Protective Effects of Chungkookjang Extract on High Glucose Induced Oxidative Stress in LLC-PK$_1$ Cells

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Abstract

This study was designed to investigate the protective effect of a methanol extract of Chungkookjang (CKJ) on high glucose induced oxidative stress in LLC-PK$_1$ cells (renal tubular epithelial cells), which are susceptible to oxidative stress. Freeze dried CKJ powder was extracted with methanol, and the extract solution was concentrated, and then used in this study. To determine the protective effect of CKJ extract, oxidative stress was induced by exposing of LLC-PK$_1$ cells to high glucose (30 mM) or normal glucose (5 mM) for 24 hr. Exposure of LLC-PK$_1$ cells to high glucose for 24 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 mM glucose. CKJ extract 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 LLC-PK$_1$ cells. These results suggest that CKJ extract may be able to protect LLC-PK$_1$ cells from high glucose-induced oxidative stress, partially through the antioxidative defense systems.

Key words: Chungkookjang, LLC-PK$_1$ cells, high glucose, oxidative stress

INTRODUCTION

Oxidative stress is caused by an imbalance between the antioxidant system and the production of oxidants including reactive oxygen species (ROS). The accumulation of these ROS can result in oxidative stress that has been related to human disease such as cardiovascular diseases, cancers, aging, diabetes, and atherosclerosis (1-3). Many natural products are known to produce significant amounts of antioxidants to control the oxidative stress caused by ROS (4,5).

Free radicals and ROS generated in cells are effectively scavenged by the antioxidant defense system which consists of antioxidant enzymes such as catalase, SOD and GSH-px. When the activity of antioxidant defense system decreases or the ROS production increases, an oxidative stress may occur (6). Recently, compelling evidence has been provided that onset and progression of diabetes and its complications are closely associated with oxidative stress (7,8). Prolonged exposure to hyperglycemia can degrade antioxidant enzyme defenses, thereby allowing ROS to build up and result in cellular and tissue damage. Moreover, several mechanisms have been proposed to be responsible for the oxidative damage during chronic hyperglycemia, including mitochondrial ROS oveproduction (9), glucose autooxidation (10)

and synthesis of advanced glycation end-products (11). Several workers have suggested that oxygen free radicals are generated as a result of hyperglycemia and cause various complications of diabetes, such as nephropathy, retinopathy and neuropathy (12,13). Therefore, to prevent pathological damage and/or reverse abnormalities associated with diabetes mellitus, oxidative stress caused by hyperglycemia important issue.

Chungkookjang (CKJ) is a fermented soybean product and a favorite Korean traditional food made from *Bacillus* species derived from rice straw and used to ferment boiled soybean. There have been several reports describing biological activities of CKJ such as antioxidant, antimicrobial, blood pressure lowering and antidiabetic activities (14-19). During fermentation of CKJ, *B. subtilis* or *B. natto* derived enzymes hydrolyze the soy proteins into easily digestible peptides and amino acids (20). The fermented CKJ contains functional components such as isoflavones (21), phytic acid (22), saponins (23), lignins (24) and unsaturated fatty acids (25). Among its constituents, isoflavones (e.g. genistein, daidzein) are found at high concentrations and they are known to possess protective effects against oxidative damage (26). A recent study reported that CKJ is rich in antioxidants which may prevent oxidative injury (27). However, the effect of CKJ extract on high glucose in-

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duced oxidative stress related to diabetic complications, expecially diabetic nephropathy, has not been investigated to date.

Thus, in this study, we investigated protective effects of CKJ extract on oxidative stress induced by high glucose using LLC-PK$_1$ cells, which are renal tubular epithelial cells known to be susceptible to oxidative stress. The protective effect of CKJ extract was evaluated by determining cell viability, lipid peroxidation, intracellular ROS level and antioxidant enzyme activities.

MATERIALS AND METHODS

Materials

CKJ was purchased from a local market in Soonchang province, Korea and used in this study. Dulbecco's modified Eagle medium/nutrient mixture F-12 (DMEM/F-12) and fetal bovine serum (FBS) were purchased from Hyclone (Logan, UT). q3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT), 2',7'-dichlorofluorescein-diacetate (DCFH-DA) were obtained from Sigma Chemical Co. (St. Louis, MO). All other chemicals and reagent used were of analytical grade.

