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Protective Effects of the BuOH Fraction from *Laminaria japonica* Extract on High Glucose-induced Oxidative Stress in Human Umbilical Vein Endothelial Cells

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Abstract

This study investigated the protective effect of the butanol (BuOH) fraction from *Laminaria japonica* (BFLJ) extract on high glucose-induced oxidative stress in human umbilical vein endothelial cells (HUVECs). Freeze-dried *L. japonica* was extracted with distilled water, and the extracted solution was mixed with ethanol then centrifuged. The supernatant was subjected to sequential fractionation with various solvents. The BuOH fraction was used in this study because it possessed the strongest antioxidant activity among the various solvent fractions. To determine the protective effect of the BFLJ, oxidative stress was induced by exposing of HUVECs to the high glucose (30 mM) or normal glucose (5.5 mM) for 48 hr. Cell viability, lipid peroxidation, glutathione (GSH) concentration, and antioxidant enzyme activities such as catalase, superoxide dismutase (SOD), glutathione peroxidase (GSH-px), and glutathion reductase (GSH-re) were measured. Exposure of HUVECs to high glucose for 48 hr resulted in a significant ($p<0.05$) decrease in cell viability, SOD, GSH-px and GSH-re and a significant ($p<0.05$) increase in thiobarbituric acid reactive substances (TBARS) formation in comparison to the cells treated with 5.5 mM glucose or untreated with glucose. BFLJ treatment decreased TBARS formation and increased cell viability, GSH concentration, and activities of antioxidant enzymes including catalase, SOD, GSH-px, and GSH-re in high glucose pretreated HUVECs. These results suggest that BFLJ may be able to protect HUVECs from high glucose-induced oxidative stress, partially through the antioxidative defence systems.

Key words: HUVECs, *Laminaria japonica*, high glucose, oxidative stress

INTRODUCTION

Free radicals and reactive oxygen species generated in cells are effectively scavenged by the antioxidant defense system which consists of antioxidant enzymes such as catalase, SOD, GSH-px, and GR. When the activity of the antioxidant defense system decreases or the ROS production increases an oxidative stress may occur (1). Recently, compelling evidence has been provided that onset and progression of diabetes and its complications are closely associated with oxidative stress (2,3). Hyperglycemia-induced by oxidative stress is detrimental to endothelial cells and contributes to the vascular complications of diabetes (4-6). Prolonged hyperglycemia is the major factor in the etiology of atherogenic pathogenesis in diabetes, which causes 80% of total mortality in diabetic patients. Micro- and macrovascular complication of diabetes have complexity of pathogenesis involving dysfunction of and damage to vascular endothelial cells (7), which are susceptible to stimulatory factors such as increased glucose concentration, oxidative stress, and advanced glycation end products (8).

The characteristics of human vascular lesion and experimental diabetes suggest a prime involvement of vascular endothelium. A growing body of evidence suggests a close correlation between hyperglycemia and abnormalities in endothelial function and morphology. Exposure of endothelial cells to a high glucose medium resulted in an increased production of free radicals and reduced capacity of antioxidant enzymes such as catalase, SOD, GSH-px, and non-enzymatic antioxidants including glutathione (9). Antioxidants have prevented the high glucose-mediated oxidative stress and normalize endothelial Ca^{2+}/cGMP responses in high glucose pretreated endothelial cells (10).

Seaweeds have demonstrated free radical scavenging activities, and thus may help slow aging and prevent some chronic diseases. Almost all seaweed species have

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substantial ability to scavenge hydroxyl radicals (11) and are considered to be a rich source of antioxidants (12). *Laminaria japonica*, a kind of brown algae, has long been used as a food to promote health. Studies have shown that the dietary supplement containing *Laminaria japonicas* exhibit scavenging activity against free radicals (13) and hypoglycemic effect in diabetic rats (14). In recent years, Jin et al. (15,16) reported the effects of *L. japonica* aqueous extract on oxidative damage which was induced by streptozotocin in rat liver with regard to the GSH system, xanthine oxidase (XO) activity, and conversion of xanthine dehydrogenase (XD) to the XO isotype. The results suggested that *L. japonica* would have great value in preventing complications of diabetes mellitus, possibly by its antioxidant activity.

