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Extracts from the Red Algae *Gracilaria vermiculophylla* have Antioxidant Effects in Human Bone Marrow Mesenchymal Stem Cells

Sin-Gu Jeong[1,2], Jae-Joon Lee[3], Ho-Tae Kim[1,2], Min-Ji Ahn[1,2], Hee-Kyoung Son[3], Jun Sik Lee[1,2], Won Keun Oh[4], Tae Oh Cho[1], and Goang-Won Cho[1,2,†]

Abstract

The red algae *Gracilaria vermiculophylla* is widely spread around seaside areas across the globe, and has been used as a food resource in Southeast Asian countries. Previous studies have shown that *Gracilaria* red algae extracts have beneficial antihypercholesterolemic, antioxidant, anti-inflammatory, and antimicrobial effects. In this study, we investigated the antioxidant effects of *Gracilaria vermiculophylla* extracts (GV-Ex) on human bone marrow mesenchymal stem cells (hBM-MSCs). The acetone and DMSO/ethanol solvents of the tested GV contain higher total flavonoid and polyphenolic contents that can strongly scavenge reactive oxygen species (ROS). Pre-treatment with GV-Ex protected hBM-MSCs against oxidative stress induced by hydrogen peroxide treatment. The protective effects of GV-Ex treatment were confirmed by MTT assay. The elevated levels of ROS in hBM-MSCs caused by hydrogen peroxide induced oxidative stress were significantly decreased by GV extract treatment. The levels of the antioxidant proteins superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2), and catalase (CAT) were also restored or protected by GV-Ex treatment, suggesting that GV extracts moderate excess ROS levels and prevent cells from oxidative damage.

Keywords: *Gracilaria vermiculophylla*; Anti-oxidant; Mesenchymal Stem Cells; Reactive Oxygen Species; ROS

1. Introduction

Tissue homeostasis maintains the balance between cell death and cell proliferation within tissues and organs, and is one of the most important phenomena for human health[1,2]. Adult stem cells and their microenvironments play crucial roles in tissue homeostasis[3,4]. However, most stem cell populations will gradually decline due to cellular senescence during the lifetime of an organism, eventually breaking the homeostatic balance and causing progressive tissue ageing[5,6]. A strategy to prevent adult stem cell senescence would be useful to attenuate the functional decline of tissues and organs, and help keep the aging body healthy.

An excess of reactive oxygen species (ROS) can lead to senescence and cellular pathologies such as protein misfolding, DNA damage, and gene dysregulation[7,8]. Our previous studies have shown that a functional decline in stem cells can be caused by the accumulation of ROS during in vitro expansion, and that treatment with antioxidants such as ascorbic acid can delay stem cell senescence[9]. Thus, the accumulation of harmful intracellular ROS (iROS) causes a functional decline in aging cells, including stem cells. Living cells normally produce excess ROS through aerobic energy metabolism[10]. Cells use antioxidant enzymes such as superoxide dismutase 1 (SOD1), superoxide dismutase 2 (SOD2), and catalase (CAT) to dispose of harmful ROS[11,12]. Functional defects in these enzymes can lead to severe cellular damage resulting from the accumulation of iROS[13-15]. Cellular senescence may then accompany the chronic oxidative damage resulting from the abnormal accumulation of iROS. The elimination of excessive ROS is therefore crucial for maintaining proper cellular function.

Red algae of the genus *Gracilaria* are commonly

[1]Department of Biology, College of Natural Science, Chosun University, Gwangju 501-759, Korea
[2]Department of Life Science, BK21-Plus Research Team for Bioactive Control Technology, Chosun University, Gwangju 501-759, Korea
[3]Department of Food and Nutrition, College of Natural Science, Chosun University, Gwangju 501-759, Korea
[4]Korea Bioactive Natural Material Bank, Research Institute of Pharmaceutical Science, College of Pharmacy, Seoul National University, Seoul, Korea

[†]Corresponding author : gwcho@chosun.ac.kr
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found in bodies of water around the world. *Gracilaria* has been used as an agarophyte and an ingredient in Southeast Asian cuisine. Various species belonging to this genus have been commercially cultivated for human consumption[16]. *Gracilaria* extracts have been found to have anti-hypercholesterolemic, antioxidant, anti-inflammatory, and antimicrobial effects[17,18]. In this study, GV extracts reduced the ROS levels in human bone marrow mesenchymal stem cells (hBM-MSCs) following chemically induced oxidative stress.