Preparation of Chungkookjang (CKJ) extract

CKJ was freeze dried, powdered, and extracted with methanol for 72 hr at room temperature. The filtrate of the extracted solution was evaporated under reduced pressure yielding the 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

LLC-PK$_1$, a porcine proximal tubule cell line, was maintained on culture plates containing 5% FBS-supplemented DMEM/F-12 medium (pH 7.2) at 37°C in a humidified atmosphere of 5% CO$_2$ in air. All subsequent procedures were carried out under these conditions.

Cell viability

For cell viability analysis, LLC-PK$_1$ cells were seeded at 2×10^4/mL in 96-well plates and preincubated for 24 hr. The cells were pretreated with 5 mM glucose for 24 hr before treatment with 30 mM glucose and CKJ extract. Cell viability was evaluated by a colorimetric MTT assay, which is based on the conversion of MTT to MTT-formazan by mitochondrial enzymes (28). 100 µL MTT solution (5 mg/mL in PBS) was added to each well containing 1 mL medium. After 4 hr of incubation, the media were removed and formazan crystals were solubilized with 100 µL dimethyl sulfoxide (DMSO). The absorbance of each well was read at 540 nm using a microplate reader.

Assay of lipid peroxidation

Lipid peroxidation was measured by determining thiobarbituric acid reactive substances (TBARS) production (29). Cells (2×10^4 cells/mL) in 96-well plates were first incubated with 5 mM for 24 hr, and then incubated with 30 mM glucose alone or with CKJ extract for 24 hr. 200 µL of each medium supernatant was mixed with 400 µL of TBARS solution then boiled at 95°C for 20 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).

Assay of intracellular ROS levels

Intracellular ROS levels were measured by the dichlorofluorescein assay (30). DCF-DA can be deacetylated in cells, where it can react quantitatively with intracellular radicals (mainly hydrogen peroxide) and is converted to 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/mL) were plated on 96-well plates and pretreated with 5 mM glucose for 24 hr, then treated with 30 mM glucose alone or with CKJ extract for 24 hr, after that the cells were washed with phosphate buffered saline (PBS) and incubated with 5 µM DCF-DA for 30 min at room temperature. Fluorescence was measured using a fluorescence plate reader.

Assay of nitric oxide (NO) levels

Cells (2×10^4 cells/mL) were grown in 96-well plates and pretreated with 5 mM glucose for 24 hr, and then treated with 30 mM glucose alone or with CKJ extract for 24 hr. The nitrite accumulation in the supernatant was assessed by Griess reaction (31). 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.

Assay of antioxidant enzyme

Cells (2×10^4 cells/mL) in 24-well plates were pretreated with 5 mM glucose for 24 hr, and then treated with 30 mM glucose alone or with CKJ extract for 24 hr. The medium was removed and the cells were washed twice with PBS. One milliliter of 50 mM potassium phosphate buffer with 1 mM 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 (32) with bovine serum albumin as the standard. SOD activity was determined by monitoring the auto-oxidation of pyrogallol (33). A unit of SOD activity was defined as the amount of enzyme that inhibited the rate of oxidation of pyrogallol by 50%. Catalase activity was measured according to the method of Aebi (34) by following the decreased absorbance of H_2O_2. The decrease of absorbance at 240 nm was measured for 2 min. Standard containing 0, 0.2, 0.5, 1 and 2 mM of H_2O_2 were used for the standard curve. GSH-px activity was measured by using the method of Lawrence and Burk (35). One unit of GSH-px was defined as the amount of enzyme that oxidizes 1 nmol of NADPH per consumed per minute.
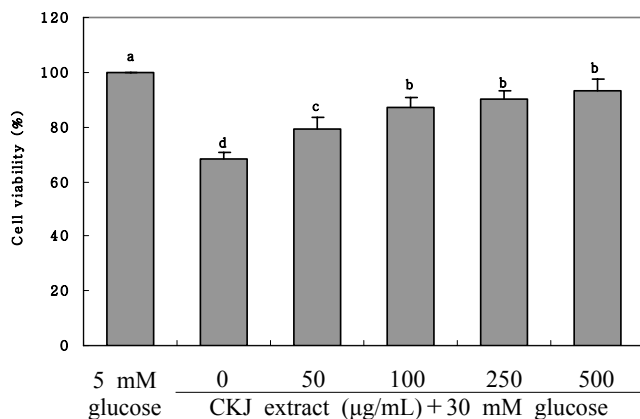
Statistical analysis

