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Antioxidant Effects of Cranberry Powder in Lipopolysaccharide Treated Hypercholesterolemic Rats

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ABSTRACT: This study was conducted to investigate the effects of cranberry power on antioxidant defense system in rats fed an atherogenic diet and injected with lipopolysaccharide (LPS). Sprague-Dawley rats were divided into the following 5 groups: normal diet+saline (NS), atherogenic diet+saline (AS), atherogenic diet+LPS (AL), atherogenic diet with 5% cranberry powder+LPS (AL-C5), and atherogenic diet with 10% cranberry powder+LPS (AL-C10). Total antioxidant status measured by ferric reducing ability of plasma (FRAP) was significantly reduced by LPS injection (24%) and was restored by the cranberry powder treatment (P<0.05). In addition, the mean level of plasma total phenolics was significantly decreased by LPS injection (P<0.05) and tended to be increased when cranberry powder was incorporated in to the diet. Activity of serum superoxide dismutase (SOD) tended to be lowered by LPS injection and declined further in cranberry powder fortified groups. Overall results indicate that dietary cranberry powder may provide appropriate antioxidants to counter the diminished antioxidant status induced by exposing hypercholesterolemic rats to LPS.

Keywords: cranberry powder, atherogenic diet, rat, lipopolysaccharide, oxidative stress

INTRODUCTION

Oxidative stress reflects the imbalance between the generation and detoxification of reactive oxygen species (ROS), resulting in excess free radicals. Oxidative stress is thought to be responsible for the oxidative damage observed in various human diseases, including cardiovascular disease, atherosclerosis, and dyslipidemia (1,2). In animal models, atherogenic diets containing high fat and cholesterol have been shown to cause oxidative damage to endogenous tissues (3) and induce hypercholesterolemia (4,5). In a study by Yang et al. (2), oxidative stress was proposed to be an early event in the evolution of hyperlipidemia. In addition to the atherogenic diet, lipopolysaccharide (LPS) injection has been shown to induce not only cardiovascular events, including atherogenesis (6,7) and dyslipidemia, but also oxidative stress. In a study by Kukongviriyapan et al. (8), LPS injection increased biomarkers of lipid and protein oxidation, while cellular redox status was suppressed. Appropriate antioxidant treatment has been suggested as a potential remedy for reducing lipid peroxidation and restoring the body's antioxidant capacity in individuals with a high risk for developing hyperlipidemia.

Nutritional guidelines (9) have suggested that an increase in the consumption of foods rich in antioxidant nutrients may decrease or prevent the risk of many diseases caused by oxidative stress. Fruits and vegetables have a large selection of flavonoids (10), and consuming these foods results in improved total antioxidant capacity (11). Cranberries, in particular, contain a large amount of flavonoids, including quercetin and anthocyanins (12). Quercetin has been shown to alleviate LPS induced oxidative stress in animals (8). Furthermore, the antioxidant effect of cranberry powder has been verified in atherogenic diet induced hypercholesterolemic rats (4). However, while the antioxidant potency of cranberry flavonoids and cranberry products themselves has been well recognized, it is not well known how cranberry products modulate biomarkers of oxidative stress in the hypercholesterolemic condition following LPS exposure. Therefore, we aimed to investigate whether cranberry powder positively alters antioxidant status and some biomarkers of oxidative stress in hypercholesterolemic rats treated with LPS.

