

Protective Effects of Fermented Soymilk Extract on High Glucose-Induced Oxidative Stress in Human Umbilical Vein Endothelial Cells

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Abstract

We investigated whether the fermented soymilk extract (FSE) has protective effects against high glucose-induced oxidative stress in human umbilical vein endothelial cells (HUVECs). FSE was prepared via fermentation of soymilk with *Bacillus subtilis* followed by methanol extraction. To determine the protective effect of FSE, oxidative stress was induced by exposing of HUVECs to the high glucose (30 mM) for 48 hr. Exposure of HUVECs to high glucose for 48 hr resulted in a significant ($p < 0.05$) decrease in cell viability, catalase, SOD and GSH-px activity and a significant ($p < 0.05$) increase in intracellular ROS level and thiobarbituric acid reactive substances (TBARS) formation in comparison to the cells treated with 5.5 mM glucose. However, at concentration of 0.1 mg/mL, FSE treatment decreased intracellular ROS level and TBARS formation, and increased cell viability and activities of antioxidant enzymes including catalase, SOD and GSH-px in high glucose pretreated HUVEC. These results suggest that FSE may be able to protect HUVECs from high glucose-induced oxidative stress, partially through the antioxidative defense systems.

Key words: fermented soymilk extract, HUVECs, 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 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).

Soybean, the most important legume in the Asian diet, is rich in high-quality protein, including lysine and other essential amino acids (9). In addition to proteins, soy beans contain various nutritional and functional components such as isoflavones. The interest in the potential

health effects of soybean and soy isoflavones is growing as epidemiological studies have associated a diet rich in isoflavones with a lower risk of certain diseases (10-12). Recently, soybean and soy protein have received much attention for their preventive effects on chronic disease (13-15). However, two of the main oligosaccharides in soybeans, raffinose and stachyose, are not nutritionally useful because these are fermented by microbes present in the gut, resulting in flatulence and discomfort. Fermentation is a chemical reaction that splits complex organic compounds into relatively simple substances. Fermentation of soymilk by mixed cultures of bifidobacteria and lactic acid bacteria has been shown to effectively decrease the content of the two non-digestible oligosaccharides. Interestingly, fermentation of legumes has been reported to improve their nutritional value by increasing protein digestibility, monosaccharide content, and vitamin B family biosynthesis, as well as decreasing non-nutritive factors (16). Soybean products fermented with *Bacillus subtilis* are widely consumed in Asia, including Chungkookjang and natto.

B. subtilis, which is safe for human consumption and which grows rapidly, making it easy to scale-up for mass culture, has been a good organism to develop as a probiotic diet adjunct. Kuo et al. (17) reported that *Bacillus subtilis*-fermented natto hydrolyzed daidzin and genistin

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to daidzein and genistein, respectively, in black soymilk. Soymilk, the water extract from ground soybeans, is a colloidal dispersion and most of the components present in the soybean are also present in soymilk. During fermentation, the active compounds in soymilk are exposed. Therefore, this study was designed to examine whether the soymilk fermented with *Bacillus subtilis* has protective effects against high glucose-induced oxidative stress in human umbilical vein endothelial cells (HUVECs).

MATERIALS AND METHODS

Preparation of FSE

Soymilk was purchased from Donghwa food, Inc. (Yangsan, Korea). Soymilk was fermented by *Bacillus subtilis* isolated from Chungkookjang for 6 hr at 40°C under aerobic conditions. Non-fermented as well as the fermented soymilk was freeze-dried, powdered and extracted with ten volumes of 100% methanol for 12 hr three times at room temperature. The filtration of the extracted solution and evaporation under reduced pressure yielded methanol extract. After the extract was thoroughly dried for complete removal of solvent, the dried extract was then stored in a deep freezer (-80°C).

Cell culture

Human umbilical vein endothelial cells (HUVECs) and endothelial cell basal medium-2 (EBM-2) with endothelial cell growth medium-2 (EGM-2) bullet kit were purchased from Clonetics Inc. (San Diego, USA). Cells in passages 3~6 were used. Cells were cultured in EGM-2 containing 2% fetal bovine serum (FBS; Gibco Inc., NT, USA) at 37°C in a humidified atmosphere containing 5% CO₂, according to the supplier's recommendation.

