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Antioxidant Property of Vitamin C - in Comparison with Vitamin B1

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Various aspects of antioxidant activity in vitamin C were evaluated in this study. Relatively high level of 1,1-diphenyl-2-picrylhydrazyl (DPPH) radical scavenging activity was detected in vitamin C, but not in non-antioxidative vitamin, vitamin B1. Vitamin C also reduced the production of lipid peroxidation in Chinese hamster lung fibroblast (V79-4) cells with IC_{50} value of 4 μg/ml. Vitamin B1 showed comparable reduction in lipid peroxidation products (IC_{50} value was about 10 μg/ml). It was shown that vitamin C also dose-dependently enhanced the activities of superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) in V79-4 cells, and these effects were not observed in vitamin B1-treated cells. Our data suggest that well-known anti-oxidant vitamin C involved in direct activation of SOD, CAT and GPX.

Keywords: Vitamin C, Radical scavenging, Lipid peroxidation, Antioxidant enzymes.

Introduction

The univalent reduction of molecular oxygen results in reactive oxygen species (ROS). ROS include free radicals such as superoxide ions ($O_2^{\cdot-}$) and hydroxy radicals ($OH^{\cdot}$) as well as non free radical species such as hydrogen peroxide (H_2O_2) (Halliwell and Gutteridge, 1998). Although the generation of ROS is an essential defence mechanism in some instances, in excessive concentrations or in the wrong location it can cause tissue degeneration and a wide range of common diseases. These include immunodeficiency syndrome, heart diseases, diabetes, and cancer (Alho and Leinonen, 1999: Tanizawa *et al.*, 1992: Duh, 1998: Frilich and Reiderer, 1995: Hertog, 1993).

All aerobic organisms, including human beings, have antioxidant defenses that protect against oxidative damages and numerous damage removal and repair enzymes are present to remove or repair damaged molecules (Halliwell and Gutteridge, 1998). As enzymatic defense mechanisms, superoxide dismutase (SOD) which catalyzes dismutation of superoxide anions to hydrogen peroxide, catalase (CAT) which convert H_2O_2 into molecular oxygen and water, and seleno-dependent glutathione peroxidase (GPX) which catalyzes the degradation of H_2O_2 and hydroperoxides originating from unsaturated fatty acids at the expense of reduced glutathione.

Dietary intake of antioxidant compounds becomes important. These non enzymatic antioxidants include vitamins such as ascorbic acid, α-tocopherol, β-carotene, and other carotenoids, glutathione, ubiquinol-10, flavonoids, as well as micronutrient elements such as zinc and selenium (Polidori *et al.*, 2001). Therefore, numerous natural antioxidants have been studied in fruits and vegetables (Martinez *et al.*, 1996; Brown and Rice-Evans, 1998; Jung *et al.*, 2005), oilseeds (Deiana *et al.*, 1999), herbs (Wang *et al.*, 2001), tea (Roedig-Penman and Gordon, 1997), berry crops (Wang and Jiao, 2000), propolis (Banskota *et al.*, 2000), *Ginkgo biloba* (Gohil *et al.*, 2000) and *Panax ginseng* (Keum *et al.*, 2000).

Vitamins are essential elements to maintain normal body functions. The amount of individual vitamin requirement varies and depends on the health status of the body. Vitamin C readily undergoes reversible oxidation and reduction and plays an important role as a redox agent in biological systems (Bsoul *et al.*, 2004). It is well understood that vitamin C involves in the synthesis of collagen, which promotes the formation of hydroxyproline (Roach *et al.*, 1985; Peterkofsky, 1991). In the absence of vitamin C, nonhydroxylated collagen is produced. This form of collagen

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is unstable and can not form the triple helix of required for normal structure of connective tissues including bone, teeth and subcutaneous tissue (Tsuchiya and Bates, 2003). Another important function of vitamin C is that it stabilizes free radicals which are thought to be involved in a number of disease process. Vitamin C is proposed to be involved in the prevention and treatment of cancer, neurodegeneration, inflammation, apoptosis and oral diseases (Head, 1998; Heo and Lee, 2004; Bsoul *et al.*, 2004; Fumeron *et al.*, 2005; Serbecic and Beutelspacher, 2005). We tried to evaluate whether vitamin C is involved in direct activation of antioxidative enzymes including SOD, CAT and GPX. Therefore, in the present study, the antioxidant property of vitamin C was examined by their abilities to scavenge 1,1-diphenyl-2-picrylhydrazyl (DPPH) free radicals, and inhibit the formation of lipid peroxides. Also, the effect of vitamin C on the activity of antioxidant enzymes such as SOD, CAT and GPX was investigated. These activities were compared with non-antioxidative vitamin B1.

