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Analysis of the Cytotoxicity of Green Pigment Produced on the Surface of Roasted and Retorted Chestnuts Using Immune Cells and Gastrointestinal Cancer Cells

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Abstract

Roasted and retorted (RR) chestnuts develop green pigment spots on their surface during storage. The purpose of this study was to evaluate the cytotoxicity of the green pigment using RAW 264.7, MOLT-4, KATOⅢ and HT-29 cells. The pigment scraped from RR chestnuts (GP), whole RR chestnuts with green pigment spots (GC), whole RR chestnuts without green pigment (WC) and roasted and frozen stored chestnuts (FC) were extracted in 10% DMSO. MOLT-4 cells were less resistant to the cytotoxicity of the chestnut extracts than the RAW 264.7 cells. The GP extracts did not show different responses against H_2O_2-induced oxidative stress and LPS-induced NO production compared to the other extracts. The chestnut extracts did not have proliferative activity on either of the KATOⅢ or HT-29 cells ($p>0.05$). Our results from the comparison of the green pigment produced on the surface of the RR chestnuts to chestnuts that do not develop the green pigment suggest that the pigment may not be harmful in terms of either cytotoxicity towards immune cells or proliferation of gastrointestinal cancer cells.

Key words: chestnut, green pigment, cytotoxicity, immune cell, cancer cell

INTRODUCTION

Chestnuts (*Castanea sativa* Mill.) are used raw or cooked (boiled or roasted) and are processed to make syrups and liquors (1). Roasted and retorted (RR) chestnuts are popularly consumed in Korea and Japan. RR chestnuts are mostly produced in a process of washing, packaging in a pouch and retorting. The RR chestnuts develop small green pigment spots on their surface after storage for more than 5 months at room temperature. The cause of the green pigment and chemical properties of the pigment are not known. Visual perception has huge impacts on consumer's perception. As the green spots on the chestnuts can be thought as a result of deterioration, this phenomenon can reduce the visual quality of the product. Moreover, this restricts the shelf-life labelling of the product to 6 months, which otherwise can stay up to two years. The chemical composition and quality of roasted chestnuts have been poorly studied. Morini and Maga (2) studied the changes in fatty acid composition of the lipids in chestnuts during roasting at 182°C. Shin et al. (3) evaluated the effect of roasting in an oven (200°C) on the composition of chestnuts.

Adams and Brown (4) reported that green discoloration in raw and processed vegetables was not a common phenomenon; however, garlic products and cooked sweet potatoes could also develop green pigments. Kubec et al. (5) reported that isoalliin of garlic is enzymatically cleaved to 1-propenyl-containing thiosulfinates in the disrupted tissue and that these compounds react with amino acids to produce green pigments. However, the formation of green pigment by this mechanism is observed only in garlic. Also, it has been demonstrated that a mixture of blue, green and brown pigments was formed in cooking water of sweet potato (6). A maximum amount of pigment was produced in the water at approximately pH 9. A similar behavior was found in a chlorogenic acid-glutamine model system, suggesting that the cause of the green discoloration was the reaction between chlorogenic acid and amino acids in the sweet potato (7). Evidence was presented that the green pigments were formed as a result of condensation of two molecules of chlorogenic acid with one molecule of an amino acid, feasibly by a free radical mechanism. The pigments formed were novel benzacridine derivatives, and they were shown to exist in blue, green and yellow forms, depending on their oxidation state. We assume that the green pigment produced on the surface of the

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RR chestnuts would be produced in a similar way to that occurring in a sweet potato.

When analyzed with MS and NMR, the green pigment on chestnuts was found to be a mixture of carbohydrates, phenolic compounds and flavonoids (data not published). The pigment was soluble in dimethylsulfoxide (DMSO), but not in ethyl acetate, chloroform, methanol or water.

