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Protective Effects of Ginseng Coffee against Hydrogen Peroxide-induced Oxidative Damage in L6 Muscle Cells

Yeonmi Lee, Kyung-Tack Kim, Hye-Min Bae, Young-Chul Lee, and Sang Yoon Choi*

Korea Food Research Institute, Seongnam 463-746, Korea

This study investigated the antioxidant effects of ginseng coffee in L6 muscle cells. Ginseng coffee was prepared by coating and digesting coffee beans with ginseng concentrate. The ginseng coffee water extract potently protected against hydrogen peroxide-induced L6 cell death and adenosine triphosphate reduction in a dose-dependent manner; in fact, these cytoprotective effects were significantly greater than those of normal coffee. However, ginseng coffee did not exhibit significant radical scavenging or catalase-like activity. These results suggest that ginseng coffee might act as a cytoprotective agent in muscles, but that the protective effects are not due to a direct radical-reduction property but rather to another intracellular signaling factor.

Keywords: Ginseng, L6, Muscle cell, Antioxidant, Coffee

INTRODUCTION

Regular and proper exercise helps to prevent and alleviate hypertension, stroke, cardiovascular disease, diabetes, hyperlipidemia, and cancer [1,2]. However, strenuous exercise produces excessive reactive oxygen compounds, which cause oxidative damage to muscle tissues [3,4]. "Reactive oxygen" is a collective term for oxygen compounds such as the superoxide radical, hydroxyl radical, hydrogen peroxide (H_2O_2), and singlet oxygen, which are generated during normal metabolic processes and perform several biological functions. However, excessively produced reactive oxygen compounds lead to tissue damage by lipid peroxidation and cause problems in DNA nucleic acid sequences [5,6]. The human body protects itself by producing antioxidant enzymes such as superoxide reductase, catalase, and glutathione peroxidase. However, the protective abilities of these enzymes differ greatly depending on a person's age and health. Vitamin C and β-carotene are known to assist in the antioxidant defensive system, and Choi *et al*. [7] reported that red ginseng reduced the serum malondialdehyde level, which is increased by reactive oxygen compounds generated during high-intensity aerobic exercise. In addition, a protective effect of maltol (2-methyl-3-hydroxy-4-pyrone) on oxygen radical-induced tissue damage was reported by Shin *et al*. [8].

Ginseng has been used not only as a traditional medicine, but also as a food; thus, it is manufactured as both health and general food products including extracts, pills, tablets, pouches, and red ginseng tea. In particular, ginseng has an "energy burst" effect in relation to athletic activity. This property has recently received increased interest in the United States, and several types of processed products with enhanced palatability, such as ginseng-laced beverages and ginseng coffee, are available on the market. As a part of such technology,

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***Corresponding author**
E-mail: sychoi@kfri.re.kr
Tel: +82-31-780-9307, Fax: +82-31-709-9876

we developed and reported a Korean ginseng coffee with excellent palatability [9]. This study was conducted to investigate ginseng coffee's antioxidant effect on muscle cells compared to normal coffee. Its influence on cell viability and adenosine triphosphate (ATP) production was comparatively analyzed in H_2O_2-treated L6 muscle cells.

MATERIALS AND METHODS

Experimental samples

The ginseng-coated coffee beans used in this study were prepared according to the method described by Kim *et al*. [9]. Five grams per milliliter hydroxypropyl methyl cellulose was dissolved in distilled water, and white ginseng concentrate (Panax ginseng C.A. Meyer; Guan Industry Co., Seoul Korea) was added to each concentration of 5 degrees Brix (5°Bx) and 20°Bx to make the coating solution. Coffee beans (Crystal Mountain, Cuba) were roasted at 250°C for 17 minutes, and the coating solution was sprayed onto the coffee beans (40 mL/100 g), which were then cooled. Ginseng-digested coffee beans were prepared by digesting 500 g of coffee beans (Crystal Mountain) in a 500 mL solution of white ginseng concentrate, diluted to each concentration of 5°Bx and 20°Bx for 4 hours, and then dried.

Extraction

Coffee beans, ginseng-digested coffee beans, and ginseng-coated coffee beans (5°Bx, 20°Bx) were extracted using distilled water through a coffee machine (BCO 120T; DeLonghi, Bedford Heights, OH, USA) at 100°C for 5 minutes, whereupon the obtained extract was passed through filter paper and freeze-dried for experimental use as samples.