Materials and Methods

Materials

The following chemicals were obtained from Sigma Chemical Co. (St Louis, MO, USA): Vitamin C, vitamin B1, [3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] (MTT), 1,1-diphenyl-2-picrylhydrazyl (DPPH), thiobarbituric acid (TBA), 2,2'-di-*p*-nitrophenyl-5,5'-diphenyl-3,3'-(3,3'-dimethoxy-4,4'-diphenylene) ditetrazolium chloride (NBT), nicotinamide adenine phosphate (NADPH), xanthine, xanthine oxidase, sodium ethylenediamine tetraacetate (Na-EDTA), pyridine, sodium azide, glutathione, glutathione reductase. Ethanol was bought from Hayman Chemical Co. (Witham, Essex, UK). Hydrogen peroxide was purchased from Fluka Chemical Co. (Buchs, Swiss). All other chemicals were of the highest analytical grade and purchased from common sources.

Cell culture

Chinese hamster lung fibroblast, V79-4 (ATCC CCL-93) cells were maintained at 37°C in an incubator with a humidified atmosphere of 5% CO_2/95% O_2. Cells were cultured in Dulbecco's modified Eagle's medium (DMEM, Gibco BRL, USA) containing 5% fetal bovine serum (FBS, BioWhittaker, USA), 100 μg/ml of streptomycin, 100 unit/ml of penicillin (Gibco BRL, USA) and 2 mM L-glutamine (Gibco BRL, USA).

DPPH free radical scavenging activity

In order to measure antioxidant activity, the DPPH free radical scavenging assay was carried out according to the procedure described by Blosi *et al.*, (1958). Vitamines at various concentrations (0.8, 4, 20, and 100 μg/ml) was added to a 1.5×10^{-4} M solution of DPPH (Sigma, St. Louis, MO, USA) in methanol and the reaction mixture was shaken vigorously. The amount of DPPH remaining was determined at 520 nm, and the radical scavenging activity was obtained from the following equation:

$$\text{Radical scavenging activity (\%)} = \{(OD_{control} - OD_{sample})/OD_{control}\}\times 100.$$

Lipid peroxidation inhibitory activity

Lipid peroxidation was assayed by the measurement of malondialdehyde (MDA) according to the method of Ohkawa *et al.*, (1979). The cells were exposed to either vitamin C or B1 at various concentrations (4, 20 and 100 μg/ml) in the incubation medium for 60 min, followed by 1 mM H_2O_2 for 60 min. Cells were then washed with cold PBS, scraped and homogenized in ice-cold 1.15% KCl. Samples containing 100 μl of cell lysates were combined with 0.2 ml of 8.1% SDS, 1.5 ml of 20% acetic acid adjusted to pH 3.5 and 1.5 ml of 0.8% thiobarbituric acid. The mixture was brought to a final volume of 4.0 ml with distilled water and heated to 95°C for 120 min. After cooling to room temperature, 5.0 ml of a mixture of n-butanol and pyridine (15:1, v/v) was added to each sample and the mixture was shaken vigorously. After centrifugation at 1500 rpm for 10 min, the supernatant fraction was isolated and the absorbance was measured at 532 nm. Inhibitory activity towards lipid peroxidation was expressed as IC_{50}.

Assays for antioxidant enzymes

The cells were treated with 4, 20 and 100 μg/ml of vitamins for 60 min. The cells were then lysed in a lysis buffer appropriate for the requirements of each assay, as described below. The method of Bradford (1976) was used to determine protein concentrations. Results are expressed as enzyme activity per mg protein compared with corresponding control cultures.

Superoxide dismutase (SOD) activity

SOD activity was assayed by the nitroblue tetrazolium (NBT) method of Beauchamp and Fridovich (1971). NBT is reduced to blue formazan by O_2^-, which has a strong absorbance at 560 nm. The presence of SOD inhibits this reaction. The cells were homogenized in 0.05 M sodium carbonate buffer (pH 10.2). The assay mixture consisted of 0.05 M sodium carbonate buffer (pH 10.2) containing 3 mM xanthine, 0.75 mM NBT, 3 mM EDTA, 1.5 mg/ml BSA and 50 μl of homogenate. The reaction was initiated by the addition 50 μl of xanthine oxidase (0.1 mg/ml) and incubated for 30 min at room temperature. The reaction was stopped by adding 6 mM copper (II) chloride and centrifuged at 1500 rpm for 10 min. The absorbance of blue formazan in the supernatants was measured at 560 nm.