Nowadays, food safety is one of the major concerns in the food industry; however, the safety of the pigment on human consumption has not been tested. Since the pigment may possess toxicity toward humans and/or negatively affect consumer's perception of the food, evaluation of the toxicity is essential. Cell study of the pigment's potential toxicity was the first step for this analysis.

The objectives of this study were to determine potential cytotoxicity of the green pigment developed on the RR chestnuts using immune cells (RAW 264.7 rat macrophage and MOLT-4 human T cells) and the pigment's effects on human gastrointestinal cancer cell proliferation (KATOⅢ gastric cancer cells and HT-29 colon cancer cells).

MATERIALS AND METHODS

Materials and reagents

The RR chestnuts were provided by CJ Cheiljedang Corporation (Seoul, Korea). DMSO and hydrogen peroxide (H_2O_2) were purchased from Samchun Pure Chemicals (Pyeongtaek, Korea). Dulbecco's modified Eagle's medium (DMEM; containing 4.5 g D-glucose/L, 4 mM L-glutamine and 110 mg sodium pyruvate/L), Rosewell Park Memorial Institute (RPMI) 1640 (containing 2.05 mM L-glutamine and 25 mM HEPES), penicillin-streptomycin, fetal bovine serum (FBS) and trypsin-EDTA were purchased from Gibco BRL (Grand Island, NY, USA). Dulbecco's phosphate buffered saline (PBS) was purchased from Welgene (Daegu, Korea). 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), lipopolysaccharides from *Escherichia coli* (LPS 0111: B4), sulfanilamide, phosphoric acid, 0.05% N-1-naphthylethylenediamine and sodium nitrite were purchased from Sigma-Aldrich (St. Louis, MO, USA).

Sample preparation

Around 2 g of green pigment was scraped from RR chestnuts (GP) and homogenized with 30 mL of distilled water and freeze-dried using a freeze-dryer (Ilsin Lab Co., Seoul, Korea), yielding approximately 1 g of freeze dried extract. Separately, whole RR chestnuts with green pigment spots (GC), whole RR chestnuts without green pigment (WC) and roasted and frozen-stored chestnuts (FC) were also homogenized with 30 mL of distilled water and freeze-dried, yielding around 5 g of freeze dried extracts. About 1 g of each freeze-dried sample was extracted for 1 hr with 10% (v/v) DMSO and filtered through Whatman No. 1 filter paper (Whatman International Ltd., Maidstone, England) with gravity. The filtrate was concentrated by a vacuum rotary evaporator (Eyela Co., Tokyo, Japan) for 20 min in 70°C water bath. The extracts were dissolved in 10 mL of 10% (v/v) DMSO aqueous solution and stored at -20°C before use. When used, the DMSO concentration was equalized for all treatment media, never exceeding 0.1% (v/v) of the final treatment media. Control cells were treated with equal amount of DMSO for all assays.

Cell lines and cell culture

RAW 264.7 rat macrophages, MOLT-4 human leukemia T cells, KATOⅢ gastric cancer cells and HT-29 colon cancer cell lines were obtained from Korean Cell Line Bank (Seoul, Korea). The cell lines were grown in media as recommended by the supplier; i.e., RAW 264.7 cells in DMEM media, and MOLT-4, KATOⅢ and HT-29 cells in RPMI 1640. Suspension cells (MOLT-4) were cultured in a 75 cm^2 T-flask. Adherent cells (RAW 264.7, KATOⅢ and HT-29) were cultured in 100 cm^2 cell culture dishes. All cell lines were maintained in culture medium supplemented with 10% heat-inactivated FBS and 1% penicillin/streptomycin in a humidified atmosphere at 37°C with 5% CO_2. The media were changed three times a week. Cell growth and morphology were determined microscopically using a hemocytometer and microscope (Olympus, Tokyo, Japan), respectively. All experiments were performed with cells in the exponential growth phase.