Assay of neutral red cell viability

Cell viability was assessed by measuring the uptake of the supravital dye neutral red (18). Cells (4×10^4 cells/well) were seeded in 24-well plate and pre-incubated with glucose (5.5 and 30 mM) in humidified atmosphere containing 5% CO₂ at 37°C for 48 hr. After 48 hr of incubation, the cells were treated with various concentrations (0.1, 0.5 and 1.0 mg/mL of FSE) and further incubated for 20 hr. Thereafter, the medium was carefully removed from each well, 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 and the cells were washed twice with phosphate buffered saline (PBS, pH 7.4). The incorporated neutral red was released from the cells by incubation in the presence of 1 mL of cell lysis buffer [50 mmol/L Tris-HCl (pH 7.4),

150 mmol/L NaCl, 5 mmol/L dithiothreitol (DTT), and 1% Triton X-100] containing 1% acetic acid and 50% ethanol at room temperature for 15 min. To measure the dye taken up, the cell lysis products were centrifuged and the absorbance of supernatant was measured spectrophotometrically at 540 nm.

Assay of intracellular ROS levels

Intracellular ROS levels were measured by the 2',7'-dichlorofluorescein diacetate (DCF-DA) assay (19). DCF-DA can be deacetylated in cells, where it can react quantitatively with intracellular radicals to convert into its fluorescent product, DCF, which is retained within the cells. Therefore, DCF-DA was used to evaluate the generation of ROS in oxidative stress. Cells (2×10^4 cells/well) were seeded in a 96-well plate and pre-incubated with glucose (5.5 and 30 mM) in humidified atmosphere containing 5% CO₂ at 37°C for 48 hr. After 48 hr of incubation, the cells were treated with various concentrations (0.1, 0.5, and 1.0 mg/mL) of FSE and further incubated for 20 hr. Thereafter, the medium was removed and the cells were washed twice with phosphate buffered saline (PBS, pH 7.4) and then were incubated with 5 μM DCF-DA for 30 min at room temperature. Fluorescence was measured using a fluorescence plate reader.

Assay of lipid peroxidation

Lipid peroxidation was measured by thiobarbituric acid reactive substance (TBARS) production (20). Cells (4×10^4 cells/well) were seeded in 24-well plate and pre-incubated with glucose (5.5 and 30 mM) in humidified atmosphere containing 5% CO₂ at 37°C for 48 hr. After 48 hr of incubation, the cells were treated with various concentrations (0.1, 0.5, and 1.0 mg/mL) of FSE and further incubated for 20 hr. 200 μL of each medium supernatant was mixed with 400 μL of TBARS solution then heated at 95°C for 20 min. The absorbance at 532 nm was measured and TBARS concentrations were extrapolated from the 1,1,3,3-tetraethoxypropane serial dilution standard curve, TBARS values were then expressed as equivalent nmoles of malondialdehyde (MDA).

Assay of nitric oxide (NO) level

Cells (2×10^4 cells/well) were seeded in 96-well plate and pre-incubated with glucose (5.5 and 30 mM) in humidified atmosphere containing 5% CO₂ at 37°C for 48 hr. After 48 hr of incubation, the cells were treated with various concentrations (0.1, 0.5 and 1.0 mg/mL) of FSE and further incubated for 20 hr. Thereafter, each 50 μL of culture supernatant was mixed with an equal volume of Griess reagent [0.1% N-(1-naphthyl)-ethylenediamine, 1% sulfanilamide in 5% phosphoric acid] and incubated

at room temperature for 10 min. The absorbance at 550 nm was measured in a microplate absorbance reader, and a series of known concentrations of sodium nitrite was used as a standard (21).

Assay for antioxidant enzymes

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 FSE 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 the 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 measuring antioxidant enzyme activities. The protein concentration was measured by using the method of Bradford (22) with bovine serum albumin as the standard. SOD activity was determined by monitoring the auto-oxidation of pyrogallol (23). 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 (24) 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 (25). One unit of GSH-px was defined as the amount of enzyme that oxidizes 1 nmol of NADPH per consumed per minute.