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MATERIALS AND METHODS

Animals and diets

Forty male Sprague-Dawley rats with a mean weight of 160±10 g were purchased from Animals Inc. (Central Lab., Seoul, Korea). Rats were housed in cages under temperature- (23±2°C), humidity- (50±10%), and light- (12-h cycle beginning at 07:00) controlled conditions. After a 7 day acclimation period, rats were divided into the following five groups by randomized complete block design: (1) normal diet and saline injected group (NS, n=8), (2) atherogenic diet and saline injected group (AS, n=8), (3) atherogenic diet and LPS (lipopolysacchardies from *Escherichia coli* O26:B6; Sigma Co., St. Louis, MO, USA) injected group (AL, n=8), (4) 5% cranberry fortified atherogenic diet and LPS injected group (AL-C5, n=7), and (5) 10% cranberry fortified atherogenic diet and LPS injected group (AL-C10, n=8). The amounts of cranberry powder in the experimental diets (5 and 10% w/w) would be equivalent to 26 and 51 g in the daily human diet, based on the body surface area normalization method (13). All diets were based on the modified AIN-76 diet (American Institute of Nutrition, 1977) (Table 1). The experimental diets and water were provided *ad libitum*, no limits were placed on caloric intake, and all diets were in a mixed powder form. Cranberry powder was manufactured by Kiantama Oy Co. (Suomussalmi, Finland). All animal experiments were performed based on the guidelines approved by the Animal Ethics Committee of Seoul Women's University.

Blood and tissue preparation

Eighteen hours prior to the postmortem exam, NS and AS groups were injected with a 0.9% physiological saline solution (0.5 mg/kg) and AL, AL-C5, AL-C10 groups were injected with LPS (0.5 mg/kg body weight) into their abdominal cavity. Following a 12-h fast on the last day of the 6-week experimental period, blood was drawn from the saphenous vein of etherized rats. Blood samples were collected in vacuum tubes (Becton Dickinson, Meylan, France) without anticoagulant for biochemical analysis and with heparin for erythrocyte and plasma analyses.

All blood samples were immediately centrifuged at 3,000 rpm (4°C) for 15 min (Sovall ST16R Centrifuge, Thermo Scientific Co., Rockford, IL, USA) to separate serum, plasma, and erythrocytes. Erythrocyte samples were lysed in 4 volumes of ice cold HPLC-grade water, centrifuged (1,000 *g*, 15 min, 4°C), and the supernatant (erythrocyte lystate) was collected for later analysis. The liver was rinsed with physiological saline after blood collection, exterior moisture was removed, weight was measured, and the sample was frozen in liquid nitrogen for storage until analysis.

Sample collection and preparation

Rat liver samples were selected from each lobe of the liver, and the tissues were homogenized with buffer [0.3 mM sucrose, 2 mM ethylenediaminetetraacetic acid (EDTA), 10% glycerol, and 20 mM N-(2-hydroxyethyl)piperazine-N'-2-ethane sulfonic acid (HEPES), pH 7.5]. A portion of each homogenate was centrifuged at 1,000 *g* for 15 min and the pellets were discarded. The supernatant was re-centrifuged at 100,000 *g* for an hour (Optima™ LE-80K Ultracentrifuge, Beckman Coulter Inc., Pasadena, CA, USA). This supernatant (cytosolic fraction) was used to measure the activities of catalase, glutathione reductase (GSH-R), and glutathione peroxidase (GSH-Px). All

Table 1. Composition of experimental diets (%)

Ingredient	Group[1]				
	NS	AS	AL	AL-C5	AL-C10
Casein	20	20	20	20	20
DL-methionine	0.3	0.3	0.3	0.3	0.3
Corn starch	29	17.5	17.5	12.5	7.5
Sucrose	40	40	40	40	40
Cellulose	1	1	1	1	1
Corn oil	5	5	5	5	5
Lard	0	10	10	10	10
AIN-mineral mix[2]	3.5	3.5	3.5	3.5	3.5
AIN-vitamin mix[3]	1	1	1	1	1
Choline chloride	0.2	0.2	0.2	0.2	0.2
Cholesterol	–	1	1	1	1
Sodium cholate	–	0.5	0.5	0.5	0.5
Cranberry powder[4]	–	–	–	5	10

[1]NS, normal diet+saline; AS, atherogenic diet+saline; AL, atherogenic diet+LPS; AL-C5, atherogenic diet with 5% cranberry powder+LPS; AL-C10, atherogenic diet with 10% cranberry powder+LPS.
[2]Composition of AIN-76 salt mixture.
[3]Composition of AIN-76 vitamin mixture.
[4]Manufacturer reported that anthocyanin and proanthocyanidin contents in cranberry powder were 120 mg/100 g and 2,600 mg/100 g, respectively.

experiments were performed at a temperature of 4°C, and samples were stored at −80°C (14).