MTT assay

Cytotoxicity of the extracts was measured by MTT assay (8) with minor modifications. Suspension cells were collected by centrifugation at 1,000 rpm for 3 min to pellet the cells and adherent cells were harvested after trypsinization. All cells were washed in PBS and counted using the hemocytometer. The cells were resuspended in each culture medium and seeded in flat (adherent cells) or round-bottom (suspension cells) 96-well culture plates at 5×10^4 to 1×10^5 cells/mL, with a final volume of 100 μL per well. After a 24 hr pre-incubation, the medium was gently removed from each well and 100 μL of the serum-free media containing various concentrations of the extracts were added to each well. After 24 to 48 hr, 20 μL of MTT solution (5 mg/mL) were added to each well. Following 4 hr of incubation at 37°C, the media was removed and formazan crystals, which resulted from the reduction of MTT by active cells, were

dissolved in 200 μL DMSO. The optical density at 570 nm was measured by an ELISA microplate reader (Bio-Rad, Richmond, CA, USA). Cell viability was calculated as % viability = (sample absorbance/control absorbance) × 100. All experiments were performed in triplicate or more.

H_2O_2 induced apoptosis

The MOLT-4 cells were plated at a density of 1×10^5 cells in a round-bottom 96-well culture plate with 100 μL of culture medium and pre-incubated for 24 hr. The culture medium was aspirated from each well after centrifugation (1,000 rpm, 3 min) and replenished with 100 μL of the serum-free media containing various concentrations of the extracts. After 1 hr, cells were treated with H_2O_2 in serum-free medium at 1～2 pmol/cell for 24 hr, and the cell viability was determined using MTT assay.

NO assay

To study the effects of the extracts on NO production, the Griess method (9) was used. The MOLT-4 cells were pre-incubated for 24 hr, pretreated with the extracts for 1 hr, exposed to LPS (100 ng/mL) and incubated for 48 hr. The plate was centrifuged at 3,000 rpm for 10 min and 100 μL of supernatant were taken for the assay. To measure nitrite, the collected supernatant were mixed with an equal volume of Griess reagent (0.5% sulfanilamide, 2.5% phosphoric acid and 0.05% N-1-naphthylethylenediamine) and incubated for 20 min at room temperature. NO production was determined measuring the absorbance at 540 nm with the ELISA microplate reader.

Statistical analysis

The significance ($p<0.05$) of difference among the groups was assessed using one-way analysis of variance and Duncan's multiple range tests by SAS program (version 9.1, SAS Inc, Cary, NC, USA). Student's *t*-tests were used to compare sample treated cells with the control.

RESULTS AND DISCUSSION

Effects of chestnut extracts on immune cells

Our study was more focused on whether the immune activities of GP extract are similar to or different from those activities of other chestnut extracts. The macrophage and T cells used in this study play a crucial role in immune functions as effectors and regulators (10). To assess the effect of the green pigments and chestnut extracts on the proliferation of the cells, MTT was assayed. Viabilities of RAW 264.7 and MOLT-4 cells treated with the chestnut extracts are shown in Fig. 1. Treatment with the chestnut extracts and green pigment from the chestnuts dose-dependently decreased the cell viabilities of both RAW 264.7 and MOLT-4 cells when 0.2 mg/mL or more of the extracts was used. The FC (the most fresh chestnut) extract had significantly lower cytotoxicity than the other extracts with 1 mg/mL or more concentrations on the RAW 264.7 cells ($p<0.05$) (Fig. 1A). The extracts did not show much difference in cytotoxicities on the MOLT-4 cells (Fig. 1B). These data were used to calculate the concentrations of the chestnut and green pigment extracts required to cause a 50% reduction (IC_{50}) in growth (cell number) for each cell line. The IC_{50} values of the RAW 264.7 (1.77, 1.89, 1.54 and 2.49 mg/mL) were higher than MOLT-4 (1.45, 1.58, 1.46 and 1.49 mg/mL) treated with GP, GC, WC and FC extracts, respectively. The results suggest that MOLT-4 cells are