The aim of this work was to evaluate protective effect of the BFLJ on oxidative stress induced by the exposure of HUVECs to high glucose. The protective effect of the BFLJ was evaluated by determining cell viability, lipid peroxidation, GSH concentration and antioxidant enzyme activities.

MATERIALS AND METHODS

Materials

L. japonica was purchased from a local market in Pusan, Korea and used in this study. All chemicals were obtained from Sigma (St. Louis, MO).

Sample preparation and extraction

L. japonica was extracted with distilled water and the extracted solution was mixed with ethanol and centrifuged. The supernatant contained the ethanol soluble non-polysaccharide fraction and the precipitate the ethanol insoluble polysaccharide fraction (17). The non-polysaccharide fraction was subjected to sequential fractionation with dichloromethane, ethyl acetate, butanol and water. Our preliminary study showed that the butanol fraction possessed the strongest antioxidant activity among those various solvent fractions. Therefore, the butanol fraction was dried and placed in a plastic bottle, and then stored at -80°C (18,19).

Cell culture and treatment

HUVECs and EBM-2 growth media with EGM-2 bullet kit were purchased from Clonetics Inc. (San Diego, CA). Cells in passage 3~6 were used. Cells in 10 mm dishes (1×10^6 cells/dish) or 24 well plates (4×10^4 cells/well) were preincubated with glucose (5.5 mM or 30 mM) for 48 hr, and then incubated with or without indicated concentrations (25, 50, 100 μg/mL) of butanol fraction from *L. japonica* (BFLJ) in a humidified atmosphere containing 5% CO_2 at 37°C for 20 hr. Cell not treated with glucose were used as negative controls.

Neutral red cell viability

Cell viability was assessed by measuring the supravital dye neutral red uptake (20). After the culturing of cells, as described previously, the medium was removed and replaced with 0.5 mL of fresh medium containing 1.14 mmol/L neutral red. After 3 hr of incubation, the medium was removed then the cells were washed twice with phosphate buffered saline solution (PBS, pH 7.4). The incorporated neutral red was released from the cells by incubation in the presence of 1 mL of the cell lysis buffer [50 mmol/L Tris-HCl (pH 7.4), 150 mmol/L NaCl, 5 mmol/L dithiothreitol (DTT), and Triton X-100 (1%, v/v)] containing acetic acid (1%, v/v) and ethanol (50%, v/v) at room temperature for 15 min. To measure the dye taken up, the cell lysis products were centrifuged and absorbance of supernatant was measured spectrophotometrically at 540 nm.

Lipid peroxidation

Lipid peroxidation was measured by thiobarbituric acid reactive substances (TBARS) production (21). Cells (4×10^4 cells/well) in 24-well plates were first incubated with glucose (5.5 mM, 30 mM) for 48 hr, and then incubated with or without the indicated concentrations of BFLJ for 20 hr. 200 μL of each medium supernatant was mixed with 400 μL of TBARS solution then boiled at 95°C for 30 min. The absorbance at 532 nm was measured and TBARS concentrations extrapolated from the 1,1,3,3-tetraethoxypropane serial dilution standard curve, TBARS values were then expressed as equivalent nmoles of malondialdehyde (MDA).

GSH concentration

GSH was measured by an enzymatic recycling procedure of Tietze (22) in which GSH is sequentially oxidized by 5,5″-dithiobis (2-nitrobenzoic acid) and is reduced by NADPH in the presence of glutathione reductase. The medium was removed from the cultured HUVECs and the cells were washed twice with PBS. One milliliter of PBS was added and cells were scraped. Cell suspensions were sonicated three times for 5 sec on ice then the cell sonicates were centrifuged at $2300\times g$ for 10 min. 400 μL of cell supernatant was mixed with 200 μL of 5% sulfosalicylic acid then centrifuged at $2,300\times g$ for 10 min. A 50 μL aliquot of supernatant was mixed with 100 μL of the reaction mixture [100 mmol/L sodium phosphate buffer with 1 mmol/L EDTA (pH 7.5), 1 mmol/L dithiolnitirobezene, 1 mmol/L NADPH, 1.6 U GSH reductase]. The rate of 2-nitro-5-thio-benzoic

acid formation was spectrophotometrically monitored wavelength at 412 nm. GSH content was determined by extrapolation from the standard curve obtained from known amounts of GSH.

