Original Article

No correlation is found for vegetables between antioxidant capacity and potential benefits in improving antioxidant function in aged rats

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Vegetables vary greatly in antioxidant capacity *in vitro*. This study was to investigate the actions of three vegetables different remarkably in antioxidant capacity *in vitro* on antioxidant function in aged rats. Sixty female aged Wistar rats were randomly assigned to the control, lotus root, rape and cucumber (high, moderate and low in antioxidant capacity, respectively) treated groups. After 6 weeks of feeding, there were no significant differences in plasma FRAP value and contents of vitamin C, vitamin E, uric acid and total phenolics among different groups, whereas the content of reduced glutathione was significantly higher in the rape and cucumber groups. Plasma superoxide dismutase activity also was significantly increased in the rape and cucumber groups. Plasma contents of malondialdehyde, carbonyls and hemolysis were decreased significantly in 3 vegetable-treated groups. Meanwhile, urinary 8-hydroxy-2'-deoxyguanosine excretion was lower significantly in the rape group and the ratio of comet tail length to total length of blood mononuclear cells was decreased significantly in 3 vegetables treated groups. These results suggest that 3 vegetables tested are effective in improving antioxidant function to some extent in aged rats and no correlation is found between antioxidant capacity *in vitro* and improvements of antioxidant function. The benefits observed in this study may come from additive or synergistic combinations of antioxidants contained in vegetables.

***Key Words*: lotus root, rape, cucumber, antioxidant function, aged rats**

Aging is a complex process and the underlying mechanism remains poorly understood. Oxidative stress has been recognized to be involved in the aging process and development of age-related diseases. It has been demonstrated that the antioxidant function decreases and production of reactive oxygen species (ROS) and reactive nitrogen species (RNS) increases with aging.[1,2] Therefore, supplementation of antioxidants is highly recommended as one of strategies in combating the oxidative damages accumulated in the aging process. However, the use of synthetic antioxidants has been criticized. It is suggested that regular consumption of natural antioxidants from vegetables, fruits, tea and herbs may be more beneficial in improving the antioxidant function in aged subjects.[3–5]

Epidemiological studies have provided abundant evidence in the past decades that high intake of vegetables and fruits is generally associated with low incidence of oxidative stress related diseases, such as coronary heart disease, diabetes mellitus and cancers. This protective effect has been attributed frequently to the antioxidants contained in vegetables and fruits, such as vitamin C (VC), vitamin E (VE), carotenoids and phenolics (including flavonoids).[6–8] Previously, we measured the antioxidant capacity of 17 vegetables sampled in China using ferric reducing antioxidant power (FRAP) assay and found that vegetables vary greatly in antioxidant capacity. The lotus root ranked the highest in FRAP value among all vegetables tested.[9] Du *et al.*[10] also reported that lotus root had a high antioxidant capacity as measured by a 1,1-diphenyl-2-picryl-hydrazyl (DPPH) radical scavenging assay and displayed significant hepatic protective effects in high fat diet induced obese rats. On the other hand, several vegetables were found to be relatively lower in antioxidant capacity, such as cucumber, pumpkin, celery and romaine lettuce.[9] Similar results also were reported by others using different free radical scavenging assays.[11–14] Antioxidant analysis by a high performance liquid chromatography procedure coupled with coulorimetric array detection revealed that the vegetables low in antioxidant capacity contained less antioxidants than those high in antioxidant capacity.[15] It is, therefore, plausible to hypothesize that different vegetables may provide protection differently against oxidative damages because they are different in antioxidant capacity. Vegetables high in antioxidant capacity may be more effective than those low in antioxidant capacity in reducing oxidative damage associated with the aging process. To our knowledge, there is no comparative study so far carried out on the antioxidant action of different vegetables *in vivo*.

In the present study, we investigated the effects of lotus root, rape and cucumber on several markers of antioxidant function in aged rats. The objective of this study is to provide evidence that the antioxidant capacity of vegetables *in vitro* may be correlated with their potential benefits in improving antioxidant function in aged rats and lay a basis for further study.