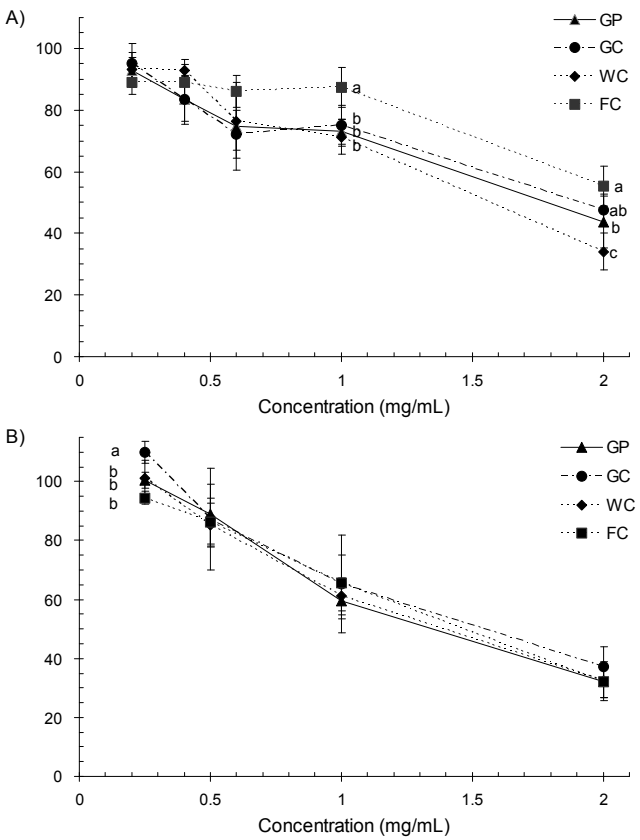

Fig. 1. Viabilities of RAW 264.7 (A) and MOLT-4 (B) cells treated with chestnut extracts. Expressed as % of cell viability compared to the control. Values expressed are mean ± standard deviation (n=5). [a-c]Values with different letters are significantly different at the same concentrations ($p<0.05$); one way ANOVA and Duncan's multiple range test. GP, green pigment scraped from roasted and retorted (RR) chestnuts; GC, whole RR chestnuts with green pigment spots; WC, whole RR chestnuts without green pigment; FC, roasted and frozen stored chestnuts. mg/mL: freeze-dried chestnut and/or green pigment/culture medium.

less resistant to the cytotoxicity of the chestnut extracts than RAW 264.7 cells.

Recently, cell culture has been used extensively as an *in vitro* method to assess the toxicity of the new food materials (11); however, *in vitro* study on chestnuts is scarce. Lee et al. (12) reported that water extract of chestnut kernel had antioxidant and immunomodulatory activities on RAW 264.7 macrophage cells. They used the same cell line and the same amount of the extracts (0.2 mg/mL) as those in our study. They suggested that the chestnut might provide a natural source of antioxidants and active immunity. No research has been done on the antioxidant and immunomodulatory activities of the RR chestnuts.

The MOLT-4 cells were used to determine cytotoxicity and oxidative damage response of the chestnut extracts. To assess the effect of the green pigment and chestnut extracts on the proliferation of the cells exposed to H_2O_2, MTT assays were performed. In this study, since H_2O_2 induced oxidative damage in MOLT-4 cells at dose of 1 pmol H_2O_2/cell or more, the cells were treated with 1 pmol H_2O_2/cell or more. The MOLT- 4 cells with or without (control) treatments with chestnut extracts did not respond differently. The H_2O_2 caused a dose-dependent reduction in the cell viability for both 0.2 mg/mL and 0.4 mg/mL concentrations of chestnut extracts (Fig. 2). The GC extract (0.2 mg/mL) had significantly lower cytotoxicity than FC and GP extracts ($p<0.05$), suggesting at least the green pigment itself is not as toxic as the fresher chestnuts.