Catalase (CAT) activity

The reaction mixture contained 12 μl 3% (v/v) H_2O_2 and

100 μl of cell lysates in 50 mM phosphate buffer (pH 7.0) at a final volume of 1.0 ml. Samples were incubated for 2 min at 37°C and the absorbance of the samples were monitored for 5 min at 240 nm. The change in absorbance is proportional to the breakdown of H_2O_2 (Carrillo *et al.*, 1991).

Glutathione peroxidase (GPX) activity

GPX was assayed by the method of Paglia and Valentine (1967). The reaction mixture contained 0.1 M phosphate buffer (pH 7.0), 1 mM EDTA, 10 mM glutathione (GSH), 1 mM NaN_3, 1 unit of glutathione reductase, 1.5 mM NADPH and 0.1 ml of cell lysates. After incubation for 10 min at 37°C, H_2O_2 was added to each sample at a final concentration of 1 mM. GPX activity was measured as the rate of NADPH oxidation at 340 nm.

Statistics

All data represent means S.E. Statistical analysis was performed using analysis of variants followed by the Student's *t*-test.

Results

Radical scavenging activity

The DPPH radical scavenging activity of vitamin C is shown in Fig. 1. The activity was compared with vitamin B1. Vitamin C showed relatively high DPPH radical scavenging activity. Approximately 50% of DPPH was scavenged at 0.8 μg/ml of vitamin C. And More than 90% of scavenging activity was shown at higher than 4 μg/ml of vitamin C. Vitamin B1 showed slight increase in DPPH radical scavenging activity but it was not significant.

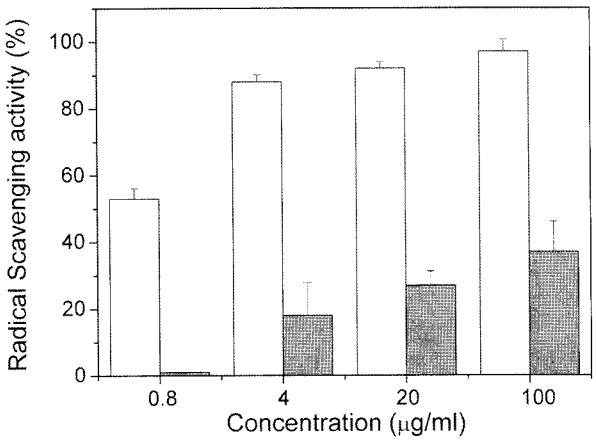

Fig. 1. Comparison of DPPH radical scavenging effect. Either Vitamin C (□) or vitamin B1 (■) was added to a methanolic solution of DPPH and radical scavenging activity was measured at 520 nm. Each experiment was performed 3 times and data were expressed as average percent changes from versus the control± S.D.

Lipid peroxidation inhibition

We also tested the ability of vitamin C to inhibit lipid peroxidation in H_2O_2-treated V79-4 cells (Fig. 2). About 50% of lipid peroxidation was inhibited at 4 μg/ml of vitamin C. Vitamin B1 showed significant lipid inhibitory activity than DPPH radical scavenging activity, with IC_{50} value of approximately 10 μg/ml.

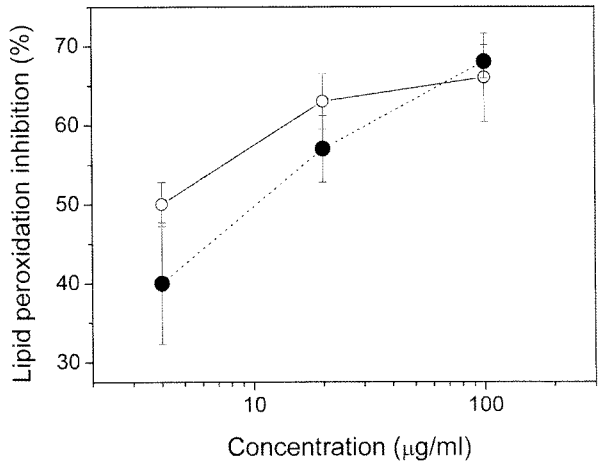

Fig. 2. Lipid peroxidation inhibitory effect of vitamin C (○) and vitamin B1(●). Cells were incubated with 4, 20 and 100 μg/ml of either vitamin C or vitamin B1 and 1 mM of H_2O_2 was added to after 1 hr incubation. The amounts of MDA were measured at 532 nm. Each experiment was performed 3 times and data were expressed as average percent changes from versus the control ± S.D.

