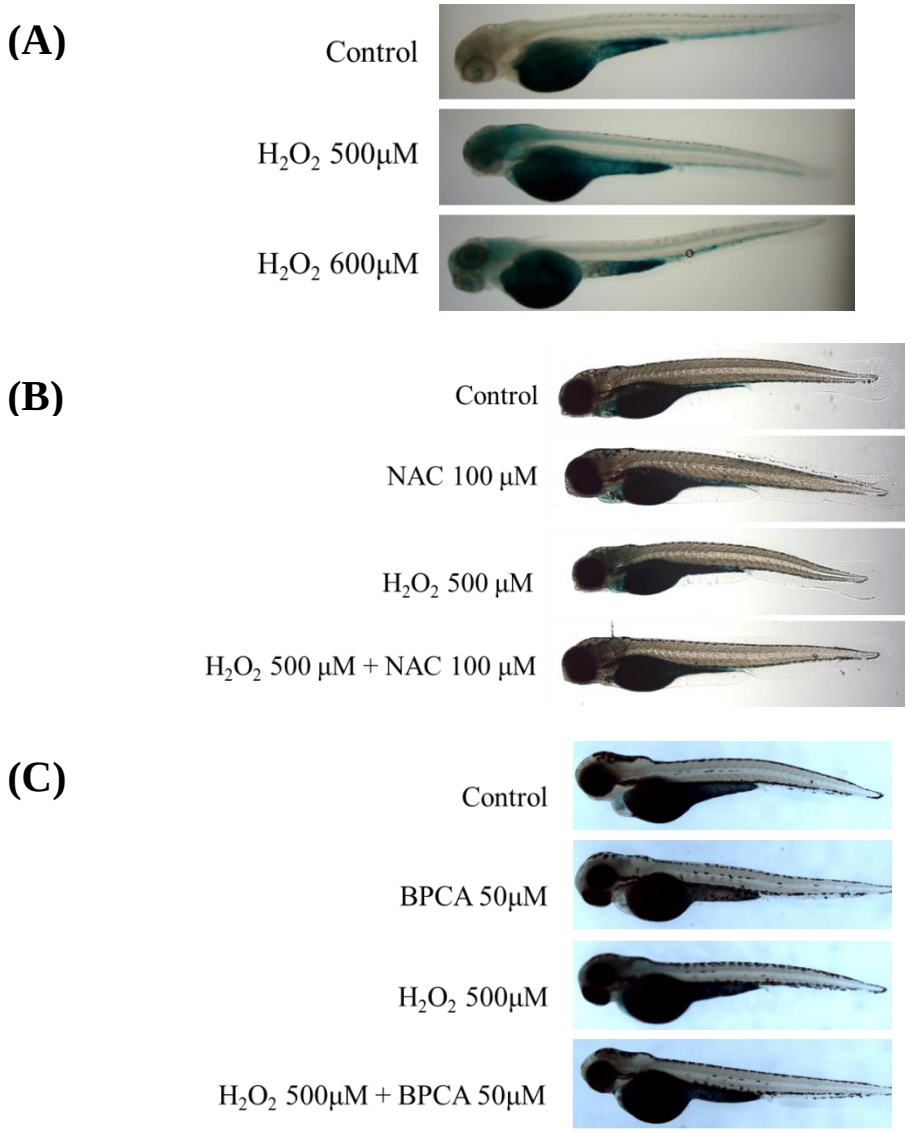


Fig. 2-6. Effect of BPCA on SA-β-gal activity in H_2O_2-stimulated zebrafish. Zebrafish was fixed and stained using SA-β-gal solution after treatment of H_2O_2 and BPCA. Imaging was performed using the inverted microscope. (A) Zebrafish embryos were treated H_2O_2 (500 and 600 μM) for 3 dpf. 500 μM of H_2O_2 induced SA-β-gal activity (B) Zebrafish embryos were pre-treated NAC (100 μM) ant then treated H_2O_2 (500 μM) for 3 dpf. NAC suppressed the SA-β-gal activity in H_2O_2-stimulated zebrafish. (C) Zebrafish embryos were pretreated BPCA (50 μM) and then treated H_2O_2 (500 μM) for 3 dpf.

4. Discussion

The overexpression of ROS leads the injury of organisms due to oxidative damage of DNA, RNA, protein, and lipid, and hinders damaged nucleus and mitochondrial DNA repair, contributing to cellular imbalance (Aybek et al., 2008; Li et al., 2013; Weyemi and Dupuy, 2012). Eventually, it leads the cellular and tissue damage and finally causes the various diseases such as Parkinson's disease, cancer, inflammation, and aging (Barbara S. Berlett and Earl R. Stadtman, 1997; Hong et al., 2011; Kang et al., 2012; Heo et al., 2006). In previous studies, it is demonstrated that H_2O_2 accelerates the aging by inducing oxidative stress (Yu et al., 2010; Satoh et al, 2005). For that reason, in this study, we evaluated the anti-aging effect of BPCA on H_2O_2-induced premature senescence in HDF. We first examined the cytotoxicity of BPAC in HDF cells and found that it was not cytotoxic at the highest concentration (50 μM). Thus doses without cytotoxicity were chosen to determine the anti-aging effect of BPCA via regulation of antioxidant enzyme. . Next, we confirmed that BPCA significantly inhibited the intracellular ROS production. The results show that BPCA can protect from oxidative stress in H_2O_2-stimulated HDF.