2. Materials and Methods

2.1. Characteristics of Primary hBM-MSCs and Cell Culture

Human bone marrow mesenchymal stem cells (hBM-MSCs) were purchased from the Cell Engineering for Origin Company (CEFO, Seoul, Korea), and maintained according to the previous study's instructions[9]. Passage-seven (P-7) hBM-MSCs were used for the experiments.

2.2. Preparation of Gracilaria Vermiculophylla Extract

G. vermiculophylla was harvested from Busan of Korea (TC17055; deposited in the Marine Brown Algae Resources Bank, Chosun University, Gwangju, Korea), washed three times with tap water to remove residual salts, and dried at room temperature. The seaweed powder was soaked in 80% ethanol (20 times volume) for 7 days, then filtering them through a Qualitative Filter Paper (150 mm diameter; Hyundai Micro, Gyeonggi-do, Korea). After evaporation of the ethanol using a Rotary Evaporator (Eyela, Tokyo, Japan), the extracts were stored at -70 °C for a day. After freezing, the extract was obtained by lyophilization for 3 days in a freeze dryer (Ilshin Lab. Co. Ltd., Gyeonggi-do, Korea). The solutions for analysis were prepared by solubilizing dried extract (20 mg) with 1 mL of either a mixture of equal parts dimethyl sulfoxide and ethanol (GV-crude; GV-Cr), or distilled water (GV-H_2O; GV-H), or acetone (GV-acetone; GV-Ac).

2.3. Determination of Total Polyphenolics Compounds and Flavonoids Contents

The total polyphenolic content (TPC) of GV extracts was assessed using the Folin-Ciocalteu method[19] with some modification. Briefly, 1 ml of GV extracts, 1 ml of 95% ethanol, 5 ml distilled water, and 0.5 ml of Folin-Ciocalteu reagent were mixed and allowed to react at room temperature for 5 min. One ml of 5% Na_2CO_3 was added, and then the reaction mixture was incubated for 90 min at room temperature in the dark. Absorbance at 750 nm was measured using a spectrophotometer (Shimadzu UV mini 1240, Duisburg, Germany). Total phenolic content of the extracts is expressed as mg tannic acid equivalents (TAE) per gram of sample.

Total flavonoid content (TFC) was determined using the aluminium chloride ($AlCl_3$) colorimetric assay[20]. Briefly, 1 ml of the extracts at 1 mg/ml was diluted in equal volumes of a solution of 2% $AlCl_3$ solution in the well of a 96 wellplate. The plate was incubated at room temperature for 15 min. The absorbance at 435 nm was measured using a microplate reader. Total flavonoid content is expressed as mg rutin equivalents (RE) per g dry plant material. Samples were analyzed in triplicate.

2.4. Antioxidant Assays

The 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging activity assay was performed as described by the method of Shimada[21] with some modification. Briefly, 0.5 ml of the GV extract solution (1 μg/ml) was mixed with to 3.5 ml of 0.15 mM DPPH. The reaction mixture was shaken and left to stand at room temperature for 30 min. The absorbance of the resulting solution was measured at 517 nm using a spectrophotometer (Shimadzu UV mini 1240, Duisburg, Germany). A reference with butylated hydroxytoluene (BHT) was used as a DPPH scavenging positive control. The scavenging effects were calculated using the equation as follows: DPPH scavenging effects (%) = [1 – (absorbance of sample/absorbance of control)] × 100.

