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Combination of Vitamin C and Rutin on Neuropathy and Lung Damage of Diabetes Mellitus Rats

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We investigated the role of vitamin C or rutin on neuropathy and lung damage of diabetic mellitus (DM) rats. Norepinephrine content was significantly decreased in sciatic nerves of DM rats compared with non-DM controls but vitamin C had no effect on decreases of norepinephrine. 2,4-dinitrophenylhydrazine (DNPH) incorporation, which is biomarker of protein oxidation, was increased in sciatic nerve of DM rats as compared with normal control. However, vitamin C had no effects on increases of DNPH incorporation. We measured the content of conjugated dienes (CD) as a biomarker of lipid oxidation in sciatic nerve. CD was increased in DM as compared with normal control. Vitamin C or rutin had no effects on increases of CD. However, Rutin plus vitamin C significantly decreased the content of CD as compared with DM rats. In lung of DM rats, DNPH incorporation or CD was increased as compared with normal control. Vitamin C or Rutin had no effects on increases of CD. However, Rutin plus vitamin C significantly decreased the content of DNPH incorporation or CD in lung tissue. Vitamin C caused marked pathological changes such as the increases of parenchyma and the thickening of alveolar septa in the lung of DM. Rutin had protective effects on the pathological changes in the lung of DM rats. In conclusion, Vitamin C had no effects on oxidative parameter, such as DNPH incorporation or CD, and on the decreases of norepinephrine content in DM rats. Vitamin C caused the marked pathological changes in the lung of DM rats but rutin had protective effects against the pathological changes.

Key words: Diabetes mellitus, DNPH incorporation, Conjugated Dienes, Vitamin C, Rutin

INTRODUCTION

Diabetes mellitus (DM) is a heterogeneous primary disorder of carbohydrate metabolism with multiple etiologic factors that generally involve absolute or relative insulin deficiency or insulin resistance. Oxidative stress is present in the diabetic state. The indexes of oxidative stress include an increase in nerve, dorsal root, and sympathetic ganglia lipid hydroperoxides and conjugated dienes (Low *et al.*, 1997). The oxidation of unsaturated fatty acid side-chains is accompanied by the formation of conjugated diene structures, which absorb ultraviolet light in the wavelength range 230-235 nm (Kinder *et al.*, 1997). Measurement of this UV absorbance as an index of peroxidation is extremely useful in studies upon pure lipids and it measures an early stage in the peroxidation process. However, it often cannot be used directly on biological materials because many of the other substances present, such as haem proteins, chlorophylls, purines, and pyrimidines, absorb strongly in the ultraviolet, and create such a high background that spectrophotometric measurements become grossly inaccurate.

Although the roles of vitamin C in a number of enzymatic systems have come to light, still its physiological functions have not yet been fully described in a scientifically satisfactory manner. It acts as a scavenger for oxidizing free radicals and harmful oxygen-derived species, such as the hydroxyl radical, hydrogen peroxide, and singlet oxygen. Under certain circumstances, however, vitamin C may act as a prooxidant to promote the production of reactive free radicals and oxygen-derived species (Zhang and Omaye, 2001). Iron-vitamin C mixtures are frequently used to stimulate lipid peroxidation in vitro; again, the vitamin C functions mainly by reducing iron ions. Vitamin C is a vital substance for humans, existing as a reduced form, ascorbic

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acid, and an oxidized form, dehydroascorbic acid, *in vivo*.

Flavonoids consist of at east two phenyl rings separated by a pyran rings. The antioxidant activity of flavonoids critically depends on the part of the polyphenol molecule with better electron-donating properties (Pulido *et al.*, 2000). Flavonoids are representatives of a multitude of phenolic compounds, exclusively present in plants. Examples include silymarin, quercetin (Afanas'ev *et al.*, 1989), rutin (Afanas'ev *et al.*, 1989; Afanas'ev *et al.*, 1998; Grinberg *et al.*, 1994), and kaempferol. The high chemical reactivity of flavonoids appear to be expressed in their binding affinity to biological polymers and heavy metal ions as well as in their ability to catalyze electron transport and to scavenge free radical (Bors *et al.*, 1990; Robak and Gryglewski, 1988). A number of these products exert powerful antioxidant actions *in vitro*, such as scavenging of O_2- and inhibition of lipid peroxidation but whether they act as an antioxidant in vivo is unknown. The antioxidant effect of vitamin C in DM is controversial at best, with even the aggravation of tissue damage vitamin C being suspected (Kang *et al.*, 1998). The determining factor that directs the effect of vitamin C either to antioxidant or to prooxidant has been suggested to be the presence of free transition metal ions (Kang *et al.*, 1998; Samuni *et al.*, 1983).