The data are represented as mean ± SD. The statistical analysis was performed using SAS software. 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 effect of CKJ extract on cell viability of LLC-PK₁ cells treated with high glucose. Exposure of LLC-PK₁ cells to 30 mM glucose led to a loss of cell viability, but treatment with each CKJ extract protected against the cellular damage induced by 30 mM glucose. 30 mM glucose exposed cells showed significantly reduced cell viability compared with the cells treated with 5 mM glucose ($p < 0.05$). Cell viability was decreased to 68% in 30 mM glucose treated LLC-PK₁ cells compared to that of 5 mM glucose, but treatment with 100 μg/mL CKJ extract together with high glucose exposure resulted in a significant increase to 87% in cell viability, suggesting that CKJ extract protected LLC-PK₁ cells from high glucose induced cytotoxicity.

Lipid peroxidation

Inhibitory effect of CKJ extract on lipid peroxidation in high glucose treated LLC-PK₁ cells was determined by measuring TBARS, a lipid peroxidation product (Fig. 2). When LLC-PK₁ cells were incubated with 30 mM glucose for 24 hr, TBARS were significantly increased ($p < 0.05$) in comparison to the cells treated with 5 mM glucose. Treatment with more than 100 μg/mL of CKJ extract together with high glucose significantly ($p < 0.05$) suppressed TBARS formation, indicating protection against lipid peroxidation. When the cells were treated with 250 μg/mL CKJ extract, TBARS was significantly decreased by 0.41 nmol MDA ($p < 0.05$). Sevanian & Hochstein suggested that lipid peroxidation is a type of cell damage mediated by free radicals (36). One of the serious consequences of lipid peroxidation is the damage to biomembranes such as mitochondrial and plasma membranes. TBARS being produced by lipid perox-

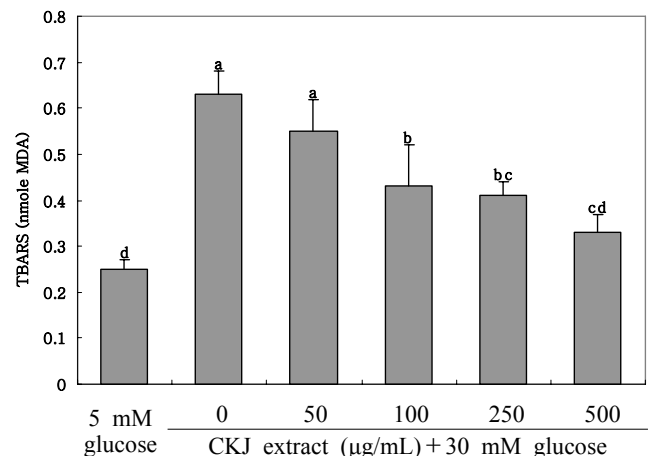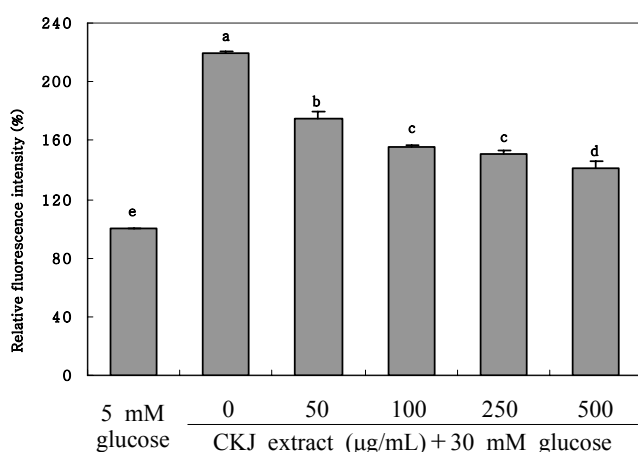

Fig. 1. Effects of Chungkookjang (CKJ) extract on cell viability in high glucose treated LLC-PK₁ cells. Cells in 96 well plates (2×10^4 cells/mL) were preincubated with 5 mM glucose for 24 hr, and then incubated with CKJ extract together with 30 mM glucose. After further incubation for 24 hr, cell viability was measured by MTT assay. Each value is expressed as mean ± SD ($n=3$). A value sharing the same superscript is not significantly different at $p < 0.05$.