Cell culture

An L6 line of muscle cells was obtained from the Korean Cell Line Bank (Seoul, Korea) and incubated at 37°C in 5% CO_2 using Dulbecco's modified Eagle's medium (Gibco, Grand Island, NY, USA) containing 10% fetal bovine serum (Gibco) and 1% antibiotic–antimycotic (Gibco).

Cell viability measurement

A measurement of cell viability was performed using an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide] assay. To measure the protective effect of ginseng coffee on muscle cells, L6 cells were cultured for 24 hours by seeding them onto a 96-well plate at a concentration of 1×10^5 cells/mL and treating them with each concentration of ginseng coffee extract and 1.5 mM H_2O_2 simultaneously. After culturing for an additional 24 hour, the cells were stained with MTT (0.5 mg/mL in phosphate-buffered saline [PBS]) solution, and absorbance was measured at 540 nm. The L6 protective effect of ginseng coffee extract was expressed as a percentage by calculating the recovery rate from the ginseng coffee extract treatment and the cell death rate from the blank treatment.

ATP production measurement

ATP production in L6 cells was measured using the ATP Bioluminescence Assay Kit HS II (Roche, Mannheim, Germany). L6 cells were cultured for 24 hours by seeding them onto a 96-well plate at a concentration of 1×10^5 cells/mL, treating them with each concentration of ginseng coffee extract and 1.5 mM H_2O_2 simultaneously, and culturing them for an additional 24 hour. The cells were then lysed for an ATP bioluminescence assay, and absorbance was measured with a luminometer (Molecular Devices, Sunnyvale, CA, USA).

Catalase-like activity

The catalase-like activity of ginseng coffee extract was measured using a catalase assay kit (Sigma-Aldrich, St Louis, MO, USA). Twenty-five microliters of 200 mM H_2O_2 and 75 μL of ginseng coffee extract dissolved in 50 mM potassium phosphate buffer were mixed and then incubated at room temperature for 5 minutes, whereupon 900 μL of 15 mM sodium azide solution was added. Ten microliters of this reactant solution was mixed with 1 mL of 0.25 mM 4-aminoantipyrine and 2 mM 3,5-dichloro-2-hydroxybenzenesulfonic acid dissolved in a 150 mM potassium phosphate buffer (pH 7.0), then incubated at room temperature for 15 minutes. The absorbance was measured at 520 nm.

DPPH radical scavenging activity

Each 0.2 mL of ginseng coffee extract dissolved in 80% ethanol was added to 3 mL of ethanol, to which 0.8 mL of 4×10^{-4} M DPPH (2,2-diphenyl-1-picrylhydrazyl; Sigma-Aldrich) dissolved in ethanol was then added. This was vortexed for 10 seconds and left at room temperature for 10 minutes, and absorbance was measured at 517 nm [10,11]. The DPPH radical scavenging activity was expressed as a percentage of the absorbance of the group to which DPPH was not added. Vitamin C was used as a positive control.

ABTS radical scavenging activity

The ABTS [2,2'-azino-bis (3-ethylbenzthiazoline-6-sulfonic acid)] radical scavenging activity of ginseng coffee extract was measured using a slight modification of the method proposed by van den Berg *et al*. [12]. To 0.1 M PBS (pH 7.4), 2.5 mM ABTS, and 1.0 mM AAPH [2,2'-azobis(2-methylpropionamidine) dihydrochloride] were added and left at 68°C for 12 minutes in a dark room, then quickly cooled to make an $ABTS^+$ solution. Each 20 μL of ginseng coffee extract dissolved in PBS was added to 980 μL of $ABTS^+$ solution and incubated at 37°C for 10 minutes, whereupon absorbance was measured at 734 nm.

Statistical analysis

Each experimental result was expressed as the mean±SD. Statistical comparison between different treatments was calculated with ANOVA.

RESULTS

L6 cell protective effect

Strenuous exercise produces excessive reactive oxygen compounds, which cause oxidative damage to muscle tissues. The defensive effect of ginseng coffee against H_2O_2-induced oxidative damage was measured in L6 muscle cells to determine how ginseng coffee's protective effect compares to that of normal coffee. First, L6 cells were treated with 10 ppm of ginseng coffee extract for 24 hours, but no cell death was observed (Fig. 1), and 0.1, 1, and 10 ppm ginseng coffee extracts were treated for 24 hours along with 1.5 mM H_2O_2. L6 cells treated only with 1.5 mM H_2O_2 showed a rate of cell death of 52.5%, whereas those treated with 1.5 mM H_2O_2 along with ginseng coffee extract demonstrated decreased cell death in a dose-dependent manner (Fig. 2). The rates of reduction of cell death in the 20°Bx ginseng-coated coffee extract were 13.5, 32.8, and 38.3% at 0.1, 1, and 10 ppm, respectively. Moreover, the ginseng coffee with a high concentration (20°Bx) of ginseng displayed an excellent cell death inhibitory effect compared to ginseng coffee with a low ginseng concentration (5°Bx). This was true for both the ginseng-coated and ginseng-digested coffee, suggesting no significant difference in the processing method of coating or digesting.