The 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid (ABTS) radical scavenging activity was performed according to the method described by Przygodzka[22]. The ABTS•+ working solution was prepared by mixing 7 mM ABTS and 2.45 mM potassium persulfate at 8:12 volume/volume ratio. The reaction mixture was previously activated in the dark for 12-16 h at room temperature. The ABTS•+ working solution was diluted with ethanol to give an absorbance at 734 nm of approximately 0.70. After adding 0.5 mL of GV extracts to 3mL of the diluted ABTS solution, the absorbance was

measured at 734 nm using a spectrophotometer and the result was calculated in terms of its IC_{50} values by regression analysis. BHT was used as an ABTS scavenging positive control.

2.5. MTT Assay

The experiment was performed as described in the previous study[9]. Briefly, 4×10^4 cells/well or 4×10^4 cells/well MSCs were seeded in both 24-well plates and 96-well plates. Cells were incubated one more day at 37°C (reach to 80% confluent), and then treated with GV-Ex for 24 h at 37°C and incubated with 20 μM DCFH-DA at 37°C for 1 h. Images were taken by a DS-Rl1 digital camera (Nikon).

2.6. Detection of Intracellular ROS

50 μg of total proteins were extracted from P-7 or -17 MSCs. Antibodies for SOD1, CAT, p53, p21, or p16 (all from Santa Cruz Biotechnology, Inc., Dallas, TX, USA) were used at a 1:500 dilution, SOD2 (Abcam, Cambridge, UK) was used at a 1:5000 dilution, cleaved caspase-3 (Merck Millipore, Darmstadt, Germany) was used at 1:500 dilution, and β-actin (Sigma-Aldrich) was used at a 1:5000 dilution. The horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) appropriate for each primary antibody species were then applied, and chemiluminescence was generated using Amersham ECL reagents (GE Healthcare, Buckinghamshire, UK).

2.7. Immunoblot Analysis

50 μg of total proteins were extracted from P-7 or -17 MSCs. Antibodies for SOD1 or CAT (all from Santa Cruz Biotechnology, Inc., Dallas, TX, USA) were used at a 1:500 dilution, SOD2 (Abcam, Cambridge, UK) was used at a 1:5000 dilution, and β-actin (Sigma-Aldrich) was used at a 1:5000 dilution. The horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) appropriate for each primary antibody species were then applied, and chemiluminescence was generated using Amersham ECL reagents (GE Healthcare, Buckinghamshire, UK).

2.8. Statistical Analysis

All graphs show data as the mean ± standard deviation (SD). Statistical comparisons between groups were performed using a paired *t*-test. The total polyphenolic and flavonoids contents, DPPH and ABTS radical scavenging activities were statistical analyzed using one-way ANOVA, followed by Tukey test for *post hoc* comparison.

3. Results and Discussion

3.1. Total Polyphenolic Content (TPC) and Total Flavonoids Content (TFC)

Among the antioxidative compounds, polyphenolic compounds are known to have an excellent antioxidant activity because of the presence of phenolic rings that stabilize free radicals. Flavonoids are one of the most abundant secondary metabolites having a polyphenolic structure in plant and fruit, and has been reported to have various physiological activities, including antioxidant, antimicrobial and antimutagenic effects[23]. Several reports indicated that marine seaweed extracts, especially their polyphenols, have antioxidant activity[24-26]. Certain red algae species were reported to contain several kinds of bromophenols[27]. Significant differences were observed in total polyphenolic and total flavonoids contents among the different solvents used for dilution (Table 1). GV-Cr and GV-AC showed higher amounts of total phenolic and total flavonoids compounds when compared to GV-H.

3.2. Antioxidative Activity

It has been reported that there's a correlation between polyphenol content in seaweed extracts and its antioxidant activity[28]. Therefore, the antioxidant effects of GV extracts obtained from different solvents and the

Table 1. Total polyphenolic content (TPC) and total flavonoid content (TFC) of *Gracilaria vermiculophylla* obtained from different solvents.