Fig. 2. Inhibitory effects of Chungkookjang (CKJ) extract on lipid peroxidation in high glucose treated LLC-PK₁ cells. Cells in 96 well plates (2×10^4 cells/mL) were preincubated with 5 mM glucose for 24 hr, and then incubated with CKJ extract together with 30 mM glucose for 24 hr. Lipid peroxidation was measured by determining TBARS production. Each value is expressed as mean ± SD ($n=3$). A value sharing the same superscript is not significantly different at $p < 0.05$.

idation can cause cross-linking and polymerization of membrane components (37). 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 (38). The protective action of CKJ extract on TBARS formation was demonstrated in this study, which may be attributed to its antiperoxidative effect.

Intracellular ROS

Fig. 3 shows the effect of CKJ extract on intracellular ROS generation in LLC-PK₁ cells. When LLC-PK₁ cells were cultured with 30 mM glucose, intracellular ROS increased significantly to 220% of that of 5 mM glucose treated cells ($p < 0.05$). But treatment with 100 μg/mL of CKJ extract together with high glucose exposure resulted in a significant decrease in intracellular ROS to 155% ($p < 0.05$). ROS are oxygen containing molecules having either unpaired electrons or the ability to cleave electrons from other molecules. The development of chronic human disease is associated with increased ROS generation and excessive ROS (O_2^-, H_2O_2, ·OH) production is an important cause of cell damage such as that occurring in renal ischemia (39,40). In addition, ROS generation has an adverse affect on cell viability (41). Our results showed that high glucose treated LLC-PK₁ cells significantly increased the total intracellular ROS level compared with 5 mM glucose treated cells. However, CKJ extract significantly decreased the

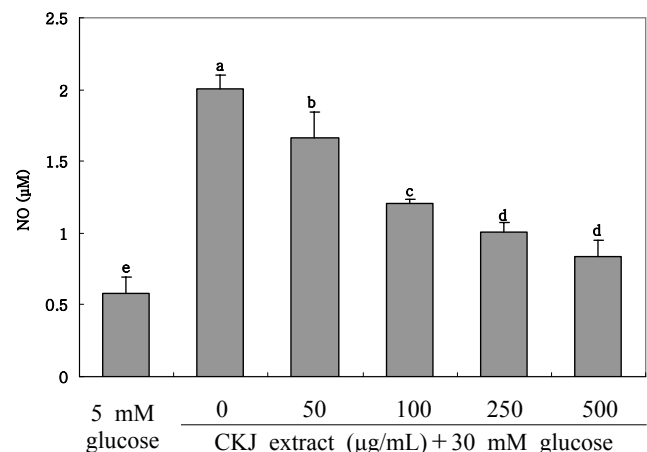elevated ROS level induced by high glucose ($p < 0.05$). These finding imply that CKJ extract alleviates oxidative stress by inhibiting ROS production induced by high glucose.

Nitric oxide (NO)

Fig. 4 shows the effects of CKJ extract on high glucose induced NO generation in LLC-PK₁ cells. The level of NO in LLC-PK₁ cells was also significantly increased by 30 mM glucose treatment compared with 5 mM glucose treatment. However, NO levels in CKJ extract treated cells significantly decreased and this effect was concentration dependent ($p < 0.05$). NO, one of the reactive nitrogen species, is also considered to be a member of the ROS family. It rapidly reacts with O_2^- to form peroxynitrite (ONOO⁻). ONOO⁻ can cross cell membranes freely and is an extremly strong and reactive oxidant (42). NO is also responsible for deleterious effects on cell function, and it interacts with O_2^- to form ·OH that leads to highly reactive oxidative damage and is associated with diabetes. Furthermore, NO targets intracellular antioxidative enzymes resulting in a loss of functionality (43). CKJ extract scavenged NO produced by high glucose induced oxidative stress. These findings suggest that CKJ extract might act as a free radical scavenger and afford important protection against the oxidative stress induced by hyperglycemia.

Antioxidant enzyme activities

Cells are protected from ROS by endogenous antioxidant enzymes such as SOD, GSH-px and catalase

Fig. 3. Effects of Chungkookjang (CKJ) extract on intracellular ROS level in high glucose treated LLC-PK₁ cells. Cells in 96 well plates (2×10^4 cells/mL) were preincubated with 5 mM glucose for 24 hr, and then incubated with CKJ extract together with 30 mM glucose. After further incubation for 24 hr, increased ROS were measured by DCF assay. Each value is expressed as mean±SD (n=3). A value sharing the same superscript is not significantly different at $p < 0.05$.

