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RESEARCH NOTE

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Hepatopotective Effects of Black Rice on Superoxide Anion Radicals in HepG2 Cells

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Abstract Cyanidin 3-glucoside (C3G) isolated from black rice was investigated for hepatoprotective effects in HepG2 cells under oxidative stress. When an increase in the production of reactive oxygen species (ROS) was induced by gramoxone, cell viability was drastically decreased by 42%. However, in the presence of C3G, no hepatocytic damage was observed in HepG2 cells treated with gramoxone. C3G was found to manifest a stronger scavenging effect (91%) on superoxide anion radical ($O_2^{\cdot-}$) than any of the other natural and synthetic antioxidants. Results suggest that C3G from black rice possesses hepatoprotective effects *in vitro*, which may be, at least in part, due to $O_2^{\cdot-}$ scavenging.

Keywords: black rice, cyanidin 3-glucoside, oxidative stress, superoxide anion radical, HepG2 cell

Introduction

Rice (*Oryza sativa* L.) is widely consumed throughout the world, particularly in Asian countries. With growing concerns regarding health, rice consumers in Asian countries have been paying a great attention to a wide range of added values for this staple food. Colored rice is broadly known as enriched rice with improved nutritional value. It has high levels of minerals and pigments that may be good for one's health. People in China have used red rice in medicinal diets from ancient times (1). However, a few have been reported about the biologically active effects of phenolics from colored rice.

Of the naturally-occurring phenolics, anthocyanins are the largest group of water-soluble secondary metabolites of plants. They are widely available in beans, cereals, and fruits and exhibit a remarkable array of biochemical and pharmaceutical actions (2). The beneficial health effects seen in anthocyanins could be due to the antioxidant action of scavenging reactive oxygen species (ROS) (3, 4). The ROS are capable of causing deleterious changes in cell function by a number of alterations such as lipid peroxidation (5), enzyme inactivation (6), and oxidative DNA damage (7). The accumulation of ROS has also been implicated in the aging process (8). The superoxide anion radical ($O_2^{\cdot-}$) initiates the ROS generation system in mitochondria by oxygen reduction. Dismutation of $O_2^{\cdot-}$ is the main mitochondrial source of H_2O_2, which is subsequently reduced to water by catalase or otherwise decomposed by glutathione peroxidase. Therefore, it is desirable to protect cells from $O_2^{\cdot-}$ and/or H_2O_2 for efficiency of antioxidant defense systems in the mitochondria.

The ROS, derived from many sources, influence macromolecules in the liver. Excessive exposure to ROS leads to hepatocytic injuries, resulting in liver diseases and eventual cell death (9). Therefore, a search for plant products or alternative medicine that could limit ROS-mediated injuries is necessary for protection of the liver from possible damage.

In the present study, we assessed the hepatoprotective potential of cyanidin 3-glucoside (C3G), an anthocyanin isolated from black rice, against oxidative stress in HepG2 cells. We also investigated its $O_2^{\cdot-}$ scavenging ability in comparison with commercially-available antioxidants. The human hepatoma-derived cell line HepG2 was chosen in this study because these cells have been reported to retain many normal hepatic metabolic functions.

Materials and Methods

Sample and chemicals The black rice used in this study was cultivated in Jin-do, Korea. Human hepatoma cell line HepG2, was purchased from American Type Culture Collection (Rockville, MD, USA). Minimum essential medium (MEM), fetal bovine serum (FBS), and antibiotics were products of Gibco BRL (Grand Island, NY, USA). Sodium salt of (2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]-2H-tetrazolium-5-carboxyanilide inner salt) or XTT, phenazine methosulfate (PMS), xanthine (XA), xanthine oxidase (XOD), gramoxone, and tetrazolium blue were obtained from Sigma Chemical Co. (St. Louis, MO, USA). Authentic anthocyanin monomer C3G was purchased from Extrasynthe (Genay, France). All other chemicals were of analytical reagent-grade.

Isolation of anthocyanin in black rice The major antho-

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cyanin, C3G, was isolated from the black rice as described by Tsuda *et al.* (10) and Ryu *et al.* (11) with some modifications. The black rice (100 g) was extracted with 0.1% HCl in methanol (2 L) overnight. The slurry was filtered and the filtrate was evaporated at 30°C using a rotary evaporator. The acidified methanolic extract was subsequently re-extracted with hexane and ethyl acetate to remove nonpolar impurities and other flavonoids. The extract was finally choromatographed and collected on an HPLC equipped with a diode-array detector and an octadecylsilica (ODS) column (100×2.1 mm). Elution solvents were (A) acidified water (0.6% perchloric acid) and (B) methanol, with a flow rate of 0.3 mL/min. Linear gradient elution was used with percentage ratios of acid/methanol descending from 100/0 (at 0 min) to 0/100 (at 40 min), with detection at 520 nm. The identification of a C3G peak was confirmed by comparison of retention time with standard. The chemical structure of the purified anthocyanin is illustrated in Fig. 1.