Sample[1]	TPC (mg TAE/g dry material)	TFC (mg RE/g dry material)
GV-Cr	160.21±1.48[2a3]	13.76±0.61[b]
GV-H	12.88±0.39[b]	2.84±0.52[c]
GV-Ac	187.29±2.92[a]	72.70±2.89[a]

[1]GV-Cr, DMSO/ethanol; GV-H: distilled water; GV-Ac: acetone. [2]All values are expressed as mean±SD of triplicate determinations. [3]Values with different superscripts in the same column are significantly different ($p<0.05$) between groups by Tukey's test.

reference compound, BHA taken as the positive control, were investigated using two most common free radical scavenging assays using DPPH and ABTS (Table 2). IC_{50} values for DPPH and ABTS radical scavenging were determined based on the concentration of the extract required for approximately 50% of the original activity. Antioxidant activities of different dilution solvents were detected in the following order; GV-Ac > GV-Cr > GV-H. This was in agreement with the previously mentioned results for the antioxidant compounds measured total polyphenolic and total flavonoid contents where GV-Ac or GV-Cr showed higher antioxidant compounds when compared with GV-H. However, the GV extracts showed a relatively low DPPH and ABTS scavenging activity as compared to reference antioxidant (BHA). Among the dilution solvents in this study, acetone had a higher antioxidant activity when compared to other dilution solvents. In general, methanol was the most efficient solvent in the extraction of compounds with antioxidant activity, particularly polyphenolic compounds[25]. However, an ethyl acetate fraction extracted from the methanolic crude extract of a red marine alga, such as *Acanthophora spicifera*, had a higher value of antioxidant activity[24].

Table 2. Free Radical Scavenging activity by DPPH and ABTS assay

Sample[1]	DPPH IC_{50} (μg/mL)[2]	ABTS IC_{50} (μg/mL)
GV-Cr	1637.24±6.231[4b5]	1073.97±4.21[b]
GV-H	3566.92±10.01[a]	3154.72±8.98[a]
GV-Ac	1233.52±7.36[b]	829.63±6.03[c]
BHA[3]	68.52±1.69[c]	52.24±0.36[d]

[1]GV-Cr, DMSO/ethanol; GV-H: distilled water; GV-Ac: acetone. [2]IC_{50} values were determined by curve-fitting the data points using nonlinear regression. [3]BHA was used as a positive control for DPPH and ABTS radical scavenging activities. [4]All values are expressed as mean±SD of triplicate determinations. [5]Values with different superscripts in the same column are significantly different ($p<0.05$) between groups by Tukey's test.

3.3. Protection by GV Extracts in H_2O_2-treated MSCs

Since several bioactive compounds have been isolated from red algae of genus *Gracilaria*[17,18], we have examined the antioxidant effects of *G. vermiculophylla* extracts. MSCs were incubated with GV-Cr, -H, or -Ac (0-50 μg/mL) for 24 h, and their viabilities were measured by MTT assay. Cellular toxicity was not observed following treatment with up to 50 μg/mL GV-Cr, 30 μg/mL GV-H, or 30 μg/mL GV-Ac (Fig. 1A). To look for possible antioxidant effects, MSCs were pre-treated with 0-10 μg/mL of GV-Cr, -H or -Ac for a day, and then exposed to 1 mM H_2O_2 for 1 h. Protective effects were observed in 1 μg/mL GV-Cr or -Ac pre-treated cells, but not in cells with GV-H (Fig. 1B). It suggests that a natural compound important for these effects is