SA-β-gal is the β-galactosidase activity and widely used as biomarker of cellular aging (Dimri et al., 1995; Lee et al., 2006). It can detect at pH 6.0 in cultured cells and mammalian tissues at the moment of replicative or induced aging and the origin is known as the lysosome (Dimri et al., 1995; Kurz et al., 2000; Debacq-Chainiaux et al., 2009). Previous study reported that the higher the induction of the aging in cells, the greater the expression level of SA-β-gal. Therefore, we measured of SA-β-gal activity to confirm that BCAP has an aging effect. In this study, HDF treated H_2O_2 increased the SA-β-gal expression thus it can know that H_2O_2 can induce the aging in HDF. However, when the cells were treated with BPCA, aging marker SA-β-gal was significantly inhibited. Therefore, the result identifies

that BPCA can inhibit oxidative stress-induced aging.

The relationship of aging and cell cycle arrest has not yet been clarified clearly. In previous study, it demonstrated that cell cycle arrest is not aging (Blagosklonny, 2011; Blagosklonny, 2012). However, in other study, aging and cell cycle arrest is related each other and cell cycle arrest induces the aging (Deursen, 2014; Dulic et al., 2000; Mao et al., 2012). It is identified that treatment of H_2O_2 induces the G_2/M phase cell cycle arrest (Li et al., 2009). Therefore, to identify the relationship of aging and cell cycle arrest, we evaluated of effect of BPCA on H_2O_2-induced the cell cycle arrest using flow cytometry analysis. When H_2O_2 were treated in HDF, G_2/M DNA content considerably increased. However, the treatment of BPCA not inhibited G_2/M cell cycle arrest. Thus, the result is demonstrated that H_2O_2 induces the cell cycle arrest but BPCA has not effect on H_2O_2-induced cell cycle arrest in HDF. These results suggest that the inhibitory effect of BPCA on cell senescence is not related to cell arrest.

In previous study, the enzymatic antioxidant defense system is related to aging (Inal et al., 2001; Tian et al., 1998). The important factors of enzymatic defense system against ROS and superoxide anion radicals, SODs convert the superoxide anion to H_2O_2 (Fridovich, 1997; Inal et al., 2001). In the previous research, SODs preserve against the ROS in the cells (Tian et al., 1998; Fridovich., 1995; Bannister J. V., 1987). Catalase plays the role of the decomposition of H_2O_2 to water and oxygen (Tian et al., 1998; Weydert and Cullen, 2010; Chelikani et al., 2004). It is also important enzymes in preserving the cell from oxidative stress by ROS (Weydert and Cullen, 2010; Chelikani et al., 2004). GPXs are family of enzymes that decrease of H_2O_2 to water or reduction of lipid hydroperoxides to corresponding alcohols by glutathione (Chen et al., 2009, Brigelius-Flohe, 2006). GPX1 is also the critical factor to remove the H_2O_2 and control ROS for metabolism in the aerobic organisms (Weydert and Cullen, 2010). Therefore, we researched the effect of BPCA on

antioxidant enzymes in H_2O_2-stimulated HDF. The treatment of H_2O_2 is impeded the GPX1 expression, but BPCA promoted the expression levels of GPX1. The expression levels of SOD1, SOD2, and catalasewere not changed by H_2O_2 and BPCA treatment than control. . Therefore, these results indicated that BPCA can protect the H_2O_2-stimulated HDF via increase of GPX1 expression. Also, we predict that BPCA can inhibit the H_2O_2-induced aging via antioxidant enzyme mechanism.

The zebrafish is a good vertebrate model to study a variety of fields. It has many advantages such as small size, similar genome to human, rapid development, and easy handling in experiment (Kim et al., 2015; Lan et al., 2013; Zou et al., 2017; Arslan-Ergul et al., 2016). Also, because it lives on average between 3 and 5 years and has an optical transparency, it is appropriate for aging animal model (Arslan-Ergul et al., 2016; Kim et al., 2015). In previous study, the zebrafish had used as aging models (Gilbert et al., 2014; Gerhard, 2003; Kishi et al., 2003). Therefore, we evaluated the effect of BPCA on H_2O_2 -induced SA-β-gal activity, an aging maker using zebrafish. First, we treated 500 and 600 μM of H_2O_2 and conducted the SA-β-gal staining in zebrafish. As a result, the treatment of 500 μM of H_2O_2 significantly induced the SA-β-gal activity than treatment of 500 μM of H_2O_2. So, we determined the treatment concentration of H_2O_2, 500 μM. Next, to confirm the direct role of ROS in aging induction, we investigated the effects of NAC (a specific inhibitor of ROS) on increasing of aging marker SA-β-gal activity in H_2O_2-induced zebrafish embryos. We found that NAC inhibited the SA-β-gal activity in H_2O_2-induced zebrafish embryo. These results suggest that the aging and H_2O_2-induced oxidative stress is related each other in zebrafish embryo. Furthermore, we confirmed the anti-aging effect of BPCA on oxidative stress-induced premature senescence in zebrafish. H_2O_2 increased the SA-β-gal activity, however BPCA decreased the SA-β-gal activity in zebrafish. We predict that BPCA can inhibit the aging by suppressing oxidative stress in zebrafish.