Statistical analysis

The data are represented as mean \pm 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

Fig. 1 shows the effects of FSE and SE on the cell viability in HUVECs treated with high glucose of 30 mM. Cell viability was examined by neutral red (NR) assay. 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 ($p < 0.05$). However, the treatment with FSE at 0.5 mg/mL increased cell viability up to 78.71% in high glucose pretreated HUVECs and this showed higher cell viability than that of 55.60% in the cells treated same concen-

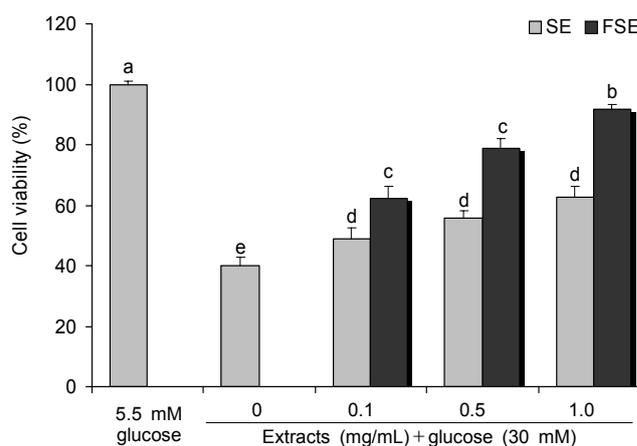


Fig. 1. Effects of FSE on cell viability in high glucose treated HUVECs. Cells in 24 well plates (4×10^4 cells/well) were preincubated with 5.5 mM normal glucose or 30 mM high glucose for 48 hr, and then incubated with or without the indicated concentrations of soymilk extract and fermented soymilk extract (0, 0.1, 0.5, 1 mg/mL) for 20 hr. Each value is expressed as mean \pm SD ($n=3$). Values with different letters are significantly different at $p < 0.05$ as analysis by Duncan's multiple rang test. SE: soymilk extract, FSE: fermented soybean milk extract.

tration of SE ($p < 0.05$), suggesting the fermentation of SE with *B. subtilis* may be useful in high-glucose treated cells.

Fermentation consists of modifying food by microorganisms that grow and reproduce and consume part of the substrate and enrich it with the products of their metabolism. Soymilk contains beneficial components for human health, such as soy protein, peptides, and isoflavones. The fermentation of soymilk was suspected to result in various compositional and functional changes as the fermentation of soybeans produce a large variety of peptides and amino acid by different kinds of microorganism. The results suggest that the amount of compounds increasing cell viability was increased during fermentation of SE.

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 FSE resulted in a dose-dependent increase of cell viability, suggesting a protective effect on the cell surface or lysosomal membranes of HUVECs.

Intracellular ROS level

As presented in Fig. 2, the generation of intracellular ROS in HUVECs increased significantly after treatment with 30 mM glucose compared with 5.5 mM glucose.

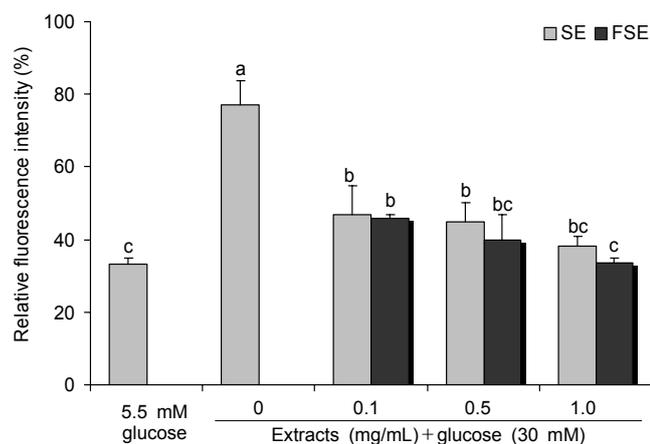


Fig. 2. Effects of FSE on intracellular ROS level in high glucose treated HUVECs. Abbreviations are the same as in Fig. 1. Each value is expressed as mean \pm SD (n=3). Values with different letters are significantly different at $p < 0.05$ as analysis by Duncan's multiple rang test. SE: soymilk extract, FSE: fermented soybean milk extract.

When HUVECs were treated with 30 mM glucose, intracellular ROS level increased significantly to 76.97%. However, treatment with FSE decreased significantly the ROS level in the cells induced with 30 mM glucose. Especially, treatment with 0.5 mg/mL of FSE resulted in a significant decrease in intracellular ROS level to 39.97% in comparison to the cells treated with the same concentration of SE (44.89%). FES significantly decreased the elevated ROS level induced by high glucose, showing that fermentation of SE with *B. subtilis* has a greater inhibitory effect than SE on the ROS generation in high glucose treated the cells.