Ferric reducing ability of plasma

Plasma antioxidant status was evaluated with the ferric reducing ability of plasma (FRAP) assay (15). Briefly, the ferric-tripyridyltriazine (Fe(III)-TPTZ) complex was reduced to ferrous tripyridyltriazine (Fe(II)-TPTZ) at a low pH, resulting in a color change that was measured by absorbance at 550 nm. The reaction varied depending on the sample. Two-hundred-seventy microliters of pre-warmed (37°C) freshly prepared FRAP reagent (25 mL of 300 mM sodium acetate buffer pH 3.6, 2.5 mL of 10 mM 2,4,6-tri(2-pyridyl)-*s*-triazine (TPTZ) in 40 mM HCl, and 2.5 mL of diluent). Absorbance at 550 nm peaked after 15 min incubation at 37°C. FRAP values were measured with a plate reader (Spectra Max 250, Molecular Devices, Sunnyvale, CA, USA) by detection of the absorbance change of the test sample due to the reducing power of antioxidants present in the plasma. Sample absorbance change was compared with the absorbance change of Trolox standards.

Total phenolics and total flavonoids assay

Total soluble phenolic compounds were determined in plasma according to the method described by Singleton and Rossi (16). Using gallic acid as a standard, total soluble phenolic compounds were determined in plasma with Folin-Ciocalteu reagent. A mixture containing 50 μL plasma and 865 μL distilled water was incubated with 75 μL Folin-Ciocalteu reagent at room temperature for 5 min. Two-hundred-twenty-five microliters of 20% Na_2CO_3 and 285 μL of distilled water were added to the reaction mixture and incubated at room temperature for 2 hours, followed by the measurement of absorbance at 760 nm. The total phenolics content is expressed as gallic acid equivalents (GAE).

In order to determine total flavonoids, 500 μL of sample was diluted with distilled water, mixed with 75 μL of 5% $NaNO_2$ solution, and incubated at room temperature for 5 min. This mixture was added to 150 μL of 10% $AlCl_3$ and allowed to stand for 5 additional min before 0.5 mL of 1 M NaOH was added. The absorbance of the solution was measured at 510 nm. The results are presented as catechin equivalents (17).

Serum and erythrocyte lysate superoxide dismutase activity

Superoxide dismutase (SOD) activities of serum and erythrocytes were evaluated using an enzyme linked immunosorbent assay (ELISA). Absorbance was measured at 440 nm. The assay method was based on the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme required to inhibit dismutation of 50% of the superoxide radicals. Commercially available kits (Superoxide Dismutase Assay Kit, Cayman Chemical Co., Ann Arbor, MI, USA) were used.

Cytosolic glutathione peroxidase, glutathione reductase, and catalase activities

Cytosolic GSH-Px activity was measured by the Paglia and Valentine method (18). A molar extinction coefficient of 6.22 $mM^{-1}cm^{-1}$ was used, and the activity was expressed as nmol oxidized NADPH/min/mg protein. This assay was performed using a commercially available kit (Glutathione Peroxidase Assay Kit, Cayman Chemical Co.).

GSH-R activity was determined by measuring the rate of NADPH oxidation and the formation of NADP. Change in absorbance was measured at 340 nm. Commercially available kits (Glutathione Peroxidase Assay Kit, Cayman Chemical Co.) were also used for this assay.

A commercially available Catalase Assay Kit (Cayman Chemical Co.) was used to measure catalase activity in cytosol. The method is based on the reaction of the catalase with methanol in the presence of an optimal concentration of H_2O_2.

Protein concentration was determined by the method of Lowry with bovine serum albumin as the standard (19).

Serum thiobarbituric acid reactive substances levels

Serum concentrations of thiobarbituric acid reactive substances (TBARS) were measured as a marker of lipid peroxidation using a TBARS Assay Kit (ZeptoMetrix Co., Buffalo, NY, USA).