The purpose of this study is to define the role of vitamin C or rutin in the sciatic nerve or lung damage of DM rats and whether vitamin C plays a protective role against the oxidative damages or aggravates them in DM rats.

MATERIALS AND METHODS

Materials

Leupeptin, pepstatin, phenylmethylsulfonyl fluoride (PMSF), aprotinin were purchased from Boehringer Mannheim (Germany). 2,3-Dinitrophenylhydrazine (DNPH) was purchased from Eastman Chemical Co (Rochester, NY, USA). Acetonitrile and methanol were purchased from Merck (Germany) and absolute ethanol was purchased from Hayman (UK). Streptozotocin, citric acid, ascorbic acid, metaphosphoric acid (MPA), perchloric acid (PCA), trichloroacetic acid (TCA), hematoxylin, eosin, Hepes, rutin and other reagents were purchased from the Sigma Chemical Co. (St. Louis, MO, USA).

Animals

Male Sprague-Dawley rats (200-250 g) were used. All rats were housed in a room maintained at a constant temperature/humidity (21 ± 2°C, 50%) on a 12/12 h light/dark cycle with food and water available *ad libitum*.

Diabetes induction and maintenance

DM was induced with streptozotocin at 65 mg/kg in 20 mM sodium citrate buffer (pH 4.5, i.p.) on 8-week-old Sprague-Dawley rats (Cay *et al.*, 2001; Piotrowski *et al.* 2001). Diabetes was confirmed by blood glucose level (>300 mg/dl) and urine glucose level (>500 mg/dl) measurement using Glucose Analyzer (Beckman, USA) after injection of streptozotocin.

Vitamin C or rutin was given at concentration of 1 *g/l* each in drinking water alone or in combination, which is equivalent to 300 mg/kg per day for control rats and 600 mg/kg per day for diabetic rats, for 4 or 8 weeks starting from 1 week after streptozotocin injection. The pH of vitamin C solution was adjusted to 7.0 by adding sodium bicarbo-nate (2 *g/l*).

Sciatic nerve and lung preparation

Lipid extract for conjugated dienes and norepinephrine was adapted from the method of Folch for peripheral nerve. After complete removal of perineurium and connective tissue including blood vessel from sciatic nerve, it was homogenized in 1 ml ice-cold 0.9% NaCl.

The lungs were first perfused *in situ* via the inferior vena cava with 250 ml of ice-cold heparinized saline and then left ventricle was incised (Freeman and Crapo, 1981). Following complete removal of blood from lung, it was removed and inflated with 10% buffered formalin at a constant pressure of 20 cm H_2O for 24 hours (Thrall *et al.*, 1979). Paraffin-embedded sections were obtained in the usual manner and stained with hematoxylin-eosin (HE) and with Mallory trichrome stains (Thrall *et al.*, 1979).

Norepinephrine quantification

Isolated sciatic nerve was homogenized in 0.1 M perchloric acid containing 0.4 mM sodium metabisulphite and 35 pg/μl dihydroxybenzylamine (the internal standard) then centrifuged (3,000 × g). The supernatant was then added to alumina to adsorb norepinephrine and washed with distilled water. Norepinephrine was eluted with acetic acid and injected into HPLC column. Electrochemical detection was performed on a Shimadzu L-ECD-6A electrochemical detector set at +8.5V. The mobile phase consisted of 0.1 M phosphate buffer pH 3.6 containing 1.0 mM sodium octylsulphate, 0.1 mM EDTA and 9% methanol at a flow rate of 2 ml/min on an octadecylsilyl reverse phase column. Calculating the peak area and multiplying it by the con-version factor obtained from the standard curves of nor-epinephrine and dihydroxybenzylamine did quantification of norepinephrine.