In conclusion, the present study suggests that BPCA inhibits premature senescence by inhibiting ROS production by restoring GPX antioxidant enzyme in H_2O_2-induced HDF cells. Also, BPCA inhibited the oxidative stress-induced aging in zebrafish embryos. Taken together, BPCA can suppress the oxidative stress-caused aging in *in vitro* and *in vivo*. These results demonstrated the potential of BPAC, providing a basis for further studies on applying it to combat aging related diseases.

Reference

Adams J. M. and Cory S., 1998, The Bcl-2 protein family: arbiters of cell survival. Science, 281, 1322-1326

Alcorta D. A., Xiong Y., Phelps D., Hannon G., Beach D., and Barrett J. C., 1996, Involvement of the cyclin-dependent kinase inhibitor p16 (INK4a) in replicative senescence of normal human fibroblasts. Biochemistry, 93, 13741-13747

Alfadda, A. A., and Sallam, R. M., 2012, Reactive oxygen species in health and disease. Journal of Biomedicine and Biotechnology, 1-14.

Arslan-Ergul A., Erbaba B., Karoglu E. T., Halim D. O., and Adams M. M., 2016, Short-term dietary restriction in old zebrafish changes cell senescence mechanisms. Neuroscience, 334, 64-75

Aybek H., Aybek Z., Rota S., Sen N., and Akbulut M., 2008, The effects of diabetes mellitus, age, and vitamin E on testicular oxidative stress. American Society for Reproductive Medicine, 90, 755-760

Baek S. O., Kim H. J., Jeong H. J., Ju E. S., Kang C. S., Seo Y. W., 2015, Antioxidant acitivity of the Halophyte Ligustrum japonicum. Journal of Korean Society for Biotechnology and Bioengineering. 30, 275-282

Barbara S. Berlett and Earl R. Stadtman, 1997, Protein oxidation in aging, disease, and oxidative stress. The Journal of Biological Chemistry, 272, 20313-20316

Baeuerle P. A., Pro-inflammatory signaling: last pieces in the NF-κB puzzle? Current Biology, 8, 19-22

Blagoskonny M. V., 2011, Cell cycle arrest is not senescence. Aging, 3, 94-101

Blagoshlonny M. V., 2012, Cell cycle arrest is not yet senescence, which is not just cell cycle arrest: terminology for TOR-driven aging. Aging, 4, 160-165

Branen A. L., 1975, Toxicological and biochemistry of butylated hydroxyanisole and butylated hydroxytoluene. JAOCS, 52, 59-62

Brigelius-Flohe R., 2006, Glutathione peroxidases and redox-regulated transcription factors. The Journal of Biological Chemistry, 387, 1329-1335

Burtke T. M., and Sandstrom P. A., 1994Oxidative stress as a mediator of apoptosis, Immunology. 15, 7–10

Caruso C., Lio D., Cavallone L., and Franceschi C., 2004, Aging, longevity, inflammation, and cancer. Annals of the New York Academy of Sciences, 1208, 1-13

Cha S.H., Ahn G.N., Heo S.J., Kim K.N., Lee K.W., Song C.B., Kim S.M., Jeon Y.J., 2006, Screening of extracts from marine green and brown algae in Jeju for potential marine angiotensin-1 converting enzyme (ACE) inhibitory activity. Journal of Korean Society of Food Science and Nutrition, 35, 307-314

Chelikani P., Fita I., and Loewen P. C., 2004, Diversity of structures and properties among catlases. Cellular and Molecular Life Sciences, 61, 192-208

Chen A. C. H., Arany P. R., Huang Y. Y., Tomkinson E. M., Sharma S. K., Kharkwal G. B., Saleem T., Mooney D., Yull F. E., Blackwell T. S., and Hamblin M. R., 2011, Low-level laser therapy activates NF-κB via generation of reactive oxygen species in mouse embryonic fibroblasts. PLoS ONE, 6, 1-8

Chen Q. M., Bartholomew J. C., Campisi J., Acosta M., Reagan J. D., and Ames B. N., 1998, Molecular analysis of H_2O_2-induced senescence-like growth arrest in normal human fibroblasts: p53 and Rb control G_1 arrest but not cell replication. Biochemical Journal, 43-50

Chen Y., Mehta G., and Vasilious V., 2009, Antioxidant defenses in the ocular surface. HHS Public Acess, 7, 176-185

Choi M. J., Kim H. Y., and Cho E. J., 2012, Anti-aging effect of black rice against H_2O_2-induced premature senescence. Journal of Medicinal Plans Research, 6, 3672-3680

Choo K. B., Tai L. H., Hymavathee K. S., Wong C. Y., Nguyen P. N. N., Huang C. J., Cheong S. K., and Kamarul T. K., 2014, Oxidative stress-induced premature senescence in Wharton's jelly-derived mesenchymal stem cells., International Journal of Medical Sciences, 11, 1201-1207

Choi W. Y., and Lee H. Y., 2014, Enhancement of antioxidant activities of Curcuma longa leaves by ultra high pressure extraction. Korean Journal of Medicinal Crop Science, 22, 121-126

Cohen G. M., 1997, Caspase: the executioners of apoptosis. Biochemical Journal, 326, 1-16

Colle D., Arantes L. P., Rauber R., de Mattos S. E., Rocha J. B., Nogueira C.W., and Soares F. A. A., 2012, Antioxidant properties of Taraxacum officinale fruit extract are involved in the protective effect against cellular death induced by sodium nitroprusside in brain of rats. Journal of Pharmaceutical Biology, 50, 833-891

da Silva F. M., Marques A., and A. Chaveiro, 2010, Reactive oxygen species: A double-edged sword in reproduction. The Open Veterinary Science Journal, 4, 127-133.