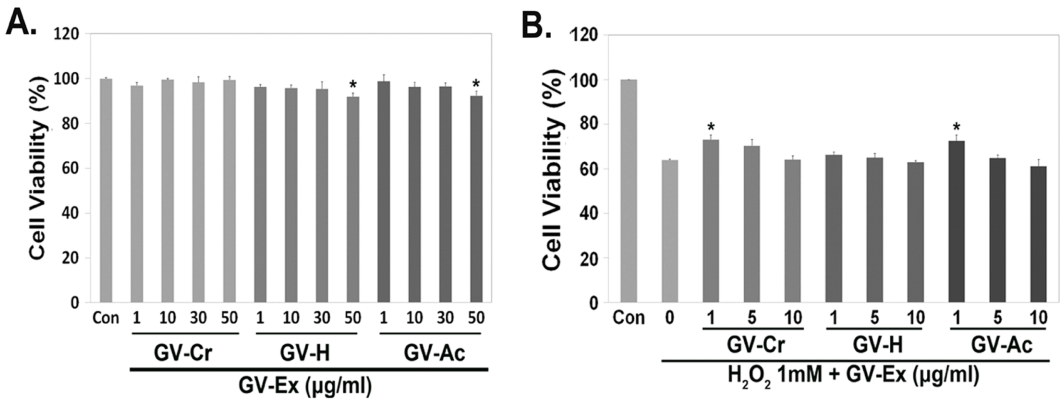

Fig. 1. Protection by GV-Ex in H_2O_2-treated MSCs. (A) Cell viabilities were determined in GV-Cr, GV-H, or GV–Ac treated MSCs by using an MTT assay. *indicates significant differences compared to the control ($p < 0.05$ in a paired *t*-test, mean±SD, n=3). (B) H_2O_2-treated MSCs fallowed by the pre-incubation with GV extracts were examined by MTT assay. *indicates significant differences compared to the cells not given GV extract ($p < 0.01$ in a paired *t*-test, mean ±SD, n=4).

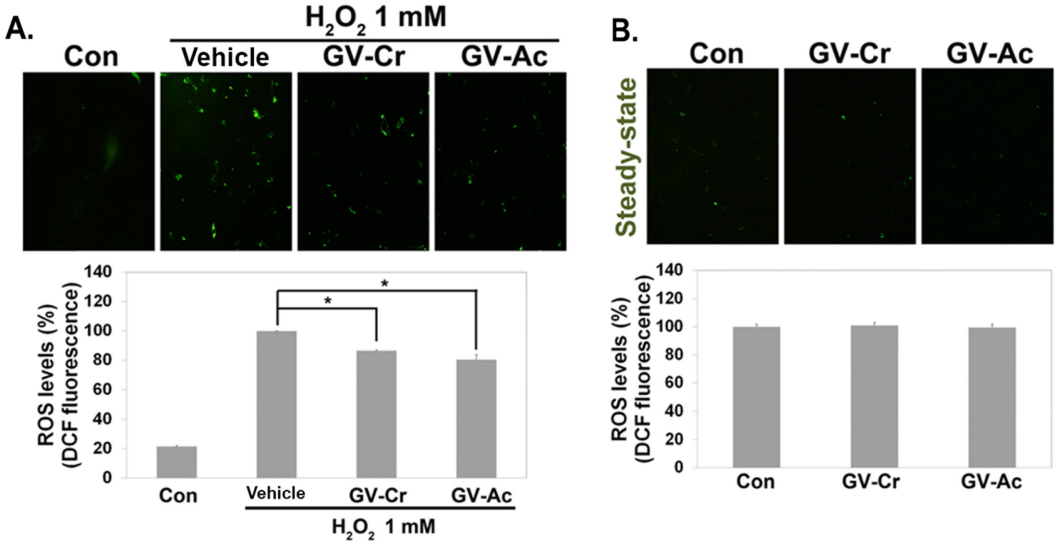

Fig. 2. Intracellular ROS levels were moderated by the recovery of antioxidant enzymes in GV-treated MSCs. (A) GV-treated MSCs were incubated with DCFH-DA and then exposed to H_2O_2. Cells were fixed and iROS levels were observed under the fluorescence microscopy (Top panel). The fluorescence intensities were quantified (Bottom panel; *p < 0.01 in a paired t-test, mean±SD, n=3). (B) The iROS levels were measured in GV-treated MSCs (Top panel), and the fluorescence intensities were quantified (Bottom panel).

soluble in organic solvents (acetone, DMSO and ethanol), not in water. This suggests that GV extracts prevents oxidative stress-induced apoptosis in hBM-MSCs.