Materials and Methods

Preparation of vegetable extracts. Based on the FRAP values of different vegetables we measured previously,[9] lotus root (high in antioxidant capacity, with FRAP value of 4.6 mmol/100 g fresh weight), rape (moderate in antioxidant capacity, with FRAP value of 1.6 mmol/100 g fresh weight) and cucumber (low in antioxidant capacity, with FRAP value of 0.4 mmol/100 g fresh weight) were chosen for comparison in improving antioxidant function in aged rats. They were purchased freshly from a local market and washed in distilled water and squeezed into juices. Lyophilized powders were obtained by freeze-drying under vacuum and stored at –20°C before being supplemented to experimental diets.

Animals and treatments. Aged Wistar female rats (19-month old), weighing 382.4 ± 27.5 g, were obtained from the

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Table 1. Composition of the AIN-93 diet

Ingredient	Content (g/kg)
Cornstarch	465.7
Casein (>85% protein)	140
Dextrinized cornstarch	155
Sucrose	100
Soybean oil	40
Fiber	50
Vitamin mix (AIN-93)	10
Mineral mix (AIN-93)	35
L-Cystine	1.8
Choline bitartate	2.5
Tert-butylhydroquinone	0.008

Laboratory Animal Center of Chinese Academy of Military Medical Sciences (Beijing, China). After being acclimated for one week, they were randomly divided into 4 groups (10 in each group) according to their body weight. Group 1 was fed an AIN-93 diet (Table 1).[16] Groups 2, 3 and 4 received the diets supplemented with different vegetable powders (AIN-93 diet supplemented with 2.5%, 0.7%, 0.5% of lotus root, rape or cucumber powders, respectively). The supplemented doses are equivalent to 20% of food intake daily as calculated based on the fresh weight of vegetables. All rats were housed individually in stainless-steel cages in a well-ventilated room and allowed free access to food and tape water. The light/dark cycles were alternated every 12 h. The experiment lasted for 6 weeks. Food intake was recorded every day and body weight measured once a week. At the end of the experiment, urine samples were collected continuously for 3 days and stored at –20°C. Finally, all rats were fasted overnight before blood samples were taken from the orbital plexus under diethyl ether anesthetization and anti-coagulated with heparin. Samples of blood cells and plasma were prepared accordingly after centrifugation. All procedures were performed in accordance with the current Chinese legislation on the care and use of laboratory animals and approved by the Department of Scientific Management of the institute.

Plasma antioxidant capacity and contents of VC, VE, reduced glutathione, total phenolics and uric acid. The FRAP assay described by Benzie and Strain was followed to analyze plasma antioxidant capacity.[17] Plasma content of VC was quantified spectrophotometrically based on the reaction with 2,4-dinitrophenydrianze.[18] An improved fluorometric method was used to determine plasma VE content.[19] Plasma reduced glutathione (GSH) was assayed spectrophotometrically by the reaction of 5,5'-dithiobis-2-nitrobenzoic acid with thiols. The reagent kit was purchased from Jiancheng Bioengineering Institute (Nanjing, China). Plasma content of total phenolics was determined according to the method of Singleton *et al.*[20] Plasma uric acid was measured using a reagent kit from BHKT Company (Beijing, China).

Plasma activities of antioxidant enzymes. Activity of plasma superoxide dismutase (SOD) was measured through the inhibition of nitroblue tetrazolium reduction by the superoxide radicals generated by the xanthine/xanthine oxidase system. One unit of SOD activity is defined as the amount of enzyme causing 50% inhibition per 1.0 ml plasma. Activity of plasma glutathione peroxidase (GSH-Px) was detected by measuring the reduction of GSH per min. Results were expressed as a decrease of 1.0 μmol/L GSH per 5 min per 0.1 ml plasma at 37°C after the nonenzymatic reaction is subtracted. Activity of plasma catalase (CAT) was determined by measuring the intensity of a yellow complex formed by molybdate and H_2O_2 at 405 nm after ammonium molybdate was added to terminate H_2O_2 degradation, which is catalyzed by CAT. One unit of CAT activity is expressed as the degradation of 1.0 μmol/L H_2O_2 per second per 1.0 ml plasma. All procedures were performed strictly in accordance to the instructions attached to the commercial assay kits obtained from Jiancheng Bioengineering Institute (Nanjing, China).