Effect of vitamins on antioxidant enzymes

In order to investigate whether these antioxidant activities of vitamin C are mediated by an increase in antioxidant enzymes, we measured SOD, CAT and GPX activities in V79-4 cells treated with vitamin C. These results were compared with those of vitamin B1. Treatment with vitamin C at doses of 4, 20 and 100 μg/ml induced 21, 40 and 51% increase, respectively, in SOD levels (Fig. 3A). The activity of SOD in control cells untreated with extract was 25.1± 1.5 U/mg protein. The enzyme activity measured was 30.4, 35.1 and 37.9 U/mg protein when cells were treated with 4, 20 and 100 μg/ml of vitamin C, respectively (Fig. 3B). Vitamin B1 showed insignificant increase SOD by 5, 13 and 19% (26.4. 28.4 and 29.9 U/mg protein) at doses of 4, 20 and 100 μg/ml (Fig. 3).

The CAT activity was also dose-dependently increased by vitamin C treatment. At doses of 4, 20 and 100 μg/ml, vitamin C activated CAT by 22, 31 and 50%, respectively. The CAT activity of control cells untreated with extract was 15.0± 1.9 U/mg protein. The enzyme activity measured was 18.3, 19.7, and 22.5 U/mg protein when cells were treated with 4, 20 and 100 μg/ml of vitamin C, respectively (Fig. 4B). Treatment with vitamin B1 induced 9, 13 and 17% increase, respectively, in CAT levels. These were corresponding to 16.4, 17.0 and 17.6 U/mg protein and are not

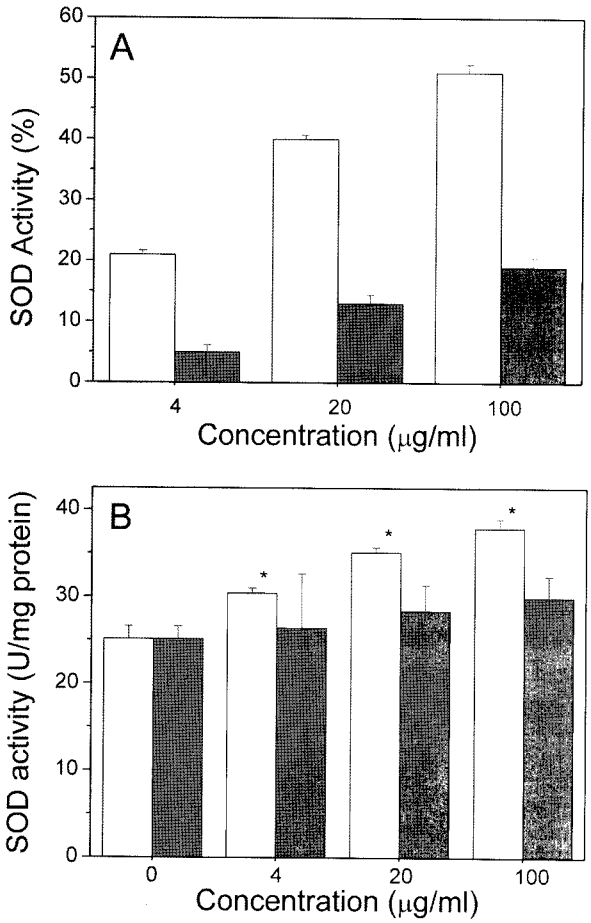

Fig. 3. Increase in superoxide dismutase (SOD) activity by vitamin treatment. Cells were treated with 4, 20 and 100 μg/ml of either vitamin C (□) or vitamin B1 (■) for 60 minutes and superoxide dismutase activity was measured at 560 nm as described in materials and methods. Each experiment was performed 3 times and the data were expressed as mean±S.D. The relative increase in enzyme activity (A) or the average enzyme activity units per mg of protein (B) were shown. (*; $p<0.001$)