Determination of DNPH incorporation

The abdomen of anesthetized rats was incised, catheter

was inserted in portal veins, and the cutting of abdominal inferior vena cava discharged perfusion fluid and blood. Ca^{2+}-free HEPES buffer was perfused by the rate of 30 ml/min. Hepatocytes were isolated by collagenase perfusion according to the method of Seglen (Seglen, 1976).

Isolated hepatocytes were suspended in HEPES buffer (137.0 mM NaCl, 4.6 mM KCl, 1.1 mM KH_2PO_4, 0.6 mM $MgSO_4$, and 1.0 mM HEPES, pH 7.4) containing the protease inhibitor leupeptin (0.5 μg/ml), phenylmethylsulfonyl fluoride (PMSF) (40 μg/ml), and pepstatin (0.7 μg/ml). The hepatocytes were broken by sonication and insoluble debris was removed by centrifugation at 15,000 × g for 30 min. The supernatant fraction was divided into equal aliquots containing 1.5 mg protein each. Both aliquots were precipitated with 10% trichloroacetic acid (TCA). One sample was treated with 2 N HCl and the other sample was treated with an equal volume of 0.2% DNPH in 2 N HCl. Both samples were incubated at 25°C for 60 min and then reprecipitated with 10% TCA. The precipitate was subsequently washed with ethanol: ethyl acetate (1:1, vol/vol) followed by 10% TCA. The precipitate was dissolved in 1 ml of 6 M guanidine hydrochloride. Insoluble debris was removed by centrifugation. The concentration of carbonyl groups was calculated from the absorbance at 365 nm. Nano moles of DNPH incorporated per miligram of protein represent Protein carbonyl content (Starke *et al.*, 1987).

Conjugated diene (CD) measurements

In order to measure the content of conjugated dienes (CD), the total lipid was extracted from cortical homogenates or mitochondria-enriched fraction with chloroform/ methanol (1:1, v/v) and chloroform/methanol/ water (86: 14:1) (Pryor and Castle, 1984). The lipid extract was dried completely under pure nitrogen gas (99.999%), which passed through oxygen trap of heated copper coil. The dried extract was redissolved in cyclohexane and its absorbance at 234 nm measured against a solvent blank by spectrophotometer. The concentration of conjugated dienes was calculated using 2.52 ± 10^{-5} $M^{-1}cm^{-1}$ as the molar extinction coefficient of conjugated dienes.

Morphology

The rat was anesthetized with urethane, and the lung was excised after perfusing with cold saline containing heparin (1 unit/ml) to remove the blood. For morphological observation under light microscope, the lung was fixed with 10% formalin, sectioned, and stained with hematoxylin-eosin (HE).

After the perfusion with heparinized saline, 2 midcoronal slices of 2 to 3 mm thickness were fixed in 10% formalin solution. Three-micrometer thick paraffin sections were stained with periodic acid Schiff reaction. All tissue samples were examined under light microscope. Quantimet 520 Image Analyzer performed Morphometry for quantification of alveolar septal thickness of the lung tissue.

Statistical analysis

All values are expressed as means ± S.E.M.. Statistical differences between groups were established using one-way analysis of variance. P<0.05 was considered as significant.

RESULTS

Neuropathy in sciatic nerves of DM rats

We measured the content of norepinephrine in sciatic nerve. Norepinephrine content was significantly decreased in sciatic nerves of DM rats compared with non-DM controls. However, vitamin C had no effect on decreases of norepinephrine (Fig. 1). The carbonyl group content, which is the biomarker of protein oxidation represented by DNPH incorporation, was significantly increased in sciatic nerve of DM rats as compared with normal rats, but vitamin C had no effects on increases of DNPH incorporation (Fig. 2). We examined the content of conjugated dienes (CD), which is the biomarker of lipid oxidation. The content of CD was significantly increased in the sciatic nerve myelin of DM rats as compared with normal controls. Vitamin C or rutin had no effects on increases of CD. However, rutin plus vitamin C significantly lowered the content of CD as compared with DM rats (Fig. 3). The result suggested that oxidative stress was increased in the sciatic nerves of DM rats and vitamin C did not affect any of above oxidation parameters significantly.