Antioxidant enzyme assays

Cells (1×10^6 cells/dish) in 10-mm dishes were preincubated with glucose (5.5 mM, 30 mM) for 48 hr then further incubated with or without the indicated concentrations of BFLJ for 20 hr. The medium was removed and the cells were washed twice with PBS. One milliliter of 50 mmol/L potassium phosphate buffer with 1 mmol/L EDTA (pH 7.0) was added and cells were scraped. Cell suspensions were sonicated three times for 5 sec on ice each time then cell sonicates were centrifuged at 10,000 $\times g$ for 20 min at 4°C. Cell supernatants were used for antioxidant enzyme activities. The protein concentration was measured by using the method of Bradford (23) with bovine serum albumin as the standard. SOD activity was determined by monitoring the auto-oxidation of pyrogallol (24). A unit of SOD activity was defined as the amount of enzyme that inhibited the rate of oxidation of pyrogallol. Catalase activity was measured according to the method of Aebi (25) by following the decreased absorbance of H_2O_2. The decrease of absorbance at 240 nm was measured for 2 min. Standards containing 0, 0.2, 0.5, 1 and 2 mmol/L of H_2O_2 were used for the standard curve. GSH-px activity was measured by using the method of Lawrence and Burk (26). One unit of GSH-px was defined as the amount of enzyme that oxidizes 1 nmol of NADPH per consumed per minute. GSH reductase activity was measured by following the oxidation of NADPH (27). A unit of GSH reductase was defined as the amount of enzyme that catalyze reduction of 1 nmol of NADPH per minute.

Statistical analysis

The data are represented as mean±SD. The statistical analysis was performed with SAS program. The values were evaluated by one-way analysis of variance (ANOVA) followed by post-hoc Duncan's multiple range tests.

RESULTS AND DISCUSSION

Cell viability

The effect of the butanol fraction from *L. japonica* (BFLJ) on cell viability in HUVECs treated with high glucose of 30 mM was examined by neutral red (NR) assay and shown in Fig. 1. When HUVECs were treated with 30 mM glucose for 48 hr, there was a significant decrease in cell viability compared with the cells treated with 5.5 mM glucose or no glucose ($p<0.05$). Cell viability was decreased to 34.88% in 30 mM glucose treated HUVECs compared to that of untreated or normal glucose. Treatment with more than 50 μg/mL BFLJ increased cell viability in high glucose pretreated HUVECs, and by 64.51% in the cells treated 100 μg/mL of BFLJ. Damage to the cell surface or sensitive lysosomal membranes decreases the uptake and binding of NR, making it possible to differentiate between viable intact cells and dead/damage cells. The high glucose induced decrease in cell viability indicated that high glucose concentration might have damaged the cell surface or lysosomal membranes of HUVECs. Treatment of HUVECs with BFLJ resulted in a dose-dependent increase of cell viability, suggesting a protective effect on the cell surface or lysosomal membranes of HUVECs.