Plasma contents of malondialdehyde, oxidized low density lipoprotein and carbonyls. The content of plasma malondialdehyde (MDA) was determined by the thiobarbituric acid reactive species assay.[21] A commercial enzyme-linked immunosorbent assay (ELISA) kit, purchased from the R & D Systems Inc., MN, was used to measure plasma oxidized low density lipoprotein (ox-LDL) content. Plasma carbonyl content was detected by the reaction with 2,4-dinitrophenylhydrazine as reported by Levine *et al.*[22]

Hemolytic sensitivity. The sensitivity of hemolysis was determined spectrophotometrically according to a method described previously by Grinberg *et al.*[23] Results were expressed as the percentage of hemolysed red blood cells after incubation with H_2O_2.

Urinary 8-hydroxy-2'-deoxyguanosine excretion and blood mononuclear cell DNA damage. The content of urinary creatinine was measured by the reaction of creatinine with picrate in alkaline medium. The 8-hydroxy-2'-deoxyguanosine (8-OH-dG) in urine was quantified by an ELISA procedure. Results were expressed as the amount of 8-OH-dG per gram of creatinine in urine. The reagent kit was purchased from Japan Institute for the Control of Aging.

Blood mononuclear deoxyribonucleic acid (DNA) damage was analyzed using the single cell micro-gel electrophoresis assay, also known as Comet assay.[24] Results were expressed as the injury rate (the percentage of DNA in tail) and the ratio of comet tail length to total length.

Statistical analysis. Data were expressed as mean and standard deviation and checked for normality before subjected to further analysis. Analysis of variance was employed for normally distributed data with post hoc Newman-Keuls test and Kruskal-Wallis H test for non-normally distributed data. The level of significance was set at $p<0.05$.

Results

Body weight and dietary intake. During the experimental period, the body weight of rats was increased slowly and a little more gain was seen in three vegetable treated groups. However, no significant difference in both dietary intake and body weight was noted among the four groups (Table 2).

Plasma FRAP value and contents of antioxidants. As shown in Table 3, there was no significant difference among the four groups in plasma FRAP value, contents of VC, VE, uric acid and total phenolics after 6 weeks of feeding, indicating that the levels of these antioxidants in plasma were not changed remarkably by vegetable treatments in aged rats. However, the content of GSH, which is not measurable by FRAP assay,[25] was increased significantly in the rape and cucumber groups compared to the control group.

Activities of plasma antioxidant enzymes. Antioxidant enzymes are one of components important in antioxidant system.

Table 2. Dietary intake and body weight during the experiment

Group	Body weight (g)		Dietary intake (g/d)
	Initial	Final	
Control	372.7 ± 32.5	387.6 ± 33.6	25.6 ± 2.2
Lotus root	370.9 ± 32.1	403.7 ± 45.1	25.7 ± 2.3
Rape	370.9 ± 31.1	400.1 ± 36.9	25.6 ± 1.9
Cucumber	371.2 ± 34.8	402.7 ± 38.7	25.5 ± 1.9

Data are expressed as mean ± standard deviation (n = 10).