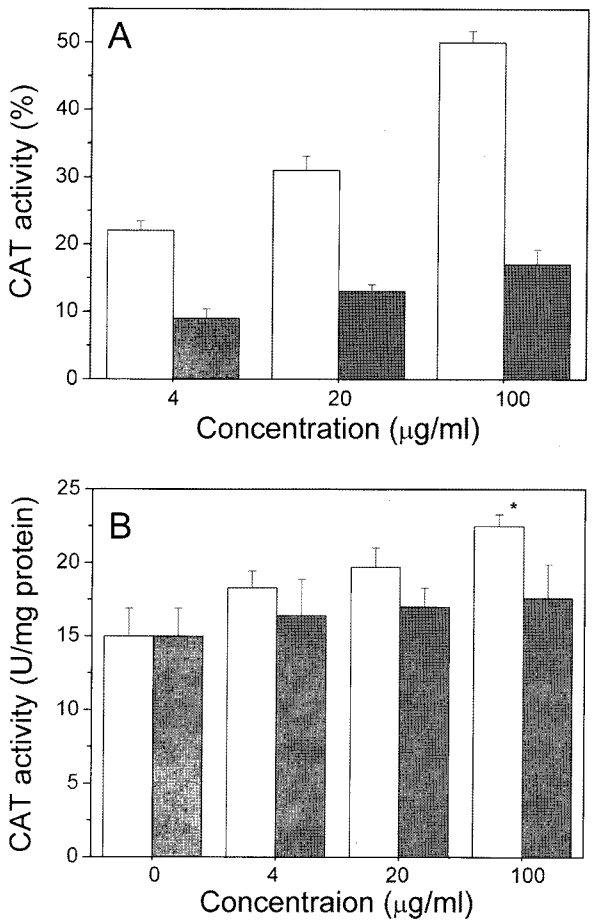

Fig. 4. Increase in catalase (CAT) activity by vitamin treatment. Cells were treated with 4, 20 and 100 μg/ml of either vitamin C (□) or vitamin B1 (■) for 60 minutes and catalase activity was measured at 560 nm as described in materials and methods. Each experiment was performed 3 times and the data were expressed as mean±S.D. The relative increase in enzyme activity (A) or the average enzyme activity units per mg of protein (B) were shown. (*; $p<0.001$)

significant increase as expected.

GPX activity was concomitantly increased when cells were treated with vitamin C. In addition, a dose-dependent increase in GPX activity was observed within the concentration range used in this study. Total extract treatment of 4, 20 and 100 μg/ml increased GPX activity by 31, 42 and 64%, respectively (Fig. 5A). The activity of GPX in untreated control cells was 11.5± 1.3 U/mg protein. The enzyme activity measured was 15.1, 16.3, and 18.9 U/mg protein when cells were treated with 4, 20 and 100 μg/ml of vitamin C, respectively (Fig. 5B). Vitamin B1 showed 5, 12 and 20% (12.1, 12.9 and 13.8 U/mg protein) increase in GPX activity, however, it was not significant.

Discussion

The free radical theory of ageing postulates that aging is caused by excessive reaction of free radicals called reactive oxygen species (ROS). That is, the higher the metabolic rate of an organism, the greater the production of ROS and hence the shorter the life span (Harman 1994). Therefore, researchers have made numerous efforts to find antioxidants. Development of antioxidants scavenging ROS is able to support biological resistance against free radicals, retard the process of aging and decrease the risk of aging-associated degenerative diseases, such as cancer, cardiovascular disease, immunesystem decline, and brain disfunction (Finkel and Holbrook, 2000).

Vitamin C seems functions as a buffer against cell damage from free radicals thus, it thought to be important in a number of disease processes. It was shown that vitamin C is the most effective aqueous-phase antioxidant in human blood plasma (Frei *et al.*, 1989). The reaction mechanism of vitamin C with oxidative enzymes and singlet molecular oxygen are reported (Chou and khan, 1983; Jalukar *et al.*,