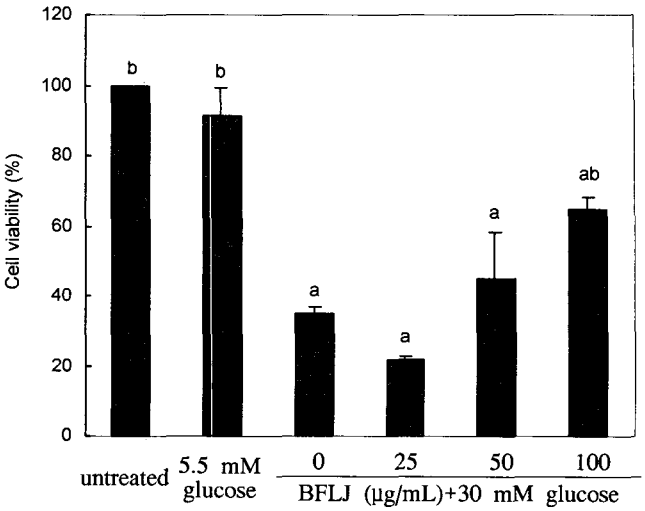

Fig. 1. Effect of the butanol fraction from *Laminaria japonica* (BFLJ) extract on cell viability in high-glucose treated HUVECs. Cells in 24 well plates (4×10^4 cells/well) were preincubated with glucose (5.5 mM, 30 mM) for 48 hr, and then incubated with or without indicated concentrations of BFLJ (25, 50, 100 μg/mL) for 20 hr. Untreated is negative control without glucose treatment, 5.5 mM is normal glucose and 30 mM is high glucose treatment. Each value is expressed as mean±SD (n=3). A value sharing same superscript is not significantly different at $p<0.05$.

Lipid peroxidation

The effect of BFLJ on lipid peroxidation in high glucose treated HUVECs was determined by measuring TBARS, a lipid peroxidation product (Fig. 2). When HUVECs were incubated with 5.5 mM or 30 mM glucose for 48 hr, TBARS in the 30 mM glucose-treated HUVECs was significantly increased ($p<0.05$) in comparison to the cells treated with 5.5 mM glucose or untreated. Treatment of HUVECs with BFLJ of 100 μg /mL significantly suppressed TBARS formation in 30 mM glucose pretreated cells, indicating protection against lipid peroxidation. When the cells were treated with 100

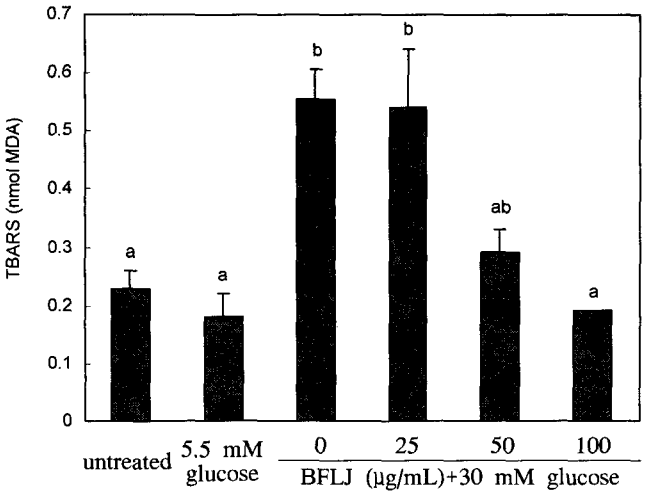

Fig. 2. Effect of the butanol fraction from *Laminaria japonica* (BFLJ) extract on TBARS generation in high-glucose treated HUVECs. Cells in 24 well plates (4×10^4 cells/well) were preincubated with glucose (5.5 mM, 30 mM) for 48 hr, and then incubated with or without indicated concentrations of BFLJ (25, 50, 100 μg/mL) for 20 hr. Untreated is negative control without glucose treatment, 5.5 mM is normal glucose and 30 mM is high glucose treatment. Each value is expressed as mean±SD (n=3). A value sharing same superscript is not significantly different at $p<0.05$.