David M. Hocekнbery, Zoltan N. Oltivai, Xiao-Ming Yin, Curt L. Milliman, and Stanley J. Korsmeyer, 1993, Bcl-2 Functions in an antioxidant pathway to prevent apoptosis. Journal of Cell, 75, 241-251

Du J., Cai J., Wang S., and You H., 2015, Oxidative stress and apoptosis to zebrafish (Danio rerio) embryos exposed to perfluorooctane sulfonate (PFOS) and Zno nanoparticles.

International Journal of Occupational Medicine and Environmental Health. 30, 213-229

Debacq-Chainiaux F., Erusalimsky J. D., Campisi J., and Toussaint O., 2009, Protocols to detect senescene-associated beta-galactosidase (SA-β-gal) activity, a biomarker of senescent cells in culture and in vivo. Nature, 4, 1798-1806

Denham Harman, 2001, Aging: Overview. Journal of Annals of the New York Academy of Sciences, 928, 1-21

Denham Harman, 2006, Free radical theory of aging: an update: increasing the functional life span, journal of Annals of New York Academy of Sciences

Deursen J. M. V., 2014, The role of senescent cells in ageing. Nature, 509, 439-446

Dimri G. P., Lee X., Basile G., Acosta M., Scott G., Roskelley C., Medrano E. E., Linskens M., Rubelj I., Pereira-Smith O., Peacocke M., Campisi J, 1995, A biomarker that identifies senescent human cells in culture and in aging skin *in vivo.* Proceedings of the National Academy of Sciences of the United States of America, 92, 9363-9367

Dimri G. P., Nakanishi M., Desprez P. Y., Smith J. R., and Campisi J., 1996, Inhibition of E2F activity by the cyclin-dependent protein kinase inhibitor p21 in cells expression or lacking a functional Retinoblastoma protein. Molecular and Cellular Biology, 16, 2987-2997

Duan J. M., Duan J. P., Zhang Z. G., and Tong T. J., 2005, Irreversible cellular senescence induced by prolonged exposure to H_2O_2 involved DNA-damage-and-repair gene and telomere shortening. The International Journal of Biochemistry & Cell Biology, 37, 1407-1420

Duan X. J., Zhang W. W., Li X. M., and Wang B. G., 2006, Evaluation of antioxidant property of extract and fractions obtained from a red alga, *Polysiphonia urceolata*. Journal of

Food Chemistry, 95, 37-43

Dubrez L., Coll J. L., Hurbin A., de Fraipont F., Lantejoul S., and Farvrot M. C., 2001, Cell cycle arrest is sufficient for p53-mediated tumor regression. Gene Therapy, 8, 1705-1712

Dulic V., Beney G. E., Frebpirg G., Drullinger L. F., and Stein G. H., 2000, Uncoupling between phenotypic senescence and cell cycle arrest in aging p21-deficient fibroblasts. Molecular and Cellular Biology, 20, 6741-6754

Eugenia Wang, 1995, Senescent human fibroblasts resist programmed cell death, and failure to suppress bcl2 is involved. Journal of Cancer Research, 55, 2284-2292

Farage M. A., Miller K. W., Elsner P., and Maibach H. I., 2008, Intrinsic and extrinsic factor in skin ageing: a review. International Journal of Cosmetic Science, 30, 87-95

Finkel T, Holbrook NJ, 2000, Oxidants, oxidative stress and the biology of ageing. Journal of Nature, 408, 239-247

Finucane D. M., Bossy-Wetzel E., Waterhouse N. J., Cotter T. G., and Green D. R., 1999, Bax-induced caspase activation and apoptosis via cytochrome *c* release from mitochondria is inhibitable by Bcl-xL. The Journal of Biological Chemistry, 274, 2225-2233

Fridovich I., 1997, Superoxide anion radical ($O^{\cdot-}_2$), superoxide dismutases, and related matters. Journal of Biological Chemistry, 272, 18515-18517

Gabriele M. Konig, Anthony D. Wright, Otto Sticher, Cindy K. Angerhofer, John M. Pezzuto, 1994, Biological activites of selected marine natural products. Journal of Planta Medica, 60, 532-537

Garbary D. J., and Clarke B., 2001, Apoptosis in trichoblast development in *Polysiphonia harveyi* (Rhodophyta). Phycologia, 40, 324-329

Gerhard G. S., 2003, Comparative aspects of zebrafish (Danio rerio) as a model for aging research. Experimental Gerontology, 38, 1333-1341

Gilbert M. J. H., Zerulla T. C., and Tierney K. B., 2014, Zebrafish (Danio rerio) as a model for the study of aging and exercise: Physical ability and trainability decrease with aging. Experimental Gerontology. 50, 106-113

Gonos E. S., Burns J. S., Mazars G. R., Koborna A., Riley T. E., Barnett S. C., Zafarana G., Ludwig R. L., Ikram Z., Powell A. J., and Jat P. S., 1996, Rat embryo fibroblasts immortalized with simian virus 40 large T antigen undergo senescence upon its inactivation. Molecular and Cellular Biology, 16, 5127-5138

Heo S. J., Cha S. H., Lee K. W., and Jeon Y. J., 2006, Antioxidant activities of red algae from Jeju Island. The Korean Society of Phycology, 21, 149-156