High ROS levels induce oxidative stress, which can result in a variety of biochemical and physiological lesions. Such cellular damage often impairs metabolic function, and leads to cell death (26). Our result showed that treatment of HUVECs with 30 mM glucose significantly increased the intracellular ROS level. However, FSE inhibited the high glucose-induced ROS generation. Mitochondrial membrane was hyperpolarized under high glucose conditions and the production of ROS was increased (27). FSE greatly decreased intracellular ROS production. It seemed that FSE significantly decreased hyperglycemia-induced mitochondrial membrane hyperpolarization and the subsequent increased ROS production.

Lipid peroxidation

The effect of FSE on lipid peroxidation in high glucose-treated HUVECs was determined by measuring TBARS, a lipid peroxidation product (Fig. 3). When HUVECs were incubated with 5.5 mM or 30 mM glucose for 48 hr, TBARS in the 30 mM glucose-treated

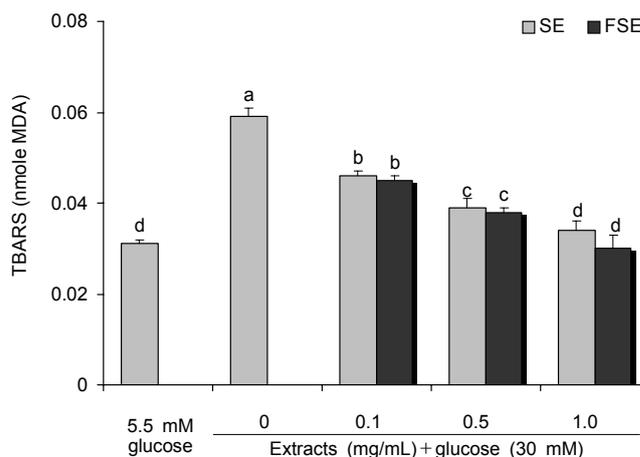


Fig. 3. Effects of FSE on TBARS generation in high glucose treated HUVECs. Abbreviations are the same as in Fig. 1. Each value is expressed as mean \pm SD (n=3). Values with different letters are significantly different at $p < 0.05$ as analysis by Duncan's multiple rang test. SE: soymilk extract, FSE: fermented soybean milk extract.

HUVECs was significantly increased ($p < 0.05$) in comparison to the cells treated with 5.5 mM glucose. Treatment of HUVECs with FSE of 0.5 mg/mL significantly suppressed TBARS formation in 30 mM glucose pretreated cells, indicating protection against lipid peroxidation. FSE showed higher protective effects against lipid peroxidation than SE overall. Sevanian & Hochstein suggest that lipid peroxidation is one of the forms of cell damage mediated by free radicals (28). In this study, high glucose (30 mM)-induced lipid peroxidation in HUVECs and FSE 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 FSE on TBARS formation, which may be attributed to its antiperoxidative effect, was demonstrated in this study.

NO generation

As shown in Fig. 4, the level of NO in HUVECs was significantly elevated by 30 mM glucose treatment compared with 5 mM glucose treatment. However, NO levels in FSE or SE treated cells were significantly decreased. The level of NO in HUVECs treated 30 mM glucose was 0.304 μ M, but treatment with 0.5 mg/mL of FSE

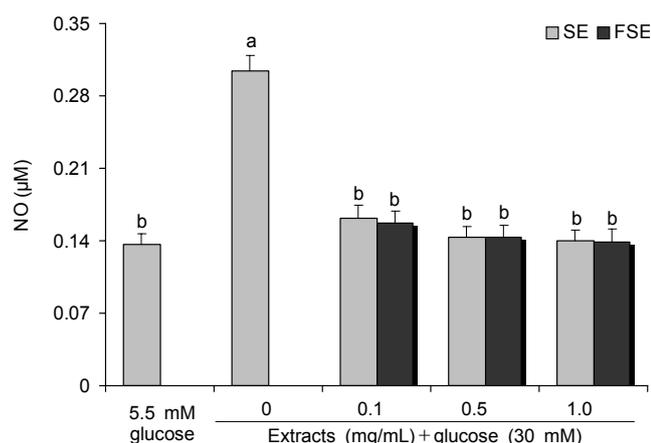


Fig. 4. Effects of FSE on NO level in high glucose treated HUVECs. Abbreviations are the same as in Fig. 1. Each value is expressed as mean \pm SD (n=3). Values with different letters are significantly different at $p < 0.05$ as analysis by Duncan's multiple rang test. SE: soymilk extract, FSE: fermented soybean milk extract.

in the cells resulted in significant decrease in NO level to 0.143 μ M. FSE scavenged NO produced by high glucose-induced oxidative stress.