Lung damage of DM rats

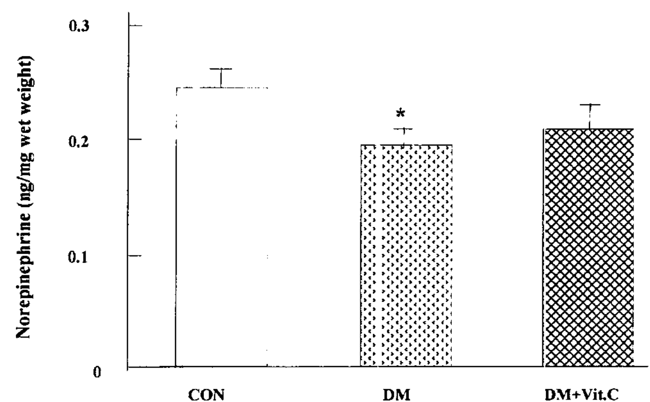

Fig. 1. The contents of norepinephrine (NE) in sciatic nerve. The contents of NE were significantly decreased in DM rats as compared with normal control. Vit. C had no effects on the change of NE. Data are expressed by mean ± S.E. statistics for 8 animals. *P<0.05 vs. control. CON: control; DM: Diabetes Mellitus; Vit.C: Vitamin C

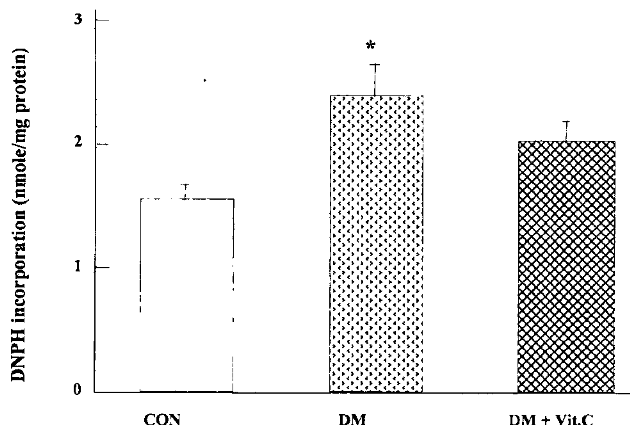

Fig. 2. Carbonyl group contents which is the biomarker of protein oxidation in the sciatic nerve represented by DNPH incorporation. DNPH incorporation was increased in DM rats as compared with control and vit. C had no effects on the increases of DNPH incorporation. Data are expressed by mean ± S.E. statistics for 8 animals. *P<0.05 vs. control. CON: control; DM: Diabetes Mellitus; Vit.C: Vitamin C.

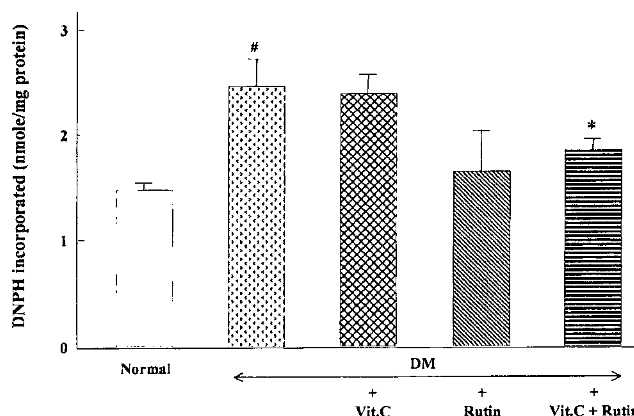

Fig. 4. Carbonyl group contents which is the biomarker of protein oxidation in the lung tissue represented by DNPH incorporation. DNPH incorporation was increased in DM rats as compared with normal and rutin plus vit. C inhibited the increases of DNPH incorporation. Data are expressed by mean ± S.E. statistics for 8 animals. # P<0.05 vs. normal. *P<0.05 vs. DM control. DM: Diabetes Mellitus; Vit.C: Vitamin C.