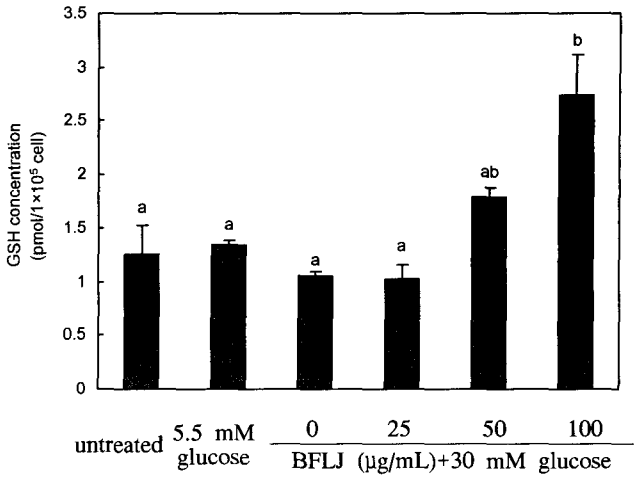

Fig. 3. Effects of the butanol fraction from *Laminaria japonica* (BFLJ) extract on GSH concentration in high-glucose treated HUVECs. Cells in 24 well plates (4×10^4 cells/well) were preincubated with glucose (5.5 mM, 30 mM) for 48 hr, and then incubated with or without indicated concentrations of BFLJ (25, 50, 100 μg/mL) for 20 hr. Untreated is negative control without glucose treatment, 5.5 mM is normal glucose and 30 mM is high glucose treatment. Each value is expressed as mean±SD (n=3). A value sharing same superscript is not significantly different at $p<0.05$.

μg/mL BFLJ, TBARS was significantly decreased by 0.19 nmol MDA (35.19% of control treatment, $p<0.05$). Sevanian & Hochstein suggested that lipid peroxidation was regarded as one of the cell damage mediated by free radicals (28). In this study, high glucose (30 mM)-induced lipid peroxidation in HUVECs and BFLJ inhibited TBARS formation effectively.

One of the serious consequences of lipid peroxidation is the damage to biomembranes such as mitochondrial and plasma membranes. TBARS being produced by lipid peroxidation can cause cross-linking and polymerization of membrane components (29). This can alter intrinsic membrane properties such as deformability, ion transport, enzyme activity, and the aggregation state of cell surface determinants. Under extreme conditions, peroxidized membranes can lose their integrity (30). The protective action of BFLJ on TBARS formation was demonstrated in this study, which may be attributed to its antiperoxidative effect.

GSH concentration

GSH is the most abundant low molecular weight thiol compound in cells and plays an important role in antioxidant defence and detoxification. Fig. 3 showed the effect of BFLJ on GSH concentration when HUVECs were treated with 5.5 mM and 30 mM glucose. 30 mM glucose caused a decrease of 1.06 pmol/L$\times10^5$ cell of GSH compared with the cells treated with 5.5 mM glucose or untreated, which were 1.34 pmol/L$\times10^5$ cell and 1.25 pmol/L$\times10^5$ cell respectively. Treatment of the 30 mM glucose-pretreated HUVECs with BFLJ increased on GSH. GSH concentration in the cell treated with BFLJ of 100 μg/mL was 2.73 pmol/L$\times10^5$ cell.

GSH (31) provides primary defence against oxidative stress by its ability to scavenge free radicals or participates in the reduction of H_2O_2 catalyzed by GSH peroxidase. Decrease in GSH can compromises cell defences against oxidative damage and may lead to cell death (32). Incubation of HUVECs with high glucose for 48 hr caused a decrease in GSH. Treatment of high glucose pretreated HUVECs with BFLJ 100 μg/mL resulted in a significant increase in GSH concentration ($p<0.05$). An increase in GSH concentration by BFLJ treatment could lead to decreased oxidative stress and thus could be part of the mechanism for the defensive effect of BFLJ against oxidative stress because GSH plays an important role in the protection of cells against oxidative stress.

Antioxidant enzyme activities

Cells are protected from activated oxygen species by endogenous antioxidant enzymes such as catalase (CAT), SOD, GSH-px and GR. The effects of BFLJ on antioxidant enzyme activities in high glucose treated HUVECs are shown in Table 1. The 30 mM glucose treatment decreased catalase activity compared with the cells treated

Table 1. Effects of the butanol fraction from *Laminaria japonica* (BFLJ) extract on antioxidant enzyme activities in high-glucose treated HUVECs