Several researchers reported that suppression of NO production by plant extracts may be attributed to direct free radical scavenging. High glucose treatment leads to overproduction of NO and ROS. When NO is produced in large amounts, it reacts rapidly with superoxide anion to form peroxynitrite, a powerful oxidant, that damages many biological molecules, including DNA and proteins, and thus can lead to amplification of inflammation and tissue injury (31,32). In addition, NO and superoxide anion induce highly reactive oxidative damage associated with diabetes (33). In this study, FSE inhibited intracellular ROS, NO and TBARS produced by high glucose induced oxidative stress, suggesting that FSE might confer protection against the oxidative stress induced by hyperglycemia.

Antioxidant enzyme activities

Cells are protected from activated oxygen species by endogenous antioxidant enzymes such as catalase, SOD and GSH-px. The effects of FSE 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 with 5.5 mM glucose ($p < 0.05$). FSE 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. Treatment of HUVECs with FSE increased SOD activity of 30 mM glucose pretreated cells. After the cells were treated with 0.5 mg/mL of FSE, SOD activity was significantly increased to 44.86 U/mg protein in comparison to high glucose treated cells (35.14 U/mg, $p < 0.05$).

GSH-px activity in HUVECs treated with high glucose was significantly decreased in comparison to normal glucose treated cells ($p < 0.05$). Treatment with FSE in high glucose pretreated HUVECs resulted in an increase of GSH-px activity and this effect was concentration dependent. These antioxidant enzyme activities were higher by FSE treatment than SE on same concentration overall.

Our results showed a distinct decrease of the activities of antioxidant enzymes such as catalase, SOD, and GSH-px in HUVECs treated with high glucose compared with normal glucose level-treated cells. Treatment with FSE restored the antioxidant enzyme activities in high glucose-pretreated HUVECs. SOD, the endogenous scavenger, catalyses the dismutation of the highly reactive superoxide anion to H_2O_2 (34). The decreased activities of both catalase 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 (35). The excess of superoxide anion

Table 1. Effects of FSE on antioxidant enzyme activities in high glucose treated HUVECs

	Catalase (μ mole/mg protein/min)	SOD (unit/mg protein)	GSH-px (unit/mg protein)
5.5 mM glucose	1.79 ± 0.04^a	64.7 ± 9.78^a	4.94 ± 0.08^a
30 mM glucose	0.47 ± 0.03^d	35.14 ± 2.71^b	1.89 ± 0.09^d
SE (mg/mL) + 30 mM glucose	0.1 0.21 ± 0.04^c	38.19 ± 0.90^b	1.47 ± 0.04^d
	0.5 0.62 ± 0.05^c	42.11 ± 8.14^b	1.78 ± 0.06^c
	1.0 1.04 ± 0.02^b	58.97 ± 6.34^a	2.45 ± 0.02^b
FSE (mg/mL) + 30 mM glucose	0.1 0.25 ± 0.01^c	38.56 ± 3.37^b	1.57 ± 0.01^d
	0.5 0.79 ± 0.07^c	44.86 ± 7.37^b	2.04 ± 0.07^c
	1.0 1.55 ± 0.04^b	61.41 ± 4.88^a	3.49 ± 0.11^b

Abbreviations are the same as in Fig. 1. Each value is expressed as mean \pm SD (n=3). Values with different letters in a column are significantly different at $p < 0.05$ as analysis by Duncan's multiple rang test. SE: soymilk extract, FSE: fermented soybean milk extract.

radical, as a consequence of a reduction in the activity of SOD, might be responsible for the decrease in the activities of catalase in high glucose treated HUVECs. These elevations of antioxidant enzyme activities by FSE might support that it contains antioxidant compounds to help the attenuation of oxidative stress.

In conclusion, we demonstrated that FSE can protect HUVECs from high glucose-induced oxidative stress by increasing antioxidant enzymes activities, cell viability and decreasing intracellular ROS generation and lipid peroxidation.

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