We used H_2O_2, which is widely regarded as a cytotoxic agent (13). It has been reported, H_2O_2 might act as an activator of immune cells (14). As the MTT test is an assay of both cell integrity and cell number, it was important to find out if it could be used as an assay to test the possible cytotoxicity of H_2O_2 (15). The Student's *t*-test results showed that the chestnut extracts did not show different response against H_2O_2- induced oxidative stress compared to the control.

The effects of the chestnut extracts on LPS-induced NO production in the MOLT-4 cells were investigated by measuring accumulated nitrite, as estimated by Griess reaction, in the culture medium. The chestnut extracts did not interfere with the reaction between nitrite and Griess reagents. The inhibition of NO production was observed without noticeable cytotoxicity in the MOLT-4 cells with the addition of the chestnut extracts (0.1, 0.2 and 0.4 mg/mL) (Fig. 3). Increased NO production was observed in the cells treated with the GC extract (0.4 mg/mL) compared to the cells treated with the other extracts ($p<0.05$); however, the chestnut extracts did not have any anti-inflammatory activities compared to the control ($p>0.05$). Among the chemical mediators, NO is important for provoking and maintaining an inflammatory condition (16). We used LPS, which is commonly used to stimulate NO production of immune cells (17). Our result revealed that the chestnut extracts did not show different responses against LPS-induced NO production compared to the control, suggesting that the green pigment may not present abnormal immune activities in either the rodent macrophages or the human T cells exposed to oxidative and inflammatory stress compared to the other chestnut extracts.

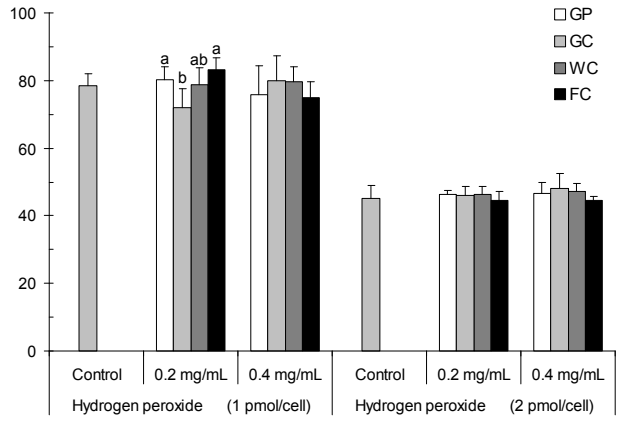

Fig. 2. Viabilities of H_2O_2 damaged MOLT-4 human leukemia T cells treated with chestnut extracts. Expressed as % of cell viability compared to the control. Control cells were treated with H_2O_2 alone. WC, FC, GC and GP: see Fig. 1. Values expressed are mean±SD (n=5). [a,b]Values with different letters are significantly different on the same concentrations ($p<0.05$).

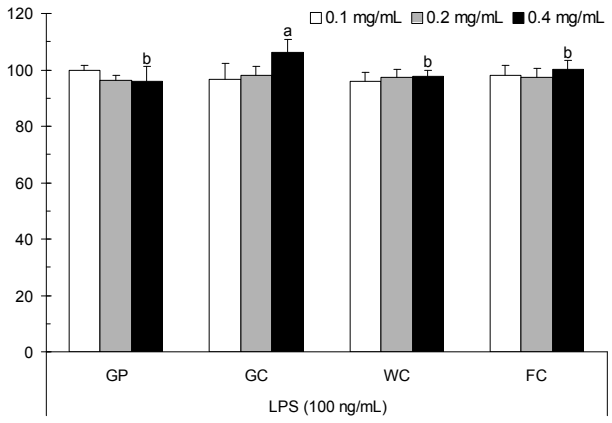

Fig. 3. Nitric oxide production (%) in LPS (100 nmol/mL) treated MOLT-4 human leukemia T cells treated with chestnut extracts. The LPS treated cells without any treatment were used as control. Expressed as % of cell viability compared to the control. WC, FC, GC and GP: see Fig. 1. Values expressed are mean±SD (n=5). [a,b]Values with different letters are significantly different on the same concentration ($p<0.05$). LPS: lipopolysaccharides from *Escherichia coli.*