Statistical analysis

SAS software (version 9.1, SAS Inc., Cary, NC, USA) was used to analyze data by one-way analysis of variance and Duncan's multiple range tests. Values are presented as the mean±standard deviation. Significance was set at $P<0.05$ unless otherwise stated.

RESULTS

Serum cholesterol

The mean total cholesterol of groups fed the atherogenic diet was between 252 mg/dL and 331 mg/dL. These values were about 156% to 236% higher than that of the group fed a normal diet.

Plasma FRAP, total phenolics and flavonoids levels

Plasma FRAP, total phenolics, and flavonoid levels were determined to examine the effects of cranberry powder on antioxidant status. Mean plasma FRAP levels were

not significantly altered by the atherogenic diet, but were significantly decreased when LPS was injected into rats consuming the atherogenic diet. Furthermore, the lowered FRAP values were restored when the diet was fortified with cranberry powder ($P<0.05$) (Table 2).

As compared to the atherogenic diet group, LPS injection lowered plasma total phenolics ($P<0.05$). These values were not fully restored but tended to be elevated with cranberry powder fortification of the diet ($P>0.05$). A similar pattern was observed for total plasma flavonoid concentrations, although they were not significantly different among groups.

Antioxidant enzyme activities and serum TBARS

Mean serum SOD activity tended to be lower in LPS injected groups, and a further decline was observed in groups fed cranberry powder (Table 3). There were no significant differences in cytosolic GSH-Px and GSH-R activities among groups. As compared to the NS group, the mean level of cytosolic catalase activity was 21% lower in the group that was fed an atherogenic diet and received a saline injection ($P>0.05$) and 50% lower in the group that was fed an atherogenic diet and received an LPS injection ($P<0.05$). In addition, cytosolic catalase activity tended to be even lower in the group fed an atherogenic diet fortified with 5% cranberry powder (Table 4). There were no differences in serum TBARS concentrations among experimental groups (Table 5).

Table 2. Effects of cranberry powder on plasma FRAP, total phenolics, and total flavonoid levels in rats[1)]

Group[2)] (n)	FRAP[3)] (μM)	Total phenolic level (mg gallic acid equivalents)	Total flavonoid level (mg catechin equivalents)
NS (8)	314.00±64.21[a]	6.96±0.55[b]	19.61±11.05[ns4)]
AS (8)	318.43±77.60[a]	8.22±1.46[a]	27.27±13.68
AL (7)	242.25±47.34[b]	7.06±0.18[b]	22.56±17.94
AL-C5 (7)	319.87±58.23[a]	7.55±0.98[ab]	28.53±13.59
AL-C10 (8)	315.65±59.04[a]	7.33±0.79[ab]	23.88±13.74

[1)]Values are mean±SD. Means with different superscripts are significantly different at $P<0.05$ by Duncan's multiple range test.
[2)]NS, normal diet+saline AS, atherogenic diet+saline AL, atherogenic diet+LPS AL-C5, atherogenic diet with 5% cranberry powder+LPS AL-C10, atherogenic diet with 10% cranberry powder+LPS.
[3)]FRAP, the ferric reducing ability of plasma.
[4)]ns, Not significant.

Table 3. Effects of cranberry powder on serum and erythrocyte SOD activity in rats[1)]

Group[2)] (n)	SOD[3)] activity in serum (U/mL)	SOD activity in erythrocytes (U/mL)
NS (8)	23.52±4.22[a]	1319.31±210.48[ns4)]
AS (8)	22.79±5.36[a]	1184.13±189.05
AL (7)	18.45±7.25[ab]	1052.59±266.98
AL-C5 (7)	14.17±2.86[b]	1031.57±219.51
AL-C10 (8)	14.62±4.19[b]	1110.69±361.94

[1)]Values are mean±SD. Means with different superscripts are significantly different at $P<0.05$ by Duncan's multiple range test.
[2)]NS, normal diet+saline; AS, atherogenic diet+saline; AL, atherogenic diet+LPS; AL-C5, atherogenic diet with 5% cranberry powder+LPS; AL-C10, atherogenic diet with 10% cranberry powder+LPS.
[3)]SOD, superoxide dismutase.
[4)]ns, Not significant.