Table 3. Plasma FRAP value and contents of some antioxidants

Group	FRAP mmol/L	VC μg/ml	VE μg/ml	GSH mg/L	UA μmol/L	Total phenolics μg/ml
Control	0.32 ± 0.06	4.7 ± 1.5	32.2 ± 9.5	0.52 ± 0.22	357.6 ± 150.0	39.5 ± 6.3
Lotus root	0.31 ± 0.04	4.2 ± 0.7	27.7 ± 4.6	0.52 ± 0.20	339.9 ± 133.4	39.2 ± 7.2
Rape	0.32 ± 0.06	4.6 ± 1.2	34.0 ± 10.4	0.92 ± 0.43[a]	335.2 ± 208.5	38.7 ± 4.8
Cucumber	0.32 ± 0.05	4.1 ± 1.4	32.9 ± 5.6	0.77 ± 0.35[a]	326.1 ± 104.1	36.0 ± 4.1

Data are expressed as mean ± standard deviation (n = 10). [a]p<0.05, compared with control. FRAP, ferric reducing antioxidant power; GSH, reduced glutathione; UA, uric acid; VC, vitamin C; VE, vitamin E.

In this study, plasma SOD activity was significantly higher in the rape and cucumber groups than in the control group. No significant difference was noted for the activities of plasma CAT and GSH-Px among the four groups (Fig. 1).

Contents of plasma MDA, ox-LDL, carbonyls and hemolysis. As indicated in Fig. 2, the contents of plasma MDA, carbonyls and hemolysis were significantly lower after lotus root, rape or cucumber treatment in comparison with the control group, suggesting that all three vegetables were effective in reducing oxidative damages to some extent in aged rats. However, the content of plasma ox-LDL was not significantly different among the four groups.

Urinary 8-OH-dG excretion and blood mononuclear cell DNA damage. Urinary excretion of 8-OH-dG was decreased in three vegetables treated groups, in which the effect of rape was significant statistically in comparison with the control group. Of the blood mononuclear cell DNA damage as determined by Comet assay, there was no significant difference in injury rate among the four groups. However, the ratio of comet tail length to total length was significantly decreased after the treatments of three vegetables (Table 4).

Discussion

ROS and RNS are constantly generated *in vivo* for physiological purposes and over-produced in some physical and pathological conditions, resulting in oxidative stress. To protect their possible damages to biological molecules, especially to DNA, lipids and proteins, all oxygen-consuming organisms are equipped with a well-integrated antioxidant system, including enzymatic and non-enzymatic components. Of the enzymatic components, SOD, GSH-Px and CAT are important antioxidant enzymes and their activities often measured for the assessment of antioxidant function. The non-enzymatic components consist of macromolecules, such as albumin, ceruloplasmin and ferritin as well as an array of small molecules, such as VC, VE, GSH, uric acid and β-carotene.[26,27] It has been reported that the rate of mitochondrial H_2O_2 production was increased and the ROS-induced macromolecular damage accumulated during the aging process. It is, therefore, indicated that more antioxidants may be required by elderly subjects.[28–30] Several studies have confirmed that supplementation of antioxidants was effective in improving antioxidant function in the aged, though controversies still exist.[31–34] Recently, the redox stress hypothesis was raised by Sohal and Orr,[2] in which they proposed that aging-associated functional losses were primarily caused by a progressive pro-oxidizing shift in the redox state and results in the overoxidation of redox-sensitive protein thiols.

The FRAP assay is a simple and reproducible method and has been applied widely in measuring the antioxidant capacity of biological materials, plant foods, beverages or pure antioxidants with results roughly comparable to those obtained with other more complex methodologies.[9,12,17,35,36] However, it should be pointed out that the data generated from different methodologies are not always well-correlated. For example, Ou *et al.*[37] compared