Also, the measurement of ATP production, which is the energy source for exercise and a critical requirement for cell survival, showed that the ginseng coffee treatment effectively inhibited the reduced ATP production

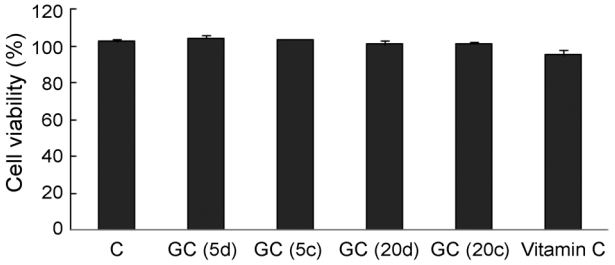

Fig. 1. Cell viabilities after treatment with ginseng coffee extract for 24 hours. L6 cells were treated with 10-ppm test samples. The data represent the mean±SD of three experiments. C, normal coffee; CG (5d), 5 degrees Brix (5°Bx) digested ginseng coffee; CG (5c), 5°Bx coated ginseng coffee; CG (20d), 20°Bx digested ginseng coffee; CG (20c), 20°Bx coated ginseng coffee.

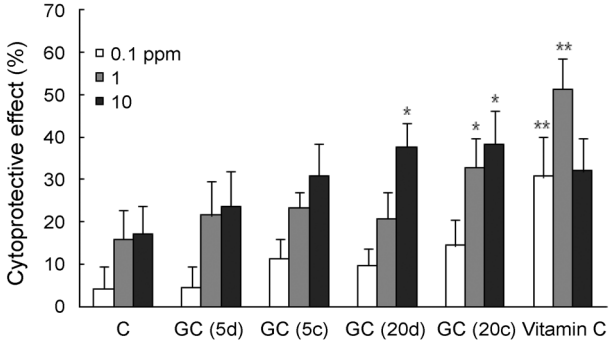

Fig. 2. Protective effect of ginseng coffee extract against H_2O_2-induced oxidative damage in L6 muscle cells. L6 cells were treated with 1.5 mM H_2O_2 and test samples for 24 hour. The data represent the mean±SD of three experiments. Asterisks indicate significant differences compared to the control. C, normal coffee; CG (5d), 5 degrees Brix (5°Bx) digested ginseng coffee; CG (5c), 5°Bx coated ginseng coffee; CG (20d), 20°Bx digested ginseng coffee; CG (20c), 20°Bx coated ginseng coffee. $^*p<0.05$; $^{**}p<0.01$.

in H_2O_2-treated L6 cells compared to normal coffee (Fig. 3).

Antioxidant enzyme-like activity

Catalase is an important antioxidant enzyme that triggers the degradation of H_2O_2 into water and oxygen [13,14]. As shown in Fig. 4, the catalase-like activities of ginseng coffee and normal coffee extract were not observed at all concentrations, which indicates that ginseng coffee was unable to degrade H_2O_2 and thereby decrease its concentration. Therefore, the protective effect of ginseng coffee extract on the toxicity of H_2O_2 in L6 cells was confirmed to not involve a direct reduction of H_2O_2 concentration.

Radical scavenging activity

DPPH and ABTS assays were performed to determine whether ginseng coffee extracts can directly scavenge free radicals. Measurement of DPPH scav-

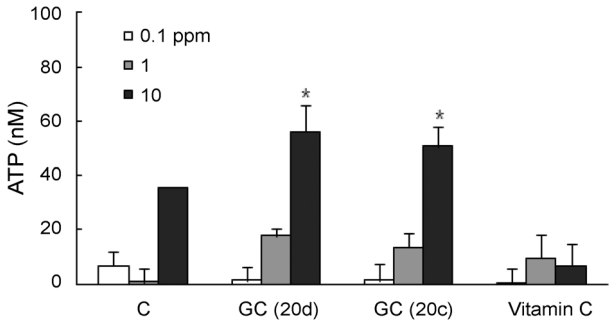

Fig. 3. Effects of ginseng coffee extracts on adenosine triphosphate (ATP) production in L6 muscle cells. L6 cells were treated with 1.5 mM H_2O_2 and test samples for 24 hour. The data represent the mean±SD of three experiments. Asterisks indicate significant difference compared to the control. C, normal coffee; CG (20d), 20 degrees Brix (20°Bx) digested ginseng coffee; CG (20c), 20°Bx coated ginseng coffee. $^*p<0.05$.