Ham Y. M., Yoon W. J., Park S. Y., Song G. P., Jung Y. H., Jeon Y. J., Kang S. M., and Kim K. N., 2012, Quercitrin protects against oxidative stress-induced injury in lung fibroblast cells via up-regulation of Bcl-xL. Journal of Functional Foods, 4, 253-262

Hara E., Smith R., Parry D., Tahara H., Stone S., and Peters G., 1996, Regulation of p16 ^{CDN2} expression and its implications for cell immortalization and senescence. Molecular and Cellular Biology, 16, 859-867

Hong C.O., Hong S.T., Koo Y.C., Yang S.Y., Lee, J.Y., Lee Y.H., Ha Y. M., and Lee K.W., 2011, Protective effect of *Plantago asiatica* L. extract against ferric nitrilotriacetate (Fe-NTA) induced renal oxidative stress in wistar rats. Journal of Food Hygiene and Safety, 26, 107-113

Hong J. K., 2009, A study on skin aging caused by free-radical and efficacy of antioxidant Vitamins. Asian Journal of Beauty and Cosmetology, 7, 51-62

Inal M. E., Kanbak G., and Sunal E., 2001, Antioxidant enzymes activities and malondialdehyde levels related to aging. Clinica Chimica, 305, 75-80

Jan Vijg, Jeanne Y. Wei, 1995, Understanding the biology of aging: The key too prevention and therapy. Journal of American Geriatrics Society, 43, 426-434

Jones N. L., Islur A., Haq Q. Mascarenhas M., Karmali M. A., Perdue M. H., Zanke B. W., and Sherman P. M., 2000, Escherichia coli Shiga toxins induce apoptosis in epithelial cells that is regulated by the Bcl-2 family. American Journal of Physiology – Gastrointestinal and Liver Physiology, 278. 811-819

Judith Campisi, 1996, Replicative senescence: An old lives' Tale?. Journal of Cell, 84, 497-500

Jung W. K., Je J. Y., and Kim S. K., 2002. A novel anticoagulant protein form Scapharca broughtonii. Journal of Biochemistry and Molecular Biology, 35, 199-205.

Kang M. C., Kim E. A., Kang S. M., Wijesinghe W. A. J. P., Yang X. D., Kang N. L., and Jeon Y. J., 2012, Thermostability of a marine polyphenolic antioxidant dieckol, derived from the brown seaweed Ecklonia cava. Journal of Algae, 27, 205-213

Kastan M. B., Onyekwere O., Sidransky D., Vogelstein B., and Craig R. W., 1991, Participation of p53 protein in the cellular response to DNA damage. Cancer Research, 51, 6304-6311

Kasuga A., Aoyagi Y., and Sugahara T., 1988, Antioxidants activiteis of edible plants. Nippon Shokuhin Kogyo Gakkaishi, 35, 22-25

Kerr, J. F. R., Wyllie, A. H., Currie, A. R., 1972, Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. British Journal of Cancer, 26, 239-257

Kim B. K., Park K. Y., Kim H. Y., Ahn S. C., and Cho E. J., 2011, Anti-aging effects and mechanisms of kimchi during fermentation under stress-induced premature senescence cellular system. Journal of Food Science and Biotechnology, 20, 643-649

Kim E. A., Kang M. C., Lee J. H., Kang N. L., Lee W. W., Oh J. Y., Yang H. W., Lee J. S., and Jeon Y. J., 2015, Protective effect of marine brown algal polyphenols against oxidative stressed zebrafish with high glucose. Royal Society of Chemistry, 5, 25738-25746

Kim J. J., Kim S. J., Kim S. H., Park H. R., Lee S. C., 2005, Antioxidant and anticancer activities of extracts from *Styela plicata*. Journal of Korean Society of Food Science and Nutrition, 34, 937-941

Kim K. L., 2007, Biology of aging: medical perspective. Journal of Korean Medical Association. 50, 216-220

Kim S. Y., Kim S. R., Oh M. J., Jung S. J., and Kang S. Y., 2011. In vitro Antiviral activity of red algae, *Polysiphonia morrowii* extract and its bromophenols against fish pathogenic infectious hematopoietic necrosis virus and infection pancreatic necrsosis virus. Journal of Microbiology, 49, 102-106

Kishi S., Uchiayma J., Baughman A. M., Goto T., Lin M. C., and Tsai S. B., 2003, The zebrafish as a vertebrate model of functional aging and very gradual senescence. Experimental Gerontology, 38, 777-786

Kohen R., 1999, Skin antioxidants: their role in aging and in oxidative stress – New approaches for their evaluation. Journal of Biomedicine & Pharmacotherapy, 53, 181-192

F.B. Johnson, D.A. Sinclair, L. Guarente, 1999, Molecular Biology of Aging. Journal of Cell, 96, 291-302

Kobayashi M., Li L., Iwamoto N., Nakajima-Takagi Y., Kaneko H., Nakayama Y., Eguchi M.,

Wada Y., Kumagai Y., and Yamamoto M., 2009, The antioxidant defense system Keap1-Nrf2 comprises a multiple sensing mechanism for responding to a wide range of chemical compounds. American Society for Microbiology, 29, 493-502

Krishnaswamy Kannan, Sushil K. Jain, 2000, Oxidative stress and apoptosis. Journal of Pathophysiology, 7, 153-163