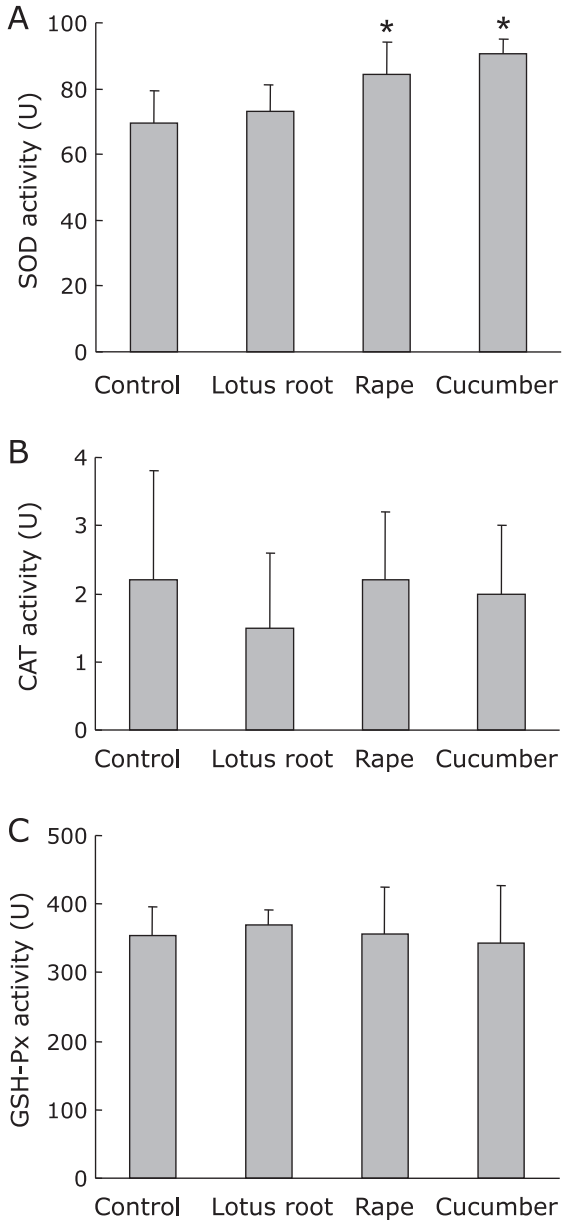

Fig. 1. Plasma activities of SOD, CAT and GSH-Px after supplementation of lotus root, rape or cucumber for 6 weeks in aged rats. Data are expressed as mean ± standard deviation (n = 10). *p<0.05, compared with control. CAT, catalase; GSH-Px, glutathione peroxidase; SOD, superoxide dismutase.

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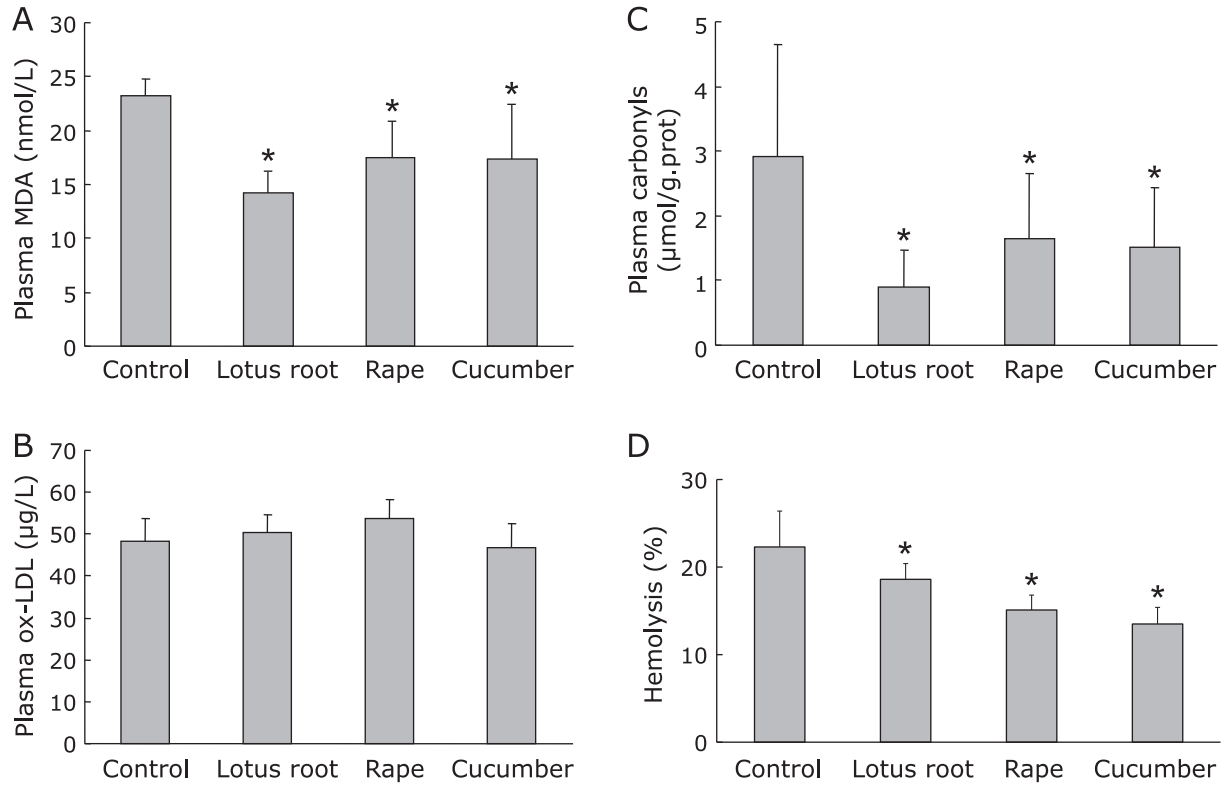