Table 4. Effects of cranberry powder on cytosolic GSH-Px, GSH-R and catalase activities in rats[1)]

Group[2)] (n)	GSH-Px[3)] (nmol NADPH/min/mg protein)	GSH-R[4)] (nmol NADPH/min/mg protein)	Catalase (nmol/mg protein/min)
NS (8)	5.05±1.76[ns5)]	2.82±1.02[ns]	321.93±148.78[a]
AS (8)	5.00±2.48	3.96±3.62	253.63±88.07[ab]
AL (8)	4.26±1.78	3.01±1.10	162.17±78.77[bc]
AL-C5 (7)	3.85±1.78	2.80±1.63	114.51±110.30[c]
AL-C10 (8)	3.19±1.11	2.72±1.60	181.63±100.10[bc]

[1)]Values are mean±SD. Means with different superscripts are significantly different at $P<0.05$ by Duncan's multiple range test.
[2)]NS, normal diet+saline; AS, atherogenic diet+saline; AL, atherogenic diet+LPS; AL-C5, atherogenic diet with 5% cranberry powder+LPS; AL-C10, atherogenic diet with 10% cranberry powder+LPS.
[3)]GSH-Px, glutathione peroxidase.
[4)]GSH-R, glutathione reductase.
[5)]ns, Not significant.

Table 5. Effects of cranberry powder on serum TBARS content in rats[1)]

Group[2)] (n)	TBARS[3)] (nmol)
NS (8)	15.47±3.00[ns4)]
AS (8)	20.56±6.46
AL (7)	21.44±9.02
AL-C5 (7)	19.46±3.62
AL-C10 (8)	18.34±4.90

[1)]Values are mean±SD. Means with different superscripts are significantly different at $P<0.05$ by Duncan's multiple range test.
[2)]NS, normal diet+saline; AS, atherogenic diet+saline; AL, atherogenic diet+LPS; AL-C5, atherogenic diet with 5% cranberry powder+LPS; AL-C10, atherogenic diet with 10% cranberry powder+LPS.
[3)]TBARS, thiobarbituric acid reactive substances.
[4)]ns, Not significant.

DISCUSSION

This study examined the antioxidant effects of cranberry powder in atherogenic diet induced hypercholesterolemic rats that had been treated with LPS. Cranberries contain abundant amounts of antioxidants, such as phenolics and flavonoids (20), and consumption of cranberry juice has been reported to be effective against oxidative stress related diseases, including cardiovascular disease (21). Cranberry juice phenolics are also known to contain antioxidants that can increase antioxidant capacity and protect LDL from oxidation (22).

In the current study, total antioxidant status measured by plasma FRAP decreased with LPS injection. LPS plays a potential role as a pro-inflammatory mediator, initiates numerous host-mediated destructive processes (23), and increases oxidative stress (8). Hsu and Liu (24) reported that LPS stimulates lipid peroxidation, and Yamada et al. (25) reported that LPS induces the generation of reactive oxygen species (ROS). In addition, glutathione redox status has been shown to be impaired by LPS (8). These previous findings support the thought that LPS injection may stimulate the production of ROS, in turn stimulating the utilization of antioxidants to scavenge the excess ROS. This results in a lower total antioxidant capacity, as measured by FRAP. In this study, cranberry powder treatment restored the LPS injection-induced lowering of FRAP to a level close that of the group that did not receive an LPS injection. In a study by Pedersen et al. (26), plasma antioxidant capacity was increased with cranberry juice intake, possibly because of the high concentrations of phenolics and vitamin C in cranberries. Cranberry powder is a rich source of bioactive components, such as flavonoids and phenolics, which are known to have highly effective antioxidant properties (27). In the current study, the levels of circulating total phenolics tended to be increased by cranberry treatments, but the magnitudes of these changes were small and did not meet statistical significance. Despite the relatively high amount of cranberry fortification, the observation of only a small increase in circulating phenolic compounds might be explained, in part, by a rapid metabolic rate (28). In addition, circulating phenolics and flavonoids are thought to be used by the body's antioxidant defense mechanism against excess ROS in situations of high oxidative stress (29).