Cytotoxicity Cell viability was measured according to the method of Rochem *et al.* (12) with some modifications. Cells were seeded to a culture plate containing 24 wells (5×10^4 cells/well) and grown in MEM plus 10% FBS (v/v) at 37°C under a humidified atmosphere of 5% CO_2-95% air. When cell growth reached to 80-90% confluence, the medium was removed and the cultured cells were washed twice with Hank's balanced salt solution (HBSS). Then, 1 mL of serum-free MEM containing 0, 25, 50, 100, 150, or 200 μg/mL of a tested sample was transferred into each well prior to incubation for 24 hr. For a colorimetric assay of cell viability, 0.25 mL of freshly prepared XTT-PMS solution (1 mg XTT and 10 mg PMS/mL of MEM without phenol red) was added to each well and incubated for an additional 2 hr. After incubation, the culture medium was collected and the absorbance was measured at a wavelength of 450 nm in a spectrophotometer. The cytotoxicity was expressed as percentage of the control, which contains no anthocyanin.

Assay for hepatoprotective activity The cells were grown in 24 wells as described above. At day 5, the growth medium was removed and cells were washed twice with Hank's balanced salt solution (HBSS). The cells were then incubated for 24 hr with 1 mL of the serum-free MEM containing 50 μg/mL of a respective tested sample. To generate free radicals, 2.0 mM gramoxone was added into each well. After another 2 hr of incubation, cytotoxicity was measured as previously stated.

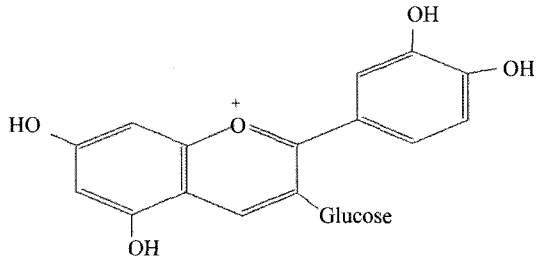

Fig. 1. Chemical structure of cyanidin 3-glucoside.

Assay for superoxide anion radical scavenging activity The O_2^- scavenging activity of the tested sample was determined by a nitroblue tetrazolium (NBT) assay according to Kim *et al.* (13) with some modifications. A 20 uL sample was added to a 980 uL of 0.5 mM XA/NBT mixture and dissolved in 50 mM potassium phosphate buffer (pH 7.4) containing 0.05 mM EDTA. After the XOD (0.07 units) was put into a test tube, the mixture was incubated for 20 min at 37°C. The reaction was terminated by the addition of 0.5 mL of 2 N HCl and the absorbance of NBT was measured at 560 nm. The scavenging activity was calculated as follows;

$$\text{Scavenging activity (\%)} = \{1 - (\text{Abs of sample}/\text{Abs of blank})\} \times 100$$

Statistical analysis Data is presented as mean±SD of three replicates. The data was statistically evaluated using Student's *t*-test or Duncan's Multiple Range test to compare the significant difference between the groups at $p < 0.05$.

Results and Discussion

The hepatoprotectant from black rice was isolated by sequential procedures with acidified methanolic extraction and HPLC. To check the purity and identity of the resulting compound, $^1H/^{13}C$-NMR and electron impact mass spectroscopy were performed (data not shown). The compound isolated from black rice was identified as cyanidin 3-glucoside (14) (Fig. 1). C3G, one of the anthocyanins abundant in colored rice (11), is known to possess diverse biological activities, such as anti-lipid peroxidation (15), anti-inflammation (16), and immune modulation (17).

The effect on cell viability in the presence of acidified methanolic extract or C3G was investigated. As expected, the purified compound exerted a higher degree of cytotoxic potency than the crude extract. Up to concentrations of 100 μg/mL, crude and purified anthocyanins exhibited no cytotoxic effects (Table 1). However, pure C3G isolated from black rice caused a significant decrease (approx. 13 %) in cell viability at 150 μg/mL ($p<0.05$), while no reduction in cell viability occurred with acidified methanolic extract

Table 1. Cytotoxic effects of acidified methanolic extract and cyanidin 3-glucoside from black rice on HepG2 cells

Concentration (μg/mL)	Cell viability (% of control)	
	Acidified methanolic extract	Cyanidin 3-glucoside
0	100	100
25	99.1±3.4[1)]	99.2±1.4
50	99.9±4.0	103.3±2.6
100	103.4±2.6	99.3±5.3
150	99.2±1.7	87.3±3.6*
200	94.4±2.5*[2)]	80.5±1.1*

[1)]Values represent the percent of control and mean±SD in triplicate experiments.
[2)]Values with an asterisk in a column are significantly different from control group at $p<0.05$.

of black rice at the same concentration. At high concentrations (200 μg/mL) of black rice extract, the cell viability was approximately 94%. On the other hand, a relatively high increase in cell cytotoxicity was observed with purified C3G (81% cell viability). Wang and Mazza (16) recently reported that approximately 200 mg/mL of C3G did not influence the viability of RAW 264.7 macrophage cells. These differences could be due to the different types of cell line used. Based on cell viability, a concentration of 50 μg/mL was chosen as a noncytotoxic level to carry out susequent experiments.