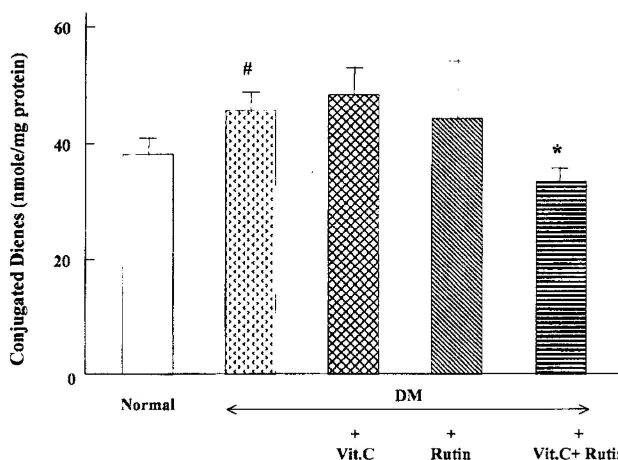

Fig. 3. The content of conjugated dienes (CD) as the biomarker of lipid oxidation in sciatic nerve. The CD content was increased in DM rats and rutin plus vit. C inhibited the increases of CD. Data are expressed by mean ± S.E. statistics for 8 animals. #P<0.05 vs. normal, *P<0.05 vs. DM control. CON: control; DM: Diabetes Mellitus; Vit.C: Vitamin C.

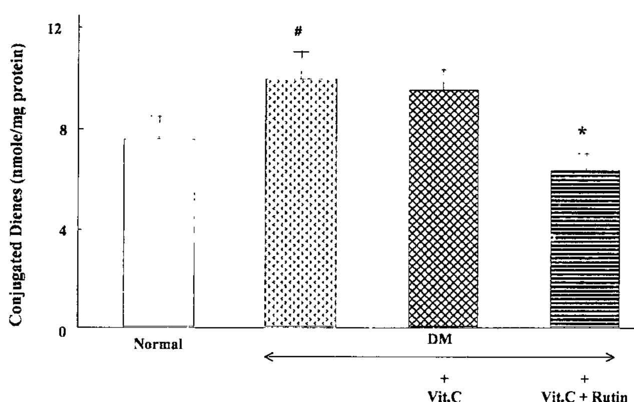

Fig. 5. The content of conjugated dienes (CD) as the biomarker of lipid oxidation in lung. The CD content was increased in DM rats and rutin inhibited the increases of CD. Data are expressed by mean ± S.E. statistics for 8 animals. #P<0.05 vs. normal. *P<0.05 vs. DM control. DM: Diabetes Mellitus; Vit.C: Vitamin C.

We measured the content of DNPH incorporation in lung. Carbonyl group content, which is the biomarker of protein oxidation represented by DNPH incorporation, was significantly increased in the lung of DM rats as compared with normal controls. However, vitamin C had no effects on increases of DNPH incorporation in lung of DM rats (Fig. 4). Rutin plus vitamin C significantly decreased the level of carbonyl group content as compared with DM rats (Fig. 4). We examined the content of CD, which is the biomarker of lipid oxidation in the lung of DM rats. Content of CD was significantly increased in the lung of DM rats as compared with normal control rats, but vitamin C had no effects on increases of CD (Fig. 5). However, rutin plus vitamin C significantly lowered the content of CD and improved on morphological changes of lung alveoli as compared with DM rats (Fig. 5, 6). The result suggests that administration of vitamin C to the DM rats causes marked pathological changes in the lung, but has no effects on the DM itself. The increase of parenchyma and the thickening of alveolar septa were observed, with the appearance of fibrocytes. However, rutin blocked such pathological changes.

DISCUSSION

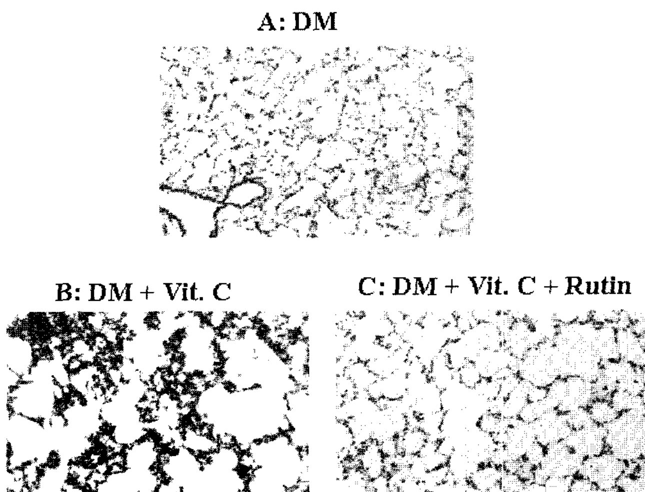

Fig. 6. Morphological changes in lung alveoli of the DM rats. Vit. C caused marked pathological changes such as the increases of parenchyma and the thickening of alveolar septa in the lung of DM. Rutin had protective effects on the pathological changes in the lung of DM rats. The lung tissue was fixed and stained with H-E as described in the method section, and observed under the light microscope at × 100 magnifications. A: DM without treatment; B: DM with vitamin C treatment; C: DM with vitamin C and rutin treatment.