Fig. 4. Effects of Chungkookjang (CKJ) extract on NO level in high glucose treated LLC-PK₁ cells. Cells in 96 well plates (2×10^4 cells/mL) were preincubated with 5 mM glucose for 24 hr, and then incubated with CKJ extract together with 30 mM glucose for 24 hr. The amounts of NO production were assayed by measuring the accumulation of nitrite. Each value is expressed as mean±SD (n=3). A value sharing the same superscript is not significantly different at $p < 0.05$.

Table 1. Effects of Chungkookjang (CKJ) extract on antioxidant enzyme activities in high glucose treated LLC-PK$_1$ cells

	5 mM glucose	CKJ extract (μg/mL) + 30 mM glucose				
		0	50	100	250	500
SOD (unit/mg protein)	59.33 ± 2.32[a]	8.11 ± 4.43[e]	17.21 ± 9.32[d]	32.12 ± 3.25[c]	41.31 ± 3.23[b]	50.62 ± 4.01[a]
GSH-px (unit/mg protein)	4.99 ± 0.59[a]	2.89 ± 0.10[c]	3.63 ± 0.26[bc]	4.31 ± 0.83[ab]	4.53 ± 0.43[ab]	4.89 ± 0.77[a]
Catalase (μmole/mg protein/min)	1.79 ± 0.55[a]	0.60 ± 0.33[b]	1.32 ± 0.14[a]	1.51 ± 0.22[a]	1.66 ± 0.35[a]	1.77 ± 0.22[a]

Cells in 24 well plates (2×10^4 cells/mL) were preincubated with 5 mM glucose for 24 hr, and then incubated with CKJ extract together with 30 mM glucose for 24 hr. SOD: super oxide dismutase, GSH-px: glutathione peroxidase. Each value is expressed as mean ± SD (n=3). A value sharing the same superscript is not significantly different at $p < 0.05$.

(CAT). The effects of CKJ extract on antioxidant enzyme activities in high glucose treated LLC-PK$_1$ cells are shown in Table 1. Treatment with 30 mM glucose significantly reduced SOD activity of LLC-PK$_1$ cells ($p < 0.05$) compared with the cells treated with 5 mM glucose, but treatment with more than 50 μg/mL of CKJ extract together with high glucose exposure resulted in an significant ($p < 0.05$) increase of SOD activity, as shown SOD activity of 32.12 and 41.31 unit/mg protein at dose of 100 and 250 μg/mL, respectively.

30 mM glucose treatment decreased GSH-px activity compared with the cells treated with 5 mM glucose. Treatment of LLC-PK$_1$ cells with CKJ extract increased GSH-px activity of high glucose treated cells. After the cells were treated with 100 μg/mL CKJ extract, GSH-px activity significantly increased to 4.31 unit/mg protein compared to 2.89 unit/mg protein of untreated cells ($p < 0.05$). CAT activity in LLC-PK$_1$ cells treated with high glucose also was significantly decreased in comparison to 5 mM glucose treated cells ($p < 0.05$). CKJ extract increased CAT activity in a dose dependent manner.

Our results showed a decrease of the activities of antioxidant enzymes such as SOD, GSH-px and catalase in LLC-PK$_1$ cells treated with high glucose compared with 5 mM glucose treated cells. Treatment with CKJ extract restored antioxidant enzyme activities in high glucose treated LLC-PK$_1$ cells. SOD, the endogenous scavenger, catalyses the dismutation of highly reactive superoxide anion to H$_2$O$_2$ (44). GSH-px catalyses the reduction of H$_2$O$_2$ at the expense of reduced GSH (i.e. H$_2$O$_2$ + 2 GSH → 2 H$_2$O + GSSG). H$_2$O$_2$ is also scavenged by catalase (45). The decreased activities of both catalase and GSH-px in the LLC-PK$_1$ treated with high glucose indicate a greatly decreased capacity to scavenge H$_2$O$_2$ produced in the cells, with an increase in ROS and oxidative stress in response to high glucose treatment (46).

In conclusion, we demonstrated that CKJ extract can protect LLC-PK$_1$ cells from high glucose induced oxidative stress by decreasing intracellular ROS level and lipid peroxidation, and increasing antioxidant enzyme activity and cell viability.

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