Chronic alcohol consumption or overexposure to drugs is associated with the increased production of ROS, which leads to severe liver damage from the imbalance between ROS production and antioxidant defenses. Therefore, oxidative stress is considered to be one of the key mechanisms responsible for liver injury. The antioxidants protect against oxidative stress (18) and may contribute to reducing the degree of hepatocytic damage. In the present study, gramoxone, which undergoes one-electron reduction to produce $O_2^{\cdot-}$ (19), was added into the cultured cells to generate free radicals. As shown in Fig. 2, cell viability decreased to 58% after treatment with 2 mM gramoxone, indicating that HepG2 cells were damaged mostly by $O_2^{\cdot-}$. The acidified methanolic extract of black rice inhibited approximately 49% of the ROS damage in HepG2 cells. The hepatotoxicity towards HepG2 cells by 2 mM gramoxone was completely prevented by C3G, suggesting that the anthocyanin possesses a strong hepatoprotective potency against oxidative stress.

The $O_2^{\cdot-}$ scavenging activity of C3G, the purified compound from black rice was assessed using an NBT reduction system and compared to commercially-available antioxidants of synthetic (buthylated hydroxytoluene) and natural (vitamin C, vitamin E, and epigallocatechin gallate) origins. The $O_2^{\cdot-}$ was reported to reduce the NBT, and to spur the formation of formazan (20). Of these antioxidants, C3G revealed the strongest scavenging ability towards $O_2^{\cdot-}$ with 91% activity at a concentration of 10 μg/mL (Fig. 3), followed by epigallocatechin gallate (79%), vitamin E (66 %), vitamin C (55%), and buthylated hydroxytoluene (31 %). Its scavenging activity was approximately 1.5-fold greater than that of vitamin E, an efficient natural $O_2^{\cdot-}$ scavenger (21). These results suggest that anthocyanin isolated from black rice shows effective antioxidative effects, may receive attention as a new naturally-occurring antioxidant.

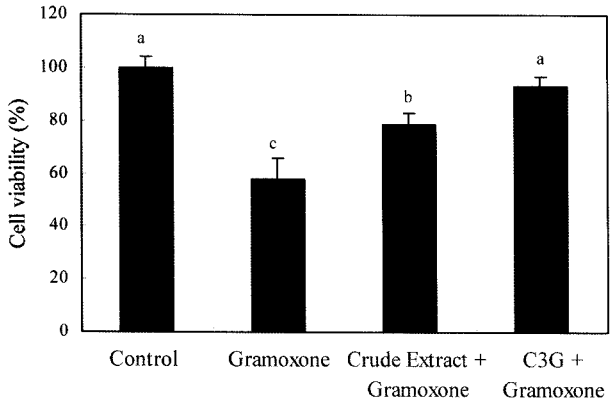

Fig. 2. Hepatoprotective effects of crude extract and Cyanidin 3-glucoside (C3G) from black rice in HepG2 cells. The values are expressed as the means±SD of three replicates. Different letters on the value are statistically different by Duncan's multiple range test ($p<0.05$).

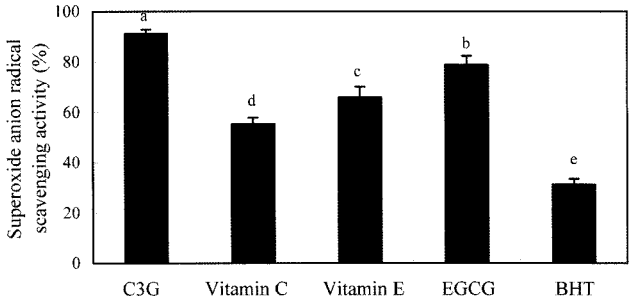

Fig. 3. Scavenging activities of cyanidin 3-glucoside (C3G) from black rice and commercial antioxidants on superoxide anion radicals. BHT: butylated hydroxytoluene, EGCG: epigallocatechin gallate. The values are expressed as the means±SD of three replicates. Different letters on the value are statistically different by Duncan's multiple range test ($p<0.05$).

Increased production of ROS in the liver leads to the deleterious changes in cell function, thereby elevating the risk of irreversible liver damage from oxidative stress (22). The elimination of ROS can successfully protect cells against such damage. Combined with the results in the present investigations, we could confirm that C3G isolated from black rice does possess potential hepatoprotective properties that may be due to $O_2^{\cdot-}$ scavenging. Research is currently underway to elucidate the further mechanism of hepatoprotective action of C3G relevant to the antioxidant system and its *in vivo* effects.

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