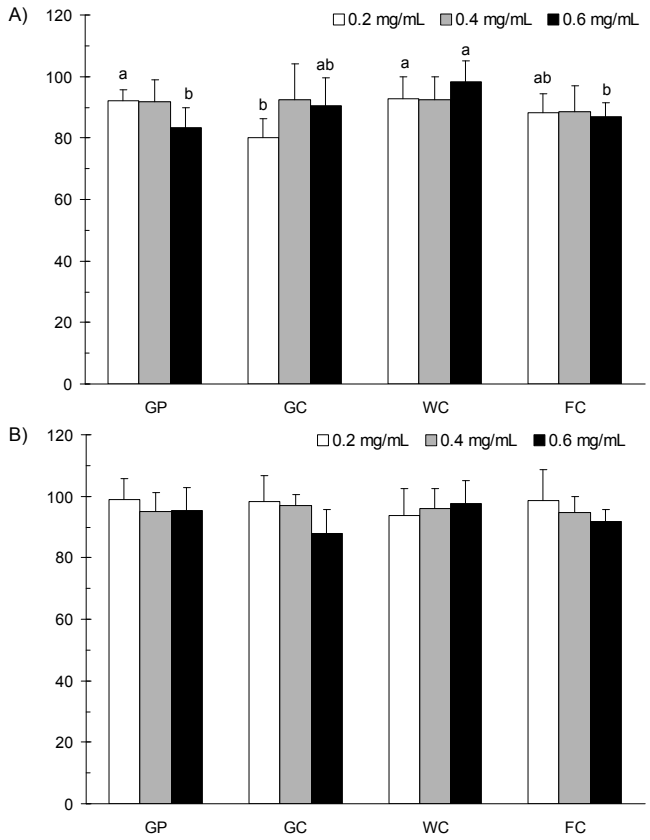

Fig. 4. Anti-proliferative activities of chestnut and green pigment extracts against KATOⅢ human gastric cancer (A) and HT-29 human colon cancer (B) cells. The cells without any treatment were used as control. Expressed as % of cell viability compared to the control. WC, FC, GC and GP: See Fig. 1. Values expressed are mean±SD (n=5). [a,b]Values with different letters are significantly different on the same concentrations ($p<0.05$).

Proliferation rates of human gastrointestinal cancer cells

The effect of GP on the proliferation rates of cancer cells was compared to the effects of the other extracts on the same cell lines. The proliferation rates of the chestnut extracts on the KATOⅢ human gastric cancer cells and HT-29 human colon cancer cells are shown in Fig. 4. The chestnut extracts did not suppress the proliferation of either KATOⅢ or HT-29 cells treated with 0.2, 0.4 and 0.6 mg/mL. The KATOⅢ cells (2～12% reduction) and HT-29 cells (1～13% reduction) were inhibited to the same extent as the other chestnut extracts. Anti-proliferative activity of the GC extract on the cells was significantly higher than those of the WC and GP extracts at 0.2 mg/mL, and that of the WC extract was significantly lower than those of the others at 0.6 mg/mL ($p<0.05$) (Fig. 4A). The chestnut extracts did not influence the cell viability of HT-29 compared with the control (without treatment) (Fig. 4B). These results suggest that the green pigment from the RR chestnuts do not have any negative effects on the cancer cells.

Based on our comparison of the effects of the different green pigment and chestnut extracts, these results suggest that the green pigment produced on the surface of RR chestnuts may not be harmful in terms of cytotoxicity in immune cells and proliferation of gastrointestinal cancer cells; however, since the cause of the pigment production and the chemical composition of the pigment are not known, the findings from this study do not mean that the green pigment is fully safe for human consumption. Therefore, further studies are needed to evaluate the pigment's chemical composition and safety.

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