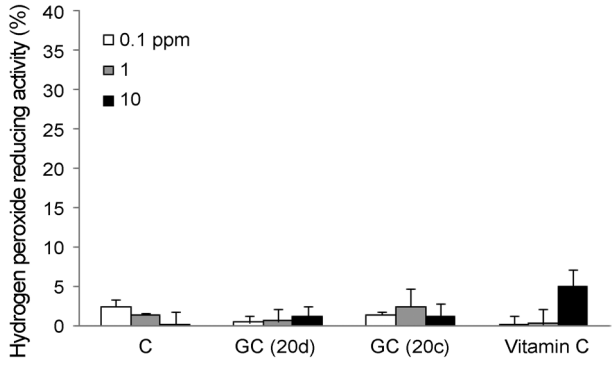

Fig. 4. Catalase-like activity of ginseng coffee extracts. The data represent the mean±SD of three experiments. C, normal coffee; CG (20d), 20 degrees Brix (20°Bx) digested ginseng coffee; CG (20c), 20°Bx coated ginseng coffee.

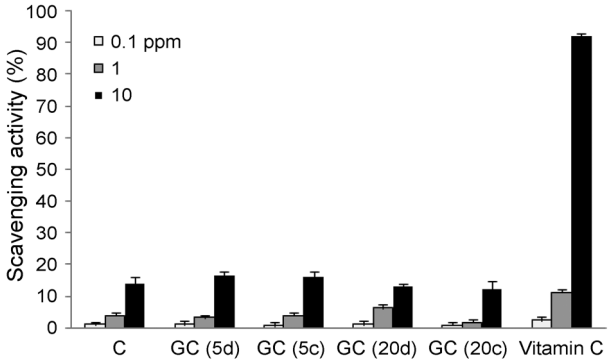

Fig. 5. DPPH radical scavenging activity of ginseng coffee extracts. The data represent the mean±SD of three experiments. C, normal coffee; CG (5d), 5 degrees Brix (5°Bx) digested ginseng coffee; CG (5c), 5°Bx coated ginseng coffee; CG (20d), 20°Bx digested ginseng coffee; CG (20c), 20°Bx coated ginseng coffee.

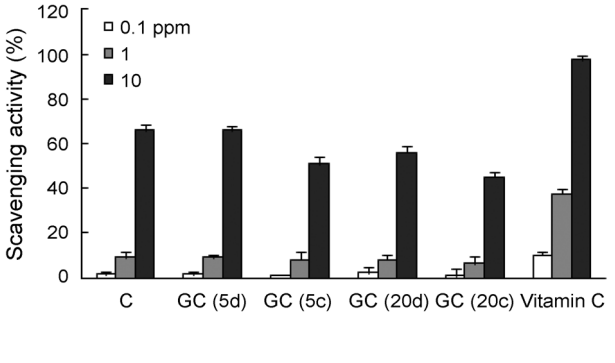

Fig. 6. ABTS radical scavenging activity of ginseng coffee extracts. The data represent the mean±SD of three experiments. C, normal coffee; CG (5d), 5 degrees Brix (5°Bx) digested ginseng coffee; CG (5c), 5°Bx coated ginseng coffee; CG (20d), 20°Bx digested ginseng coffee; CG (20c), 20°Bx coated ginseng coffee.

enging activity indicated no significant difference in the activity compared to normal coffee extract, and all the ginseng coffee extracts showed less than 20% DPPH scavenging activity at 10 ppm (Fig. 5). The ABTS scavenging activity of ginseng coffee extract at 10 ppm was 51.0 to 66.1%, which was slightly higher than the DPPH scavenging activity, but not significantly different at all concentrations compared to the scavenging activity of normal coffee extract (Fig. 6).

DISCUSSION

Ginseng coffee effectively suppressed L6 muscle cell death and reduced ATP production due to H_2O_2 compared to normal coffee. Thus, it is expected that the intake of ginseng coffee can effectively protect muscle cell damage due to increased reactive oxygen caused by strenuous exercise. However, no differences in DPPH and ABTS radical scavenging activities or catalase-like activities were observed between ginseng coffee and normal coffee; therefore, the assumption was made that the muscle cell protective effect of ginseng coffee is caused not by the direct removal of free radicals, but by other effects on cell physiology and intracellular signaling.

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