Excessive production of free radicals or inadequate antioxidant defense mechanisms can lead to damage of cellular structures and enzymes, and may thus play a significant role in pathogenesis of arteriosclerosis, cancer, rheumatoid arthritis, and diabetic complications (Cross *et al.*, 1987). Damage to entire tissues can result from free radical-mediated oxidative alteration of fatty acids, also known as lipid peroxidation.

Recently, oxidative stress has been drawing much interest regarding to the initiation and progress of diabetic complications. It has been proposed that oxidative stress contribute to development of the diabetic complications. We have been studied oxidative stress or damage in the sciatic nerves or lung of DM. The sciatic nerve is the major tissue that deteriorates in the long-lasting DM, presenting various clinical symptoms diabetic complications (Andersen *et al.*, 1983; Christiansen *et al.*, 1982; Ellis *et al.*, 1985). The oxidative injury has been suggested as one of the various mechanisms of the diabetic complication. On the other hand, the lung has not drawn much attention relating to the DM. There have been some reports of parenchymal fibrosis of the lung in type I DM (Lanng *et al.*, 1994; Strojek *et al.*, 1992). In this study, the oxidative damages occur not only in those organs vulnerable to diabetic complications like sciatic nerves but also in organs without apparent clinical sign of diabetic complications such as lung. This suggests that the oxidative stress in DM is systemic not restricted to the organs or tissues presenting the clinical symptoms.

Changes in the sciatic nerves in diabetic rats have been used as the experimental mode of diabetic neuropathy (Low and Nickander, 1991; Wright and Nukada, 1994). Decrease of norepinephrine content is due to the decreased autonomic component in the nerve, that is the most vulnerable and the first be damaged in DM. Oxidative injury of the nerve was apparent from the increased oxidized protein and lipid content.

Vitamin C is metabolized in the liver, and to some extent in the kidney, in a series of reactions. Vitamin C is oxidized by two different enzymes, ascorbate oxidase (Ihara *et al.*, 2000) and ascorbate peroxidase (Raven, 2000). The two oxidized forms of vitamin C are reduced by monodehydroascorbate reductase and dehydroascorbate reductase. Vitamin C is a strong reducing agent and readily oxidizes reversibly to dehydroascorbic acid. Disturbed vitamin C metabolisms with decreased vitamin C concentrations have been reported both in experimentally induced DM and in diabetic patients (Ceriello *et al.*, 1998). An oxidative stress is thought to be responsible for vitamin C loss and the resulting increase in dehydroascorbic acid concentration in plasma.

Beneficial effects of vitamin E have been reported repeatedly at least on the diabetic animals (Palmer *et al.*, 1998; Vinson *et al.*, 1994), while the results of vitamin C supplementation are varying from beneficial to detrimental (Mekinova *et al.*, 1995; Vinson *et al.*, 1994). This aspect of vitamin was confirmed again in the current study. However, the beneficial effects of vitamin C were not observable. Strikingly, vitamin C aggravated the lung pathology in DM rats. The lung pathology showed the interstitial cell infiltration and the extensive fibrosis, and even the organization of the alveolar exudates was apparent. Administration of rutin, a flavonoid with metal chelating activity, prevented the vitamin C-induced lung damage in DM rats. Rutin did not show any particular effect when administered alone, either to normal control or DM rats without vitamin C supplementation. The results, especially those of the lung, strongly support the previously suggested role of metal-catalyzed oxidation, or Fenton reaction, in the development of the diabetic complications. Normally, the level of transition metals like iron or copper in aqueous phase *in vivo* are infinitesimal, preventing the uncontrolled propagation of radical production and damage (Afanas'eva *et al.*, 2001).

In DM, antioxidant defense system is compensated in various ways. Probably the most important defect might be, as suggested by the current results, the increased availability of the transition metals. Direct measurement of the level of free metal in the biological samples is impossible, because the kinetics of the metal binding