Kurz D. J., Decary S., Hong Y., and Erusalimsky J. D., 2000, Senescence-associated β-galactosidase reflects an increase in lysosomal mass during replicative ageing of human endothelial cells. Journal of Cell Science, 113, 3613-3622

Kwon I. S., 2007, Understanding of aging. Journal of the Korean Medical Association, 2007, 50, 208-215

Lamming D. W., Ye L., Sabatini D. M., and Baur J. A., 2013, Rapalogs and mTOR inhibitors as anti-aging therapeutics. The Journal of Clinical Investigation, 123, 980-989

Lan C. C., Tang R. Y., Leong I. U. S., and Love D. R., 2013, Quantitative Real-Time RT-PCR (qRT-PCR) of zebrafish Transcripts: optimization of RNA extraction, quality control considerations, and data analysis. Cold Spring Harbor Protocols, 4, 5314

Lee B. Y., Han J. A., Im J. S., Morrone A., Johung K., Goodwin E. C., Kleijer W. J., DiMaio D., and Hwang E. S., 2006, Senescence-associated β-galactosidase is lysosomal β-galactosidase. Aging Cell, 5, 187-195

Leutner S., Eckert A., and Muller W. E., 2001, ROS generation, lipid peroxidation and antioxidant enzyme activities in the aging brain. Journal of Neural Transmission, 108, 955-967

Li M., Liu Z., Li Z., Wang T., Guo S., Wang S., Liu J., and Ye Z., 2013, Effects of apocynin

on oxidative stress and expression of apoptosis-related genes in testes in tests of diabetic rats. Molecular Medicine Reports, 7, 47-52

Li M., Zhao L., Liu J., Liu A. L., Zeng W. S., Luo S. Q., and Bai X. C., 2009, Hydrogen peroxide induces G_2 cell cycle arrest and inhibits cell proliferation in osteoblasts. The anatomical record, 292, 1107-1113

Li K., X. Li M., Ji N. Y., and Wang B. G., 2008, Bromophonols from the marine red alga *Polysiphonia urceolata* with DPPH radical scavenging activity. Journal of Natural Products, 71, 28-31

Manosroi A., Jantrawut P., Akihisa T., Manosroi W., and Manosroi J., 2009, In vitro anti-aging activities of Terminalia chebula gall extract. Journal of Pharmaceutical Biology, 48, 469-481

Mao Z., Ke Z., Gorbunova V., and Seluanov A., 2012, Replicatively senescent cells are arrested in G1 and G2 phase. Aging, 4, 431-435

Masaya Yamamoto, Jeremy D. Clark, Johanne V. Pastor, Prem Gurnani, Animesh Nandi, Hiroshi Kurosu, Masayoshi Miyoshi, Yasushi Ogawa, Diego H. Castrillon, Kevin P. Rosenblatt, and Makoto Kuro-o, 2005, Regulation of oxidative stress by the anti-aging hormone klotho. Journal of Biological Chemistry, 280, 38029-38034

Mayer M., Schreck R., and Baeuerle P. A., 1993, H_2O_2 and antioxidants have opposite effects on activation of NF-κB and AP-1 in intact cells: AP-1 as secondary antioxidant-responsive factor. The EMBO Journal, 12, 2005-2015

Matsui, S. M., Muizzudin, N., Arad, S. M., and Marenus, K., 2003. Sulfated polysaccharides from red microalgae anti-inflammatory properties *in vitro* and *in vivo*. Applied Biochemistry and Biotechnology., *104*, 13–22.

Michio Fujihara and Terukazu Nagumo, 1993, An influence of the structure of alginate on the chemotactic activity of macrophages and the antitumor activity. Journal of Carbohydrate Research, 243, 211-216

Melelgari S. P., Perreault F., Moukha S., Popovic R., Creppy E. E., and Matias W. G., 2012, Induction to oxidative stress by saxitoxin investigated through lipid peroxidation in Neuro 2A cells and *Chlamydomonas reinhardtii* algae. Chemosphere, 89, 38-43

Moradas-Ferreira P., and Costa V., 2000, Adaptive response of the yeast Saccharomyces cerevisiae to reactive oxygen species: defences, damage and death. Redox Report, 5, 277-285

Morgan M. J., and Liu Z. G., 2011, Crosstalk of reactive oxygen species and NF-κB signaling. Cell Research, 21, 103-115

Morris M., Hapburn P., and Wynford-Thomas D., 2002, Sequential extension of proliferative lifespan in human fibroblasts induced by over-expression of CDK4 or 6 and loss of p53 function. Oncogene, 21, 4277-4288

Morel Y., and Barouki, 1999 Repression of gene expression by oxidative stress. Biochemical Journal 342, 481–496.