Fig. 2. Contents of oxidized products in plasma and hemolytic sensitivity after supplementation of lotus root, rape or cucumber for 6 weeks in aged rats. Data are expressed as mean ± standard deviation (n = 10). *p<0.05, compared with control. MDA, malondialdehyde; ox-LDL, oxidized low density lipoprotein.

Table 4. Urinary excretion of 8-OH-dG and blood mononuclear cell DNA damage

Group	8-OH-dG (μg/g Cr)	Comet assay	
		Injury rate (%)	Tail L/Total L
Control	18.7 ± 24.9	23.6 ± 10.1	0.63 ± 0.07
Lotus root	8.2 ± 14.3	25.0 ± 12.2	0.55 ± 0.09[a]
Rape	3.4 ± 2.6[a]	28.6 ± 13.0	0.51 ± 0.13[a]
Cucumber	11.2 ± 16.8	30.7 ± 12.0	0.49 ± 0.09[a]

Data are expressed as mean ± standard deviation (n = 10). [a]p<0.05, compared with control. Cr, creatinine; 8-OH-dG, 8-hydroxy-2'-deoxyguanosine; Tail L, comet tail length; Total L, total length.

the antioxidant activities of vegetables employing oxygen radical absorbance capacity (ORAC) and FRAP assays and found that the ORAC and FRAP values were not correlated very well except for beet, onions and carrots. It is explainable because two methods are based on different principles. We have reported that lotus root, rape and cucumber were different significantly in antioxidant capacity as measured by FRAP assay.[9] Isabelle *et al.*[14] analyzed the antioxidant capacity of 66 vegetables sampled in Singapore employing ORAC assay. The results showed that lotus root was much higher than cucumber in ORAC value (21.77 μmol TE/g fresh weight vs 2.51 μmol TE/g fresh weight). The ORAC value of cabbage (Shanghai, green), a similar vegetable to rape, was 14.19 μmol TE/g fresh weight. Therefore, the rank order of the three vegetables by ORAC value is rather similar to that by FRAP value. Lotus root has been considered to be a strong astringent herb in traditional Chinese medicine and applied in treating bleeding and hematemesis.[10] Rape is one of vegetables consumed commonly in many countries and treated as a nutritionally well-balanced vegetable, because it contains carbohydrates, essential n-3 fatty acids, tocopherols, lycopene, chlorophylls, phenolics (including flavonoids).[38] Cucumber is cultivated all over the world and sometimes used for relieving headaches or as a demulcent in anti-acne lotions.[39] In the present study, the lotus root, rape and cucumber were chosen for the comparison of their actions on antioxidant function in aged rats. The results showed that three vegetables were effective in improving some aspects of antioxidant function in aged rats, as indicated by the changes in some markers of antioxidant function, though the plasma FRAP value and the contents of plasma VC, VE, uric acid and total phenolics were not changed significantly after 6 weeks of feeding. It was shown that the content of plasma GSH was increased significantly in rape and cucumber groups. The rape or cucumber treatment also increased plasma SOD activity significantly in aged rats. Moreover, plasma contents of MDA and carbonyls, markers for oxidative damages on lipids and proteins, were decreased significantly by the treatment of three vegetables. Hemolysis, resulted from the attack of free radicals on the membranes of red blood cells, also was declined after exposure to lotus root, rape or cucumber. The urinary excretion of 8-OH-dG, a marker for DNA damage, was decreased significantly in the rape group, while the ratio of comet tail length to total length was reduced significantly in all three vegetable treated groups. Based on the improvements of plasma SOD activity, GSH content, hemolysis or DNA damage in aged rats by three vegetables, it is rather surprising that the lotus root, high in antioxidant capacity, is not more potential comparatively than the rape and cucumber, which were relatively low in antioxidant capacity.