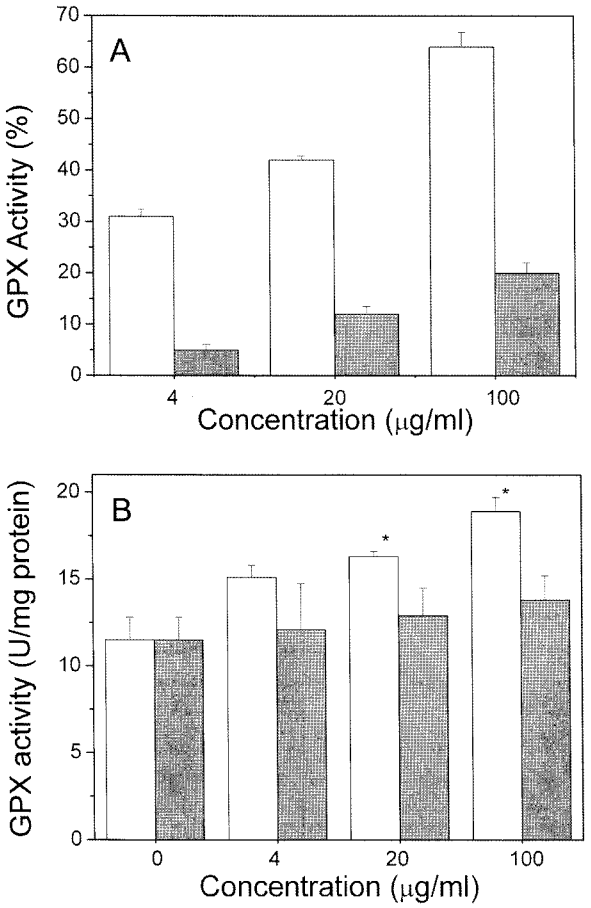

Fig. 5. Increase in glutathione peroxidase (GPX) activity by vitamin treatment. Cells were treated with 4, 20 and 100 μg/ml of either vitamin C (□) or vitamin B1 (■) for 60 minutes and glutathione peroxidase activity was measured at 340 nm as described in materials and methods. Each experiment was performed 3 times and the data were expressed as mean±S.D. The relative increase in enzyme activity (A) or the average enzyme activity units per mg of protein (B) were shown. (*; $p<0.001$)

1991). It is also suggested that vitamin C is important for protection against disease and degenerative processes caused by oxidant stress. Protective effect of vitamin C against oxidative stress-induced neurodegeneration and its relation to age-related oxidative stress and tissue aging are reported (Heo and Lee, 2004; Loo *et al.*, 2004). It is also suggested that vitamin C is involved in the prevention and treatment of cancer, neurodegeneration, inflammation, apoptosis and oral diseases (Head, 1998; Bsoul *et al.*, 2004; Mahfouz and Kummerow, 2004; Fumeron *et al.*, 2005; Serbecic and Beutelspacher, 2005).

In this study, we have characterized the antioxidant properties of vitamin C which can be easily taken by dietary uptake. Vitamin C showed significant increase in DPPH free radical scavenging activity and inhibition of lipid peroxide production. We also observed that vitamin C increased the activity of antioxidant enzymes examined, SOD, CAT and GPX. These enzymes are modulated in various diseases caused by free radical attack (Halliwell and Gutteridge, 1998). Thus, maintaining the balance between the rate of generation of radicals and scavenging of radicals is an essential part of biological homeostasis. It is of particular interest to note that SOD catalyzes the breakdown of $O_2^{\cdot}$ to O_2 and H_2O_2, prevents formation of $OH^{\cdot}$ and has thereby been implicated as an essential defense against the potential toxicity of oxygen. The ROS scavenging activity of SOD is effective only when it is followed by the actions of CAT and GPX, because the dismutase activity of SOD generates H_2O_2, which needs to be further scavenged by CAT and GPX. Vitamim C activated SOD, CAT and GPX at similar extent. This appears that vitamin C can be effectively scavenged H_2O_2 formed by SOD actions. Taken together, these results also suggest that the antioxidant activity of vitamin C may be due to degradation of H_2O_2 and other peroxides.

In this study, we used non-antioxdant vitamin, vitamin B1, which is also called thiamine, as a control. Vitamin B1 has an important role in carbohydrate metabolism. This vitamin functions as a cofactor of key metabolic enzymes such as α-ketoglutarate dehydrogenase complex and pyruvate dehydrogenase complex (Liu and Bisswanger, 2005). Vitamin B1 deficiency causes beriberi, which disturbs the central nervous and circulary system and leads to memory deficits and neurological disease (Blass *et al.*, 1992; Nagawasaki *et al.*, 2001; Bubber *et al.*, 2004). However, vitamin B1 revealed the inhibition effect of lipid peroxidation production induced by H_2O_2 treatment in this study. This finding was unexpected, but, it seems that vitamin B1 has some antioxidative property. It was reported that vitamin B1 increased the efficiency of sanazole, a radiosensitizer in the presence of air (Heinrich and Getoff, 2002). And high dose vitamin B1 therapy reversed and decreased non-HDL cholesterol and normalized triglycerides (Babaei-Jadidi *et al.*, 2004). The mechanism behind these observation of antioxidative property of vitamin B1 needs to be investigated further.

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