Oliver F. J., Rubia G. D. L., Rolli V., Ruiz-Ruiz M. C., Murcia G. D., and Murcia M. D., 1998, Importance of poly(ADP-ribose) polymerase and its cleavage in apoptosis. The Journal of Biological Chemistry, 273, 33533-33539

Palmero I. McConnell B., Parry D., Brookes S., Hara E., Bates S., Jat P., and Peter G., 1997, Accumulation of P16INK4a in mouse fibroblasts as a function of replicative senescence and not of retinoblastoma gene status. Oncogene, 15, 495-503

Park Y. C., 2003, Replicative senescence in cellular aging and oxidative stress. Official

Journal of Korean Society of Toxicology, 19, 161-172

Park J. S., 2004, Cellular senescence, apoptosis and carcinogenesis. Journal of The Korean Geriatrics Society, 8, 1-7

Roy G. Cutler, Mark P. Mattson, 2006, Introduction: The adversities of aging. Journal of Aging Research Reviews, 5, 221-238

Radonic, A., Thulke, S., Achenbach, J., Kurth, A., Vreemann, A., König, T., Walter, C., Possinger, K., and Nitsche, A., 2010. Anionic polysaccharides from phototrophic microorganisms exhibit antiviral activities to *Vaccinia* virus. J. Antivir. Antiretrovir, *2*, 51–55

Saravanakumar D. E. M., Folb P. I., Campbell B. W., and Smith P., 2008, Antimycobacterial activity of the red algae polysiphonia virgata. Journal of Pharmaceutical Biology, 46, 254-260

Satoh A., Yokozawa T., Kim Y. A., Cho E. J., Okamoto T., and Sei Y., 2005, The mechanisms underlying the anti-aging activity of the Chinese prescription Kangen-karyu in hydrogen peroxide-induced human fibroblasts. Journal of Pharmacy and Pharmacology, 57, 1335-1343

Schenk H., Klein M., Erdbrugger W., Droge W., and Schulze-Osthoff K., 1994, Distinct effects of thioredoxin and antioxidants on the activation of transcription factors NF-κB and AP-1. Proceedings of the National Academy of Sciences, 91, 1672-1676

Schreck R., Rieber P., and Baeuerle P. A., 1991, Reactive oxygen intermediates as apparently widely used messengers in the activation of the NF-κB transcription factor and HIV-1. The EMBO journal, 10, 2247-2258

Schwartzman R. A., and Cidlowski L. A., 1993, Apoptosis: the biochemistry and molecular biology of programmed cell death. Endocrine Reviews Journal, 14, 133-151

Sohal R. S., Mochett R. J., and Orr W. C., 2002, Mechanisms of aging: an appraisal of the oxidative stress hypothesis. Journal of Free Radical Biology and Medicine, 33, 575-586

Song J. C., Park N. K., Hur H. S., Bang M. H., and Baek N. I., 2000, Examination and isolation of natural antioxidants from Korean medical plants. Korean Journal of Medicinal Crop Science, 8, 94-101

Stack L. A., Reid K., Sansom O. J., Din F. V., Guichard S., Jodrell D. I., Clarke A. R., Dunlop M. G, 2007, Asprin activates the NF-κB signaling pathway and induces apoptosis in intestinal neoplasia in two in vivo models of human colorectal cancer. Carcinogenesis, 28, 968-976

Stein G. H., Beeson M., and Gordon L., 1990, Failure to phosphorylate the retinoblastoma gene product in senescent human fibroblasts. Science, 249, 666-669

Stein G, H., Drullinger L. F., Soulard A., and Dulic V., 1999, Differential roles for cyclin-dependent kinase inhibitors p21 and p16 in the mechanisms of senescence and differentiation in human fibroblasts. Molecular and Cellular Biology, 18, 2109-2117

Sun X. M., MacFarlane M., Zhuang J., Wolf B. B., Green D. R., and Cohen G. M. 1999, Distinct caspase cascades are initiated in receptor-mediated and chemical-induced apoptosis. The Journal of Biological Chemistry, 274, 5053-5060

Sung, C., Y. Hsu, C. Chen, Y. Lin, and C. Wu, 2013, Oxidative stress and nucleic acid oxidation in patients with chronic kidney disease. Oxidative Medicine and Cell Longevity. 1-15.

Suo R.,. Zhao Z. Z., Tang Z. H., Ren Z., Liu X., Liu L. S., Wang Z., Tang C. K., Wei D. H., and Jiang Z. S., 2013, Hydrogen sulfide prevents H_2O_2-induced senescence in human umbilical vein endothelial cells through SIRT1 activation. Journal of Molecular Medicine

Reports, 7, 1865-1870

Tang X. Y., Zhang W., Dai D. X., Ying H. J., Wang Q. J., and Dai Y., 2008, Effects of strontium fructose 1,6-diphosphate on expression of apoptosis-related genes and oxidative in tests of diabetic rats. International journal of Urology, 15, 251-256

Tannin-Spitz, T., Bergman, M., van Moppes, D., Grossman, S., and Arad, S.M., 2005. Antioxidant activity of the polysaccharide of the red microalga *Porphyridium* sp. Journal of Applied Phycology, 17, 215–222.

Tian L., Cai Q., and Wei H., 1998, Alterations of antioxidant enzymes and oxidative damage to macromolecules in different organs of rats during aging. Free radical Biology & Medicine, 24, 1477-1484

Tishler R. B., Calderwood S. K., Coleman C. N., and Price B. D., 1993, Increases in sequence specific DNA binding by p53 following treatment with chemotherapeutic and DNA damaging agents. Cancer Research, 53, 2212-2216

Van de Ven M, Andressoo J, Holcomb VB, Hasty P, Suh Y, van Steeg H, Garinis GA, Hoeijmaker JHJ, Michell JR, 2007, Extended longevity mechanisms in short-lived progeroid mice : identification of a preservative stress response associated with successful aging. Journal of mechanisms of Ageing And Development, 128, 58-63

Wagner H. P., 1998, Cell cycle control and cancer. Indian Journal of Pediatrics, 65, 805-814