The detoxification of ROS in biological systems is accomplished by enzymatic and non-enzymatic reactions. In enzymatic reactions, SOD converts superoxide anions to oxygen and hydrogen peroxide (H_2O_2). H_2O_2 can be rapidly degraded by catalase and GSH-Px in the liver (30). The activities of ROS scavenging enzymes (e.g., SOD, GSH-Px, GSH-R, and catalase) have been shown to be altered in various conditions, including during the consumption of a high fat diet and following exposure to endotoxin (31,32). In the current study, cytosolic catalase and serum SOD activities tended to be decreased by an atherogenic diet and LPS treatments, whereas the activities of other enzymes were not noticeably changed. Among the tested enzymes, only cytosolic catalase activity showed a considerable, but insignificant, reduction (21%) following 6 weeks of a high fat diet. In a study by Martinello et al. (33), SOD, catalase, and GSH-Px activities were reduced in the serum and livers of hamsters fed an atherogenic diet for 10 weeks. However, as shown in the current study, Rocha et al. (34) reported that hepatic GSH-Px and GSH-R activities of Wistar rats were not significantly altered by 45 days of a high fat diet, suggesting that the liver may regulate and maintain homeostasis during short-term dietary changes. LPS has also been shown to decrease the activities of antioxidant enzymes such as SOD and catalase in animals (32). In a study by Yoshikawa et al. (35), a significant reduction of tissue SOD was observed following LPS administration. Wang et al. (36) also observed that renal SOD mRNA and protein expression were decreased by LPS treatment in mice. Furthermore, Watson et al. (32) reported a decrease in hepatic total SOD and catalase activities when rats were given LPS injections, providing further support for the thought that LPS exposure induces changes in the host's response to ROS. Similarly, in the current study, LPS injection resulted in a 36% and a 19% reduction of cytosolic catalase and serum SOD activities, respectively. In addition, when LPS was administered to rats fed an atherogenic diet, there was a further reduction in cytosolic catalase activity. These results suggest that the burden of ROS may increase when those with hypercholesterolemia are exposed to endotoxin, resulting increased oxidative stress. Interestingly, there was a tendency for a further decline in serum SOD activity when cranberry powder was administered. Previous studies also report a decrease in the activities of anti-

oxidant enzymes such as SOD, catalase, and GSH-Px following consumption of flavonoid and phenolic rich diets (37,38). In addition, dietary flavonoids like quercetin and kaempferol are reported to reduce oxidative stress by lowering the production of ROS and decrease protein and mRNA expressions of SOD and GSH-Px (39). Therefore, it is assumed that flavonoid-rich cranberry powder might provide antioxidants that suppress production of oxygen radicals, leading to a decrease in the activities of antioxidant enzymes such as SOD.

Increased oxidative stress has been shown to elevate lipid peroxidation. TBARS levels, a biomarker of lipid peroxidation, are reported to be increased not only by atherogenic diet (40), but also following an LPS challenge (41). In a study by Kaur et al. (41), the LPS-induced increase in TBARS was reduced when animals were treated with the flavonoid hesperidin, which is mainly found in citrus fruits. Regrettably, in the present study, no significant changes in TBARS were observed, although there was a noteworthy trend for TBARS to be reduced.

Overall results suggested that LPS may increase oxidative stress by lowering antioxidant status, but cranberry powder provides antioxidants that may help prevent LPS-induced oxidative stress in hypercholesterolemia. However, the levels of cranberry powder incorporated in to the diet in the current study were relatively high for daily human consumption. Therefore, further studies are needed to evaluate an effective and acceptable dose for human diets to achieve the antioxidant benefits of cranberry powder.

AUTHOR DISCLOSURE STATEMENT

The authors declare no conflict of interest.

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