Antioxidant capacity of vegetables is originated from the antioxidants they contain. Of the antioxidants present in vegetables, phenolics are frequently suggested to be a class of compounds possibly contributing most to the antioxidant activity of vegetables.[40,41] We also demonstrated that phenolics were responsible

mainly for the antioxidant capacity of vegetables *in vitro*. The pulp of lotus root contained not only more total phenolics (including flavonoids) but also more VC than the pulp of cucumber (total phenolics: 34.76 mg vs 1.62 mg; flavonoids: 69.30 mg vs 0 mg; VC: 95.36 mg vs 27.11 mg based on 100 g fresh weight, respectively).[42] However, the results of this study do not support the notion that vegetables rich in antioxidants are more potential in improving antioxidant function than those not rich in antioxidants. No clear correlation was found between the FRAP value or contents of antioxidants and the improvement of antioxidant function in aged rats. It seems likely that the bioavailability of natural antioxidants may be different for different vegetables in aged rats. Although the lotus root contains more antioxidants, they may be absorbed not as efficiently as those in the rape and cucumber. This point is supported by the results obtained in this study, in which no significant difference was found in fasted plasma contents of VC, VE and total phenolics after the treatment of three vegetables in aged rats. In a preliminary study, we monitored the changes of serum FRAP value 1, 3, 5 h after oral administration of 5 ml of fresh lotus root juice in rats. However, no significant increase in serum FARP value was found, indicating that the antioxidants contained in lotus roots were not rapidly absorbed (unpublished data). It has been reported that most phenolics are poorly absorbed and metabolized extensively *in vivo* and the metabolites formed are relatively low in antioxidant activity.[43–45] Therefore, the phenolics contained in vegetables may not function as an important antioxidant *in vivo* after absorption. Further study is required to probe into the bioavailability of natural antioxidants present in vegetables in aged subjects. The antioxidant composition varies greatly among different vegetables and the possible roles of other antioxidants contained in vegetables, such as GSH and carotenoids, also should be fully investigated, because some vegetables are rich in GSH and carotenoids.[46,47] Moreover, some phenolic acids in vegetables had been demonstrated to be effective in inducing phase 2 antioxidant enzymes and increasing the biosynthesis of antioxidant and detoxification enzymes and major cellular antioxidants especially GSH.[48,49] In the present study, we also found that plasma content of GSH and SOD activity were significantly increased after the treatments of rape and cucumber. Therefore, some phenolic acids contained in vegetables may impact on antioxidant function indirectly and these actions are not measurable by antioxidant assays *in vitro*.

In conclusion, the results of this study indicate that the supplementation of lotus root, rape or cucumber is effective in improving some aspects of the antioxidant function in aged rats. However, the action is not correlated positively with the antioxidant capacity of vegetables *in vitro*. The benefits observed in this study may come from the additive or synergistic combinations of antioxidants contained in vegetables. More studies are needed to investigate the functional components in vegetables and their actions *in vivo*.

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Conflict of Interest

No potential conflicts of interest were disclosed.

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