3.4. Intracellular ROS Levels were Moderated in GV-treated MSCs

Oxidative damage and cellular senescence are mainly caused by ROS over-production[9,29]. The activities of the antioxidant enzymes SOD1, SOD2, and CAT reduce the levels of harmful iROS[12]. Since GV-Ac treatment protected cells from oxidative stress, we hypothesized that it might modulate iROS levels. To test this, iROS level was measured in GV-extracts treated MSCs. The level of iROS in MSCs after 1 mM H_2O_2 exposure was significantly decreased by pre-treatment of GV-Cr or GV-Ac (Fig. 2A). Conversely, the level of iROS in MSCs not exposed to H_2O_2 did not change in all tested condition (Fig. 2B).

3.5. Intracellular ROS Levels were Moderated by the Recovery of Antioxidant Enzymes in GV-treated MSCs

To examine the effect of GV-Ac, the level of the antioxidant enzymes such as SOD1, SOD2, and CAT was analyzed by immunoblot. The proteins were decreased

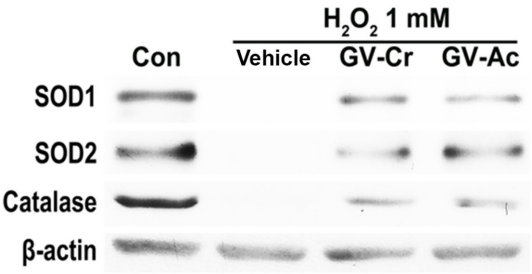

Fig. 3. Reduced intracellular ROS by GV-Ex was caused through the regulation of antioxidant enzymes. GV pre-treated MSCs were exposed to H_2O_2. Total proteins were examined for immunoblot analysis with antioxidant proteins SOD1, SOD2, and CAT. Vehicle is a mixture of equal volumes ethanol and DMSO.

in H_2O_2-treated MSCs (Vehicle), but partially recovered in GV-Cr and GV–Ac pretreated MSCs (Fig. 2C). These suggest that GV extracts may reduce excessive ROS caused by oxidative stress through moderating the levels of antioxidant enzymes.

4. Conclusions

In this study, we examined the anti-oxidative effect

of extracts from the red algae *G. vermiculophylla* using hBM-MSCs. The acetone and DMSO/ethanol solvents of the tested *G. vermiculophylla* contain higher total flavonoid and polyphenolic contents which can strongly scavenge ROS such as DPPH and ABTS free radicals. Treatment with GV-extracts moderated excessive iROS levels, increased or maintained the levels of the antioxidant enzymes SOD1, SOD2, and CAT, indicate that GV-extracts moderate excess ROS levels and prevent cells from oxidative damage. Our results suggest that GV-extract treatment may ameliorate the functional decline of stem cells and improve the therapeutic efficacy of stem cell therapy.

Abbreviations

ABTS, 2,2-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid; BHT, butylated hydroxytoluene; hBM-MSCs, human bone marrow-mesenchymal stem cells; CAT, catalase; DAPI, 4′,6-diamidino-2-phenylindole; DCF, 2',7'-dichlorodihydrofluorescein; DCFH-DA, 2',7'-dichlorofluorescein diacetate; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FBS, fetal bovine serum; MTT, by 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PBS, phosphate-buffered saline; PFA, paraformaldehyde; RIPA, radioimmunoprecipitation assay; ROS, reactive oxygen species; SD, standard deviation; SOD1, superoxide dismutase 1; SOD2, superoxide dismutase 2; TFC, total flavonoid content; TPC, total polyphenolic content; .

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