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Effect of Phytochemicals on the Antioxidative Activity of Brain Lipids in High- and Low-fat-fed Mice and Their Structural Changes during *in vitro* Digestion

Seung Jae Lee[1], Seung Yuan Lee[1], Myung-Sub Chung[2], Sun Jin Hur[1,*]

[1]Department of Animal Science and Technology, Chung-Ang University, Seodong-daero, Daeduk-myeon, Anseong-Si, Gyeonggi, Korea
[2]Department of Food Science and Technology Chung-Ang University, Seodong-daero, Daedeok-myeon, Anseong-si, Gyeonggi, Korea
*Corresponding author: hursj@cau.ac.kr

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Abstract The brain lipid samples were collected from the brains of low- and high-fat-fed mice and incubated with the *in vitro*-digested phytochemicals to determine lipid oxidation. After digestion in the mouth, the 2,2′-azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) radical-scavenging activity and ferric-reducing antioxidant power (FRAP) of quercetin and catechin were higher than those of rutin. In contrast, ABTS radical-scavenging activity and FRAP were higher in catechin and rutin than in quercetin after digestion in the stomach. The automated oxygen radical absorbance capacity (ORAC) was highest in catechin during *in vitro* digestion in the brain lipids of both high- and low-fat-fed mice. After digestion in the mouth, the inhibitory effect of rutin lipid oxidation was higher than those of quercetin and catechin, whereas after digestion in the stomach, the inhibitory effect of lipid oxidation in catechin and rutin was stronger than that of quercetin in brain lipids obtained from both low- and high-fat-fed mice.

Keywords: *phytochemicals, antioxidant activity, mouse brain lipid, in vitro digestion*

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1. Introduction

Several studies [1,2,3] have examined the effect of various phytochemicals on antioxidant activity. Therefore, the ingestion of natural phytochemicals may have important applications in the future as natural anti-aging or antioxidant agents, with significant implications for the food industry. [4] Dietary flavonoids with a catechol group were shown to inhibit the oxidation of biomolecules by acting as free radical scavengers via donation of hydrogen atoms or electrons, binding proteins and enzymes involved in the generation of reactive oxygen species. [5,6] Quercetin is the most common flavonol aglycone, and is particularly abundant in onion, which is one of the tasteful members of the genus *Allium* and a major source of antioxidants, [6] and rutin possesses several pharmacological activities, including antioxidant activity, and is widely used in treating diseases. [7] Flavonoid glycosides from the diet are believed to pass through the small intestine and then enter the cecum and colon, where they are hydrolyzed to aglycone by enterobacteria. [8] Flavonoid aglycone can be easily absorbed into epithelial cells in the large intestine because its lipophilicity facilitates its passage across the phospholipid bilayer of cellular membranes. [9] Consequently, consumption of large amounts of phytochemicals is considered to have significant nutritional or medicinal benefits. However, the effect of phytochemicals on the antioxidant activity of lipids in high-fat- and low-fat-fed mouse brain has not been studied during *in vitro* digestion, and the effects of *in vitro* digestion on changes in molecular structure and antioxidant activity have not been extensively studied. Therefore, the purpose of this study was to determine the effect of the phytochemicals quercetin, catechin, and rutin on the antioxidative activity of lipids in high and low-fat-fed mouse brain and the structural changes on these phytochemicals during *in vitro* digestion.

2. Materials and Methods

2.1. Animal and Experimental Diet

Balb/C mice (4 months old, average body weight, 50 ± 5 g) and semi-purified powdered diet ingredients were obtained from Orient Bio (Seongnam, Korea). Animals were housed in wire-bottomed individual cages in a windowless room on a 12-h light/dark cycle, under a protocol approved by the Animal Care Committee of Chung-Ang University. After 1-week adaptation, twenty female mice were fed a low-fat (10%), and twenty, a high-fat (50%) experimental diet for 10 weeks. Fresh diet was supplied at days 0, 2, and 5. Diet and water were provided

ad libitum throughout the experiment. The formulation of the experimental diets and treatment groups are listed in Table 1.