Weydert C. J., and Cullen J. J., 2010, Measurement superoxide dismutase, catalase, and glutathione peroxidase in cultured cell and tissue. National Institutes of Health, 5, 51-66

Weyemi U., Dupuy C., 2012, The emerging role of ROS-generating NADPH oxidase NOX4 in DNA-damage responses. Mutation Research, 751, 77-81

Yu S. L., Lin S. B., Yu Y. L., Chien M. H., Su K. J., Lin C. J., Way T. D., Yiang G. T., Lin C. C., Chan D. C., Harn H. J., Chen Y. S., 2010, Isochaihulactone protects PC12 cell against H_2O_2 induced oxidative stress and exerts the potent anti-aging effects in D-galactose aging mouse model. Aca Pharmacologica Sinica, 31, 1532-1540

Zelko I. N., Mariani T. J., and Folz R. J., 2002, Superoxide dismutase multigene family: a comparison of the CuZn-SOD (SOD1), Mn-SOD (SOD2), and EC-SOD (SOD3) gene structures, evolution, and expression. Free Radical Biology & Medicine, 33, 337-349

Zou Y., Zhang Y., Han L., He Q., Hou H., Han J., Wang., Li C. G., Cen J., and Liu K., 2017, Oxidative stress-mediated developmental toxicity induced by isoniazide in zebrafish embryos and larvae. Journal of Applied Toxicology, 37, 842-852

Zhou L., Chen X., Liu T., Gong Y. H., Chen S. J., Pan G. Q., Cui W. G., Luo Z. P., Pei M., Yang H. L., and He F., 2015, Melatonin reverses H_2O_2-induced premature senescence in mesenchymal stem cells via the SIRT1-dependent pathway. Journal of Pineal Research, 59, 190-205

ACKNOWLEGEMENT

저에게 있어 지난 2년은 가장 뜻 깊고 소중했던 시간이었습니다. 부족한 저를 이끌어주시고 관심을 주시고 무사히 석사과정을 마칠 수 있도록 도움을 주신 분들께 감사의 인사를 전하고자 합니다. 항상 반갑게 맞아주시고 본 논문이 있기까지 세심하고 많은 가르침과 사랑을 주신 지도 교수님이신 전유진 교수님께 먼저 깊은 감사의 인사를 드립니다. 또한 바쁘신 와중에도 제 논문이 좀 더 나은 방향으로 갈 수 있도록 심사해주시고 관심을 가져주신 이승헌 교수님, 김현구 교수님, 허문수 교수님, 여인규 교수님께도 감사의 인사를 드립니다.

그리고 올해 한국기초과학지원연구원 제주센터에서 춘천센터로 오게 되면서 낯선 환경에서 잘 적응할 수 있도록, 그리고 연구를 진행할 수 있도록 많은 도움을 주신 권승해 센터장님께 감사를 표합니다. 또한 부족한 저를 지금까지 이끌어주시고 많은 가르침과 도움을 주셨으며 논문이 옳은 방향으로 갈 수 있도록 심사해주신 김길남 박사님께도 깊은 감사를 드립니다.

이번 논문이 나오고 졸업하면서 많은 도움을 주신 허수진 박사님, 여러 가지 많은 조언을 해주신 안긴내 교수님, 실험실에 갈 때마다 반갑게 맞아주셨던 원우 오빠, 재영 오빠, 고주영 박사님, 항상 반갑게 맞아주고 많은 도움을 주셨던 현수 오빠, 실험하면서 막히거나 모르는 것들에 대해서 자세하게 알려주셨던 은아 언니, 서영 언니, 혜원 언니, 대학원에 들어오기 전부터 저에게 연구에 관하여 많이 가르쳐주시고 신경을 써주신 은이 언니, 대학원에 같이 입학하고 이번에 같이 졸업하며 저에게 많은 도움을 주었던 바로,

실험실에 다니는 동안 많은 도움을 주셨던 효근 오빠, 실험실 생활에 잘 적응할 수 있도록 편하게 대해줬던 준건이와 지민 오빠 등 모든 해양생물자원이용공학 연구실 식구들께 깊은 감사를 표합니다.

뿐만 아니라 한국기초과학지원연구원 제주센터에서 함께 지냈던 선생님들, 제주에서 춘천에 올라와 적응하고 잘 지낼 수 있게 신경을 써주신 김용운 팀장님과 모든 선생님들께도 감사의 인사를 드립니다. 함께 할 수 있어서 행복했습니다.

대학원에 들어가 공부하겠다는 딸을 위해 뒤에서 묵묵히 아낌없는 지원과 사랑, 용기를 주신 우리 가족들, 항상 감사하고 사랑합니다. 그리고 힘들 때마다 투정을 받아주고 항상 저를 응원해주는 친구들에게도 감사하고 사랑한다는 말을 전하고 싶습니다.

지면으로 일일이 언급하지 못하였지만, 저에게 많은 도움을 주셨던 분들께 모두 감사드리며, 이번 석사과정이 끝이 아니라 시작일 뿐이며, 그 동안 배웠던 것들이 앞으로 박사과정을 밟으면서 큰 힘이 될 것이라고 확신합니다. 그 동안 도움을 주신 분들께 다시 한번 감사를 표하며, 항상 겸손하고 바른 모습으로 한층 성장하며 현재의 위치에서 계속 정진하겠습니다. 감사합니다.