	Untreated	5.5 mM glucose	BFLJ (μg/mL)+30 mM glucose			
			0	25	50	100
Catalase (μmole/mg protein/min)	1.32±1.06^{ab}	1.26±0.43^{ab}	0.81±0.27^{a}	1.06±0.04^{a}	1.40±0.25^{ab}	1.85±0.14^{b}
SOD (unit/mg protein)	60.41±3.08^{b}	61.65±2.29^{b}	7.37±4.98^{a}	17.21±9.32^{ab}	37.27±10.25^{b}	52.64±4.27^{b}
GSH-px (unit/mg- protein)	5.11±0.22^{b}	4.81±0.37^{b}	2.73±0.08^{a}	3.60±0.12^{a}	4.63±0.94^{b}	4.74±0.43^{b}
GSH-reductase (unit/mg protein)	10.90±2.51^{b}	10.50±1.17^{b}	5.54±1.73^{a}	6.36±1.68^{ab}	10.54±0.66^{b}	11.05±0.50^{b}

Cells in 10 mm dishes (1×10^{6} cells/dish) were preincubated with glucose (5.5 mM, 30 mM) for 48 hr, and then incubated with or without indicated concentrations of BFLJ (25, 50, 100 μg/mL) for 20 hr. Untreated is negative control without glucose treatment, 5.5 mM is normal glucose and 30 mM is high glucose treatment. SOD: Super oxide dismtase, GSH-px: Glutathione peroxidase, GSH-reductase: Glutathion reductase. Each value is expressed as mean±SD (n=3). A value sharing same superscript is not significantly different at $p<0.05$.

with 5.5 mM glucose or not untreated with glucose ($p<0.05$). BFLJ treatment increased the catalase activity in a dose dependent manner. Treatment with 30 mM glucose for 48 hr significantly decreased SOD activity of HUVECs ($p<0.05$) compared with the cells treated with 5.5 mM glucose or untreated. Treatment of HUVECs with BFLJ increased SOD activity of 30 mM glucose pretreated cells. After the cells were treated with 100 μg/mL BFLJ, SOD activity was significanly increased to 52.64 U/mg protein in comparison to untreated or normal glucose treated cells (60.41 U/mg and 61.65 U/mg, $p<0.05$).

GSH-px activity in HUVECs treated with high glucose was significantly decreased in comparison to either the untreated or 5.5 mM glucose treated cells ($p<0.05$). Treatment of high glucose pretreated HUVECs with BFLJ resulted in an increase of GSH-px activity, as shown the GSH-px activity of 4.74 U/mg protein at dosage of 100 μg/mL. The GR activity in the cells treated with 30 mM glucose was also decreased in comparison with untreated or 5.5 mM glucose treated cells. Treatment of the cells with BFLJ resulted in an increase of GR activity. The observed decrease in GR activity after high glucose treatment in HUVECs, which catalyses NADPH-dependent conversion of GSSG to GSH, indicated a decrease in the conversion of oxidized glutathione (GSSG) back to its reduced form (GSH), with low levels in GSH (33).

Our results showed a decrease of the activities of antioxidant enzymes such as catalase, SOD, GSH-px, and GR in HUVECs treated with high glucose compared with untreated or normally treated cells. Treatment with BFLJ restored the antioxidant enzyme activities of high glucose-pretreated HUVECs. SOD, the endogenous scavenger, catalyses the dismutation of the highly reactive superoxide anion to H_2O_2 (34). GSH-px catalyses the reduction of H_2O_2 at the expense of reduced GSH (i.e. $H_2O_2+2\ GSH\rightarrow 2H_2O+GSSG$). H_2O_2 is also scavenged by CAT (35). The decreased activities of both CAT and GSH-px in the HUVECs treated with high glucose indicate a highly reduced capacity to scavenge H_2O_2 produced in the cells, with an increase in ROS and oxidative stress in response to high glucose treatment (36). Kono and Fridovich indicated that high production of superoxide anion radical inhibits CAT activity (37). The excess of superoxide anion radical, as a consequence of a reduction in the activity of SOD, might be the responsible for the decrease in the activities of CAT in high glucose treated HUVECs.

In conclusion, we demonstrated that BFLJ can protect HUVECs from high glucose-induced oxidative stress by increasing antioxidant enzymes activities, GSH concentration, cell viability and decreasing lipid peroxidation. *L. japonica* may have great value in preventing complications of diabetes mellitus and in developing a new functional food.

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