Table 1. Formulation of the experimental diet

Items (g/kg)	Treatments	
	High-fat	Low-fat
Casein	229	329
L-cystine	3	3
Sucrose	100	100
Corn Starch	128.96	228.96
Maltodextrin	132	132
Cellulose	50	50
Soybean Oil	300	100
Mineral Mix	42	42
Vitamin Mix	12	12
Choline Bitartrate	3	3
TBHQ (antioxidant)	0.04	0.04
Total	1000	1000

2.2. In Vitro Digestion Model

We used a midified version *in vitro* digestion model of that described in previous studies: [10,11]

1. *Pre-Ingestion*: The phytochemicals (quercetin, catechin and rutin).
2. *Mouth*: About 0.1 g of phytochemicals were mixed with 6 mL of simulated saliva fluid of pig (pH 6.8) and then stirred for 5 min at 37°C.
3. *Stomach*: About 12 mL of simulated gastric fluid (pH 2) was added, and then the mixture was stirred for 2 hr at 37°C.
4. *Small Intestine*: About 12 mL of duodenal juice, 6 mL of bile juice and 2 mL of bicarbonate solution (pH 6.5~7) were added, and the mixture was then stirred for 2 hr at 37°C.

The *in vitro* digested sample was then used for analysis of lipid oxidation. The compositions of the simulated saliva, gastric, duodenal, and bile fluids are listed in Table 2. During the *in vitro* digestion model, the samples were swirled (60 rpm) on a shaking water bath to simulate the motility of the gastrointestinal tract (Model HB-205SW, Hanbaek, Co., Bucheon, Korea).

Table 2. Constituents and concentrations of the various synthetic juices of the simulated human gastrointestinal digestion model representing feeding conditions

	Saliva	Gastric juice	Duodenal juice	Bile juice
Inorganic components	10ml KCl 89.6g/L	15.7ml NaCl 175.3g/ L	40ml NaCl 175.3g/ L	30ml NaCl 175.3g/ L
	10ml KSCN 20g/ L	3.0ml NaH_2PO_4 88.8g/ L	40ml $NaHCO_3$ 84.7g/ L	68.3ml $NaHCO_3$ 84.7g/ L
	10ml NaH_2PO_4 88.8g/ L	9.2ml KCl 89.6g/ L	10ml KH_2PO_4 8g/ L	4.2ml KCl 89.6g/ L
	10ml $NaSO_4$ 57g/ L	18ml $CaCl_2{\cdot}2H_2O$ 22.2g/ L	6.3ml KCl 89.6g/ L	150μl HCl 37g/ L
	1.7ml NaCl 175.3g/ L	10ml NH_4Cl 30.6g/ L	10ml $MgCl_2$ 5g/ L	
	20ml $NaHCO_3$ 84.7g/ L	6.5ml HCl 37g/ L	180μl HCl 37g/ L	
Organic components	8ml urea 25g/ L	10ml glucose 65g/ L	4ml urea 25g/ L	10ml urea 25g/ L
		10ml glucuronic acid 2g/ L		
		3.4ml urea 25g/ L		
		10ml glucosamine hydrochloride 33g/ L		
Add to mixture of organic+inorganic components	290mg α-amylase	1g BSA	9ml $CaCl_2{\cdot}2H_2O$ 22.2g/ L	10ml $CaCl_2{\cdot}2H_2O$ 22.2g/ L
	15mg uric acid	2.5g pepsin	1g BSA	1.8g BSA
	25mg mucin	3g mucin	9g pancreatin	30g bile
			1.5g lipase	
pH	6.8±0.2	1.30±0.02	8.1±0.2	8.2±0.2

The inorganic and organic solutions were augmented to 500 mL with distilled water. If necessary, the pH of the juices was adjusted to the appropriate interval.

2.3. Phytochemical Materials

Quercetin, catechin, and rutin were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA, USA).

2.4. ABTS Radical-scavenging Activity

The 2,2′ -Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) assay developed in a previous study was used. [12] ABTS radical cation ($ABTS^{\cdot}$) was produced by the 7 mM ABTS stock solution with 2.45 mM potassium persulfate ($K_2S_2O_8$) and allowing the mixture to stand in the dark at R.T. for 14 h before use. To determine the scavenging activity, 0.9 mL of ABTS reagent was mixed with 0.1 mL of extracts and the absorbance was measured at 734 nm after 6 min of reaction at R.T. [13] This activity is given as the percentage ABTS scavenging according to the following formula:

$$\%\ \text{ABTS scavenging activity} = \left[\left(\begin{array}{l}\text{control absorbance}\\ -\ \text{sample absorbance}\end{array}\right)/\left(\text{control absorbance}\right)\right]\times 100$$

2.5. Ferric-reducing Antioxidant Power (FRAP) Assay

The FRAP assay, developed in a previous study, was used. [14] To conduct the assay, a 3 mL aliquot of a FRAP reagent, a mixture of 0.3 M acetate buffer,10 mM 2,4,6-tripyridyl-S-triazine (TPTZ) in 40 mM HCl, and 20 mM ferric chloride (10:1:1 v/v/v), were combined with 50 μL of test samples and standards. This was vortex-mixed,

and the absorbance at 593 nm was read against a reagent blank at a predetermined time after sample-reagent mixing. The test was performed at 37°C and a reaction time window of 0-4 min was used.

2.6. Oxygen RADICAL-absorbance Capacity

The automated oxygen radical-absorbance capacity (ORAC) assay was performed on a spectrofluorometric centrifugal analyzer (Roche Diagnostic System Inc., Branchburg, NJ). The ORAC assay was conducted by a previously described method. [15] The collected brains were homogenized in ice-cold phosphate buffer (pH 7.00). The homogenate was centrifuged at 12,000 × *g* for 15 min at 4°C. Trolox, a water-soluble analogue of vitamin E, was used as a control standard. The experiment was conducted at 37°C under pH 7.4, with a blank sample in parallel. The analyzer was programmed to record the fluorescence of fluorescein disodium every minute after the addition of dihydrochloride and test samples. All fluorescent measurements are expressed relative to the initial reading. The final results were calculated using the differences of areas under the fluorescein disodium decay curves between the blank and a sample and were expressed as micromole Trolox equivalents (TE) per gram (μmol TE/g).

2.7. Lipid Oxidation in Mouse Brain Lipid

Twenty Balb/C mice were used to analyze the lipid oxidation. The mice were sacrificed by CO_2 gas, their skulls were opened, and the brains were collected. The lipid oxidation value was conducted by a previously described method. [16] The collected brains were homogenized in ice-cold Tris-hydrochloric acid buffer (20 mM, pH 7.4). The homogenate was centrifuged at 12,000 × *g* for 15 min at 4°C. Aliquots of 0.1 mL of the supernatant were incubated with the *in vitro* digested phytochemical samples (0.2 g) in the presence of 10 μM ferrous sulfate (0.1 mL) and 0.1 mM vitamin C (0.1 mL) at 37°C for 1 hr. The reaction was terminated by adding 0.1 mL of trichloroacetic acid (28%, w/v) and 0.3 mL of thiobarbituric acid (1%, w/v) in succession. The solution was then heated to 100°C. After 15 min, the color of MDA-thiobarbituric acid complex was measured at 532 nm. (+)-Catechin, a well-known antioxidant, was used as a positive control. The inhibition ratio (%) was calculated as follows: % inhibition = [(control absorbance – sample absorbance) / control absorbance] × 100.

2.8. Quantification of Phenolics by High-Performance Liquid Chromatography

Quercetin, catechin, and rutin contents were measured by using a diode array ultraviolet-visible detector (Agilent 1100 series, Agilent Co., Santa Clara, CA, USA). Separation was achieved with a Shiseido C18 column (250 mm × 4.6 mm id, 5 μm, Shiseido Co., Tokyo, Japan). The elution solvents were (A) 0.01 M-potassium phosphate buffer adjusted to pH 3.0 with phosphoric acid and (B) methanol. The following solvent gradient elution program was used: initial 90% (A), hold for 9.5 min; linear gradient to 68% (A) in 3.5 min; linear gradient to 67% (A) 17 min; linear gradient to 20% (A) 1 min; linear gradient to 90% (A) 1 min, and hold for 10 min. The flow rate was 1.5 mL/min. Quercetin, catechin, and rutin were identified by comparing their retention time values and ultraviolet spectra with those of known standards, and quantified based on the peak areas obtained in the chromatograms. All analyses were run in quintuplicate, and mean values were calculated. The contents of quercetin, catechin, and rutin were expressed in mg/g extract.

2.9. Statistical Analysis

The data were analyzed using the SAS software (SAS Inst. Inc., Cary, NC 2001) for the generalized linear model procedure. The Student-Newman-Keuls multiple range test was used to compare differences among means. Significance was defined at $P < 0.05$.

3. Results and Discussion

3.1. Antioxidant Activity of Phytochemicals

The antioxidant activities of the phytochemicals during *in vitro* digestion are presented in Figure 1 and Figure 2. After digestion in the mouth, the ABTS radical-scavenging activity and FRAP of quercetin and catechin were greater than those of rutin, whereas, after digestion in the stomach, ABTS radical-scavenging activity and FRAP were higher in catechin and rutin than quercetin.

3.2. ORAC and Inhibitory Effect of Lipid Oxidation in Phytochemicals in Mouse Brain Lipids

The ORAC of phytochemicals during *in vitro* digestion in brain lipids obtained from low- and high-fat-feed mice is presented in Figure 3. The ORAC of all phytochemicals during *in vitro* digestion was higher in the brains of high-fat-fed mice than in those of low-fat-fed mice. In particular, the ORAC during *in vitro* digestion was highest in catechin in the brains of both high-fat and low-fat-fed mice. The ORAC of quercetin decreased after digestion in the small intestine, whereas that of rutin increased.

The inhibitory effect of lipid oxidation of phytochemicals during *in vitro* digestion in brain lipids obtained from low-fat- and high-fat-fed mice is presented in Figure 4. The inhibitory effect of lipid oxidation of phytochemicals was higher in high-fat-fed mouse brains during *in vitro* digestion. In the brain lipids obtained from both low- and high-fat-fed mice, the inhibitory effect of lipid oxidation of quercetin and catechin was higher than that of rutin after digestion in the mouth, whereas it was higher in catechin and rutin than in quercetin after digestion in the stomach.

We simulated the mouth, stomach, and small intestine digestion of phytochemicals to determine the antioxidant activity and stability of phytochemicals under conditions of *in vitro* digestion. The study results revealed that *in vitro* digestion influenced the antioxidant activities of phytochemicals. Quercetin decreased antioxidant activity, whereas rutin increased antioxidant activity after digestion in the small intestine. Unlike quercetin and rutin, catechin had less influence on antioxidant activity by *in vitro* digestion. These findings indicate that the antioxidant activities of various phytochemicals can vary in response

to digestion. In this study, the antioxidative activities of quercetin decreased after *in vitro* digestion mimicking conditions in the human small intestine, possibly because quercetin was degraded by *in vitro* digestion, while the antioxidative activity of rutin increased after *in vitro* digestion in the small intestine. Considering these results, we assume that increased quercetin degradation by *in vitro* digestion was one of the primary reasons for the observed decrease in antioxidant activity. On the contrary, the increased antioxidative activity of rutin by *in vitro* digestion may be because rutin was changed into its aglycone form, such as quercetin. Quercetin is the aglycone form of rutin, and quercetin aglycone appears to be a more active chain-breaking antioxidant than its glycoside counterparts (e.g., rutin) because of its higher accessibility to the site of chain-initiating and chain-propagating free radicals in membranous phospholipid bilayers [17].

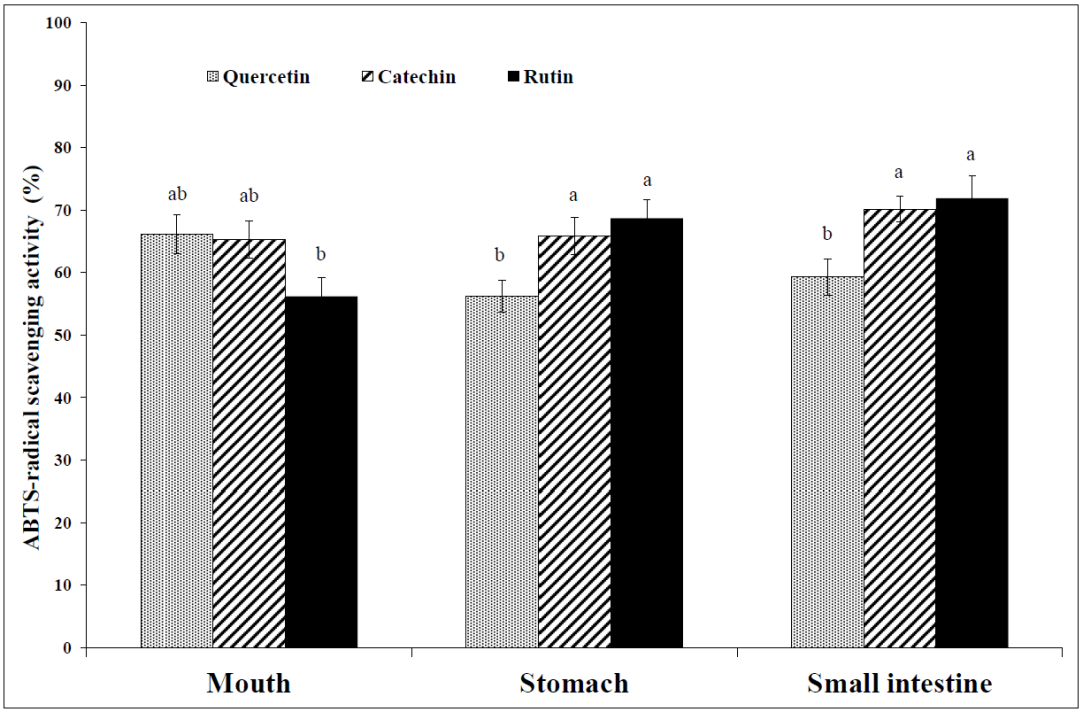

Figure 1. ABTS radical-scavenging activity of phytochemicals as they passed through an *in vitro* human digestion model

Mouth step: saliva juice after 5 min; Stomach step: gastric juice after 2 hr; Small intestine step: duodenal juice and bile juice after 2 hr. Results are presented as the mean ± SD of three independent experiments conducted in triplicate.

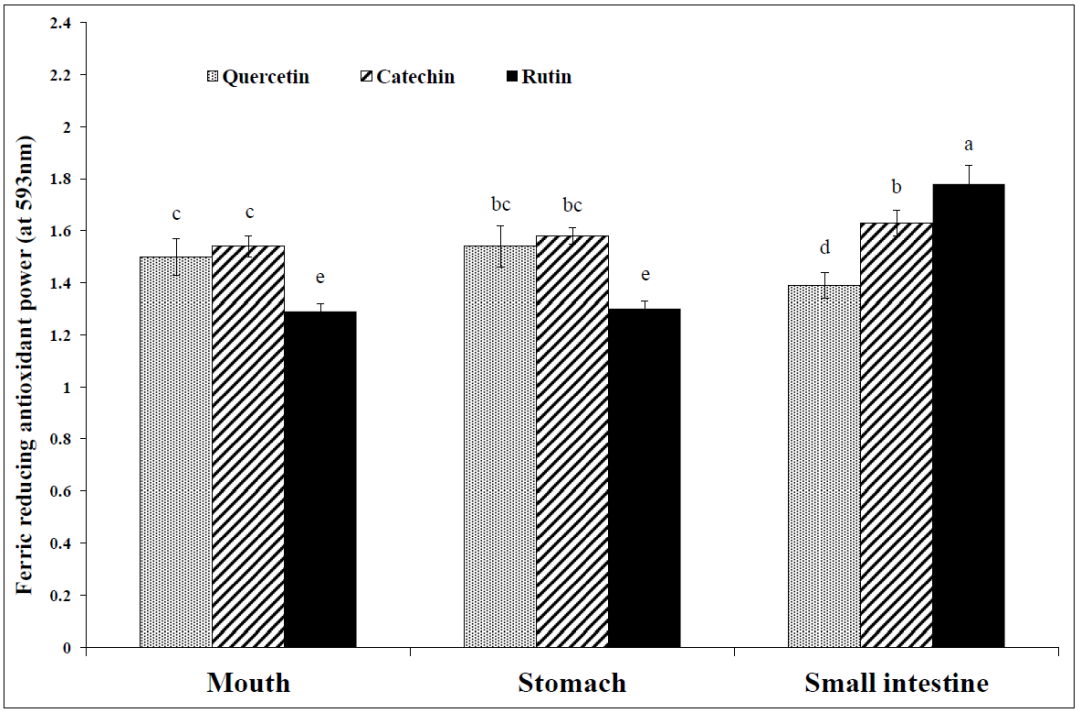

Figure 2. Ferric-reducing antioxidant power of phytochemicals as they passed through an *in vitro* human digestion model

Mouth step: saliva juice after 5 min; Stomach step: gastric juice after 2 hr; Small intestine step: duodenal juice and bile juice after 2 hr. Results are presented as the mean ± SD of three independent experiments conducted in triplicate.

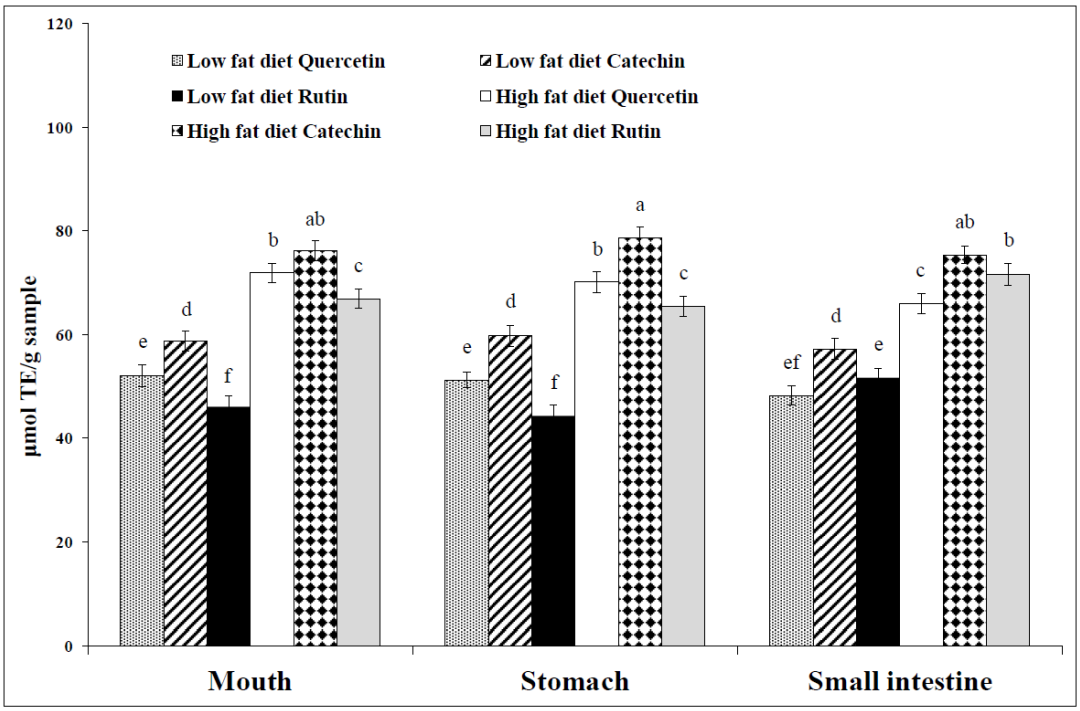

Figure 3. Oxygen radical-absorbance capacity of phytochemicals as they passed through an *in vitro* human digestion model

Mouth step: saliva juice after 5 min; Stomach step: gastric juice after 2 hr; Small intestine step: duodenal juice and bile juice after 2 hr. Results are presented as the mean ± SD of three independent experiments conducted in triplicate.

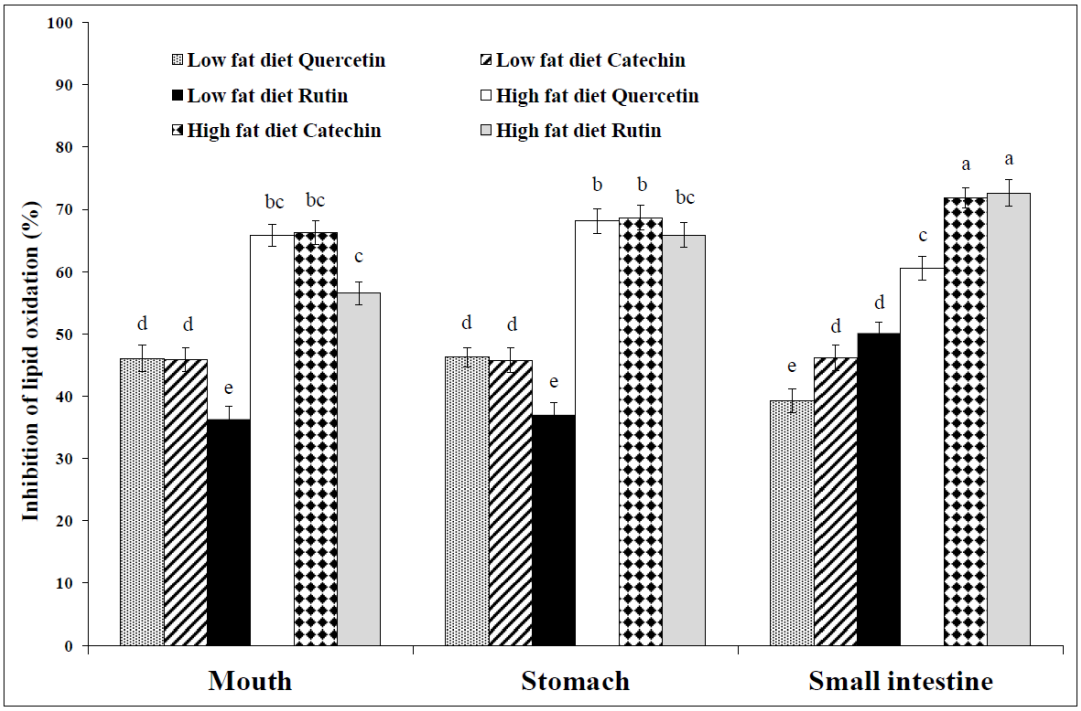

Figure 4. Inhibition of lipid oxidation of phytochemicals as they passed through an *in vitro* human digestion model

Mouth step: saliva juice after 5 min; Stomach step: gastric juice after 2 hr; Small intestine step: duodenal juice and bile juice after 2 hr. Results are presented as the mean ± SD of three independent experiments conducted in triplicate.

Most dietary polyphenols are quite stable during gastric digestion. [18] Conversely, as dietary polyphenols are highly sensitive to the mild alkaline conditions in the small intestine, some of these compounds may be transformed into different structural forms with different chemical properties during digestion in the duodenum. [18] Bermudez-Soto et al. [18] reported that a pH shift to >7.5 during pancreatic/small intestine digestion was the primary factor involved in the irreversible breakdown of the anthocyanins. The degradation of quercetin and the isolation of quercetin from the rutin sample observed after small intestine-like *in vitro* digestion in the present study may have been due to the difference in pH between the stomach and small intestine.

A previous study [19] reported that the significantly higher intracellular peroxyl radical-scavenging activity of quercetin could be explained by the difference in cell-membrane permeability between quercetin and rutin

owing to their different partition coefficients resulting from their structural characteristics. Murota and Terao [20] reported that intact flavonoid glycosides were not well absorbed by the small intestine because sugar moieties elevate their hydrophilicity, whereas flavonoid aglycone can be easily absorbed into epithelial cells in the large intestine because its lipophilicity facilitates its passage across the phospholipid bilayer of cellular membranes. [20] The results of another study [17] support our suggestion that quercetin aglycone appears to be a more active chain-breaking antioxidant than its glycoside counterparts because of its higher accessibility to the site of chain-initiating and chain-propagating free radicals in membranous phospholipid bilayers. An earlier study demonstrated that dietary quercetin could accumulate in the human circulatory system [20]. Neither quercetin aglycone nor quercetin glucosides were present in the blood plasma in an experiment using rats [21].

Quercetin is one of the most common native flavonoids, occurring primarily in glycosidic forms such as rutin. [22] Rutin possesses several pharmacological activities, including antiallergic, anti-inflammatory, vasoactive, antitumor, antibacterial, antiviral, and antiprotozoal properties, and is widely used in treating diseases. [7] However, rutin is only slightly weaker than quercetin in its ability to reduce Fe (II)-induced MDA formation in liposomes and ferrous sulfate/cysteine-induced lipid peroxidation in rat liver microsomes. [23] In our previous study [24], rutin and quercetin isolation from buckwheat and the transformation of rutin to quercetin were both increased by *in vitro* digestion. In other studies, Bermudez-Soto et al. [18] reported that dietary polyphenols are highly sensitive to the mildly alkaline conditions in the small intestine and suggested that a proportion of these compounds may be transformed into different structural forms with different chemical properties during digestion in the duodenum. Thus, the antioxidative activities of the quercetin sample may have decreased because the amount of quercetin decreased during *in vitro* digestion due to degradation, whereas the amount of quercetin isolated from the rutin sample may be increased by *in vitro* digestion and was the cause of the antioxidant effect in mouse brain lipids.

In the present study, dietary fat content was closely related with the antioxidative activity in mouse brain lipids, and the antioxidative activity of phytochemicals was increased in high-fat-fed mouse brain lipids compared to a low-fat-fed mouse brain lipids. This may have resulted from the increase in the mouse lipid content with increasing dietary fat contents, and consequently, a high-fat diet requires a high amount of antioxidants in the body. High fat intake can be associated with increased oxidative stress in mammals. [25,26] Hsu et al. [27] reported that obesity decreases the levels of antioxidant defense by lowering the levels of antioxidant enzymes (catalase, glutathione peroxidase, and glutathione reductase), and that lipid peroxide levels in the liver tissues were significantly elevated in the high-fat diet because of markedly decreased levels of superoxide dismutase, glutathione peroxidase, catalase, reduced glutathione, and thiobarbituric acid-reactive substances. [25] Moreover, high fat concentrations can increase the exposure of lipid to oxygen. [28] These are the main reasons by which increased fat intake can increase lipid peroxidation. [28] Consequently, inhibition of lipid oxidation by phytochemicals may be relatively higher in high-fat-fed mice brain lipids than in low-fat-fed mice [28].

There is still controversy over the accumulation of flavonoids in the brain because the blood-brain barrier blocks many drugs and chemicals from entering the brain. [29] However, Fiorani et al. [30] reported that quercetin aglycone is concentrated in isolated mitochondria to protect the mitochondria from oxidative stress. Ishisaka et al. [29] demonstrated that orally administered quercetin in onion accumulated in the brain tissue of rats. They also found that the concentrations of quercetin and methylquercetins almost plateaued within 1 week of administration and remained at constant levels after 1 month of administration. De Boer et al. [31] and Selvakumar et al. [32] also reported that quercetin accumulated in the brain in rats after oral administration of a quercetin diet. Thus, we assume that quercetin could directly or indirectly attenuate oxidative stress in brain lipids. Rutin is less able to penetrate cell membranes because it carries a hydrophilic disaccharide moiety [23].

Bermúdez-Soto et al. [18] suggested that, during digestion in the duodenum, a proportion of polyphenols may be transformed into different structural forms with different chemical properties. In a previous study [33], catechin losses of approximately 80%, including almost total degradation of epigallocatechin gallate, were observed during simulated digestion of simple tea infusions. Yoshino et al. [34] also reported that the content of (-)-epigallocatechin gallate decreased in authentic intestinal juice as a consequence of an oxidative dimerization. In contrast, Record and Lane [35] reported that all the catechins were stable at acidic pH for several hours. Zhu et al. [36] reported that various catechin components of green tea had different stabilities at alkaline pH. Epigallocatechin gallate and epigallocatechin were unstable and degraded rapidly under these circumstances, but the other major catechins, epicatechin and epicatechin gallate, were more stable under these conditions. [36] When examining the effects of in vitro digestion on dietary polyphenols, light, and O_2 are two important factors to consider as they can alter the structure and properties of phenolic compounds through oxidative degradation and polymerization reactions. [37] These results indicate that the stability of catechin during digestion could largely influence the kinds of catechin, pH of digestive juices, atmosphere or light. Thus, there is clearly an urgent need for more research into correlations between catechin stability and influence factors during digestion.

The major finding of this study is that the antioxidant activities of both quercetin and rutin were influenced by *in vitro* digestion, whereas that of catechin was less affected. After digestion in the small intestine, the antioxidant activity of rutin increased, whereas that of quercetin decreased by digestion in the stomach. Inhibition of lipid oxidation by phytochemicals in mouse brain lipids influenced after digestion in the stomach. In particular, the inhibitory effect of lipid oxidation of phytochemicals was higher in high-fat-fed mouse brain lipids than in those of low-fat-fed mice. A high-fat diet, therefore requires antioxidant-enriched foods, and dietary phytochemicals may have important applications as natural antioxidant agents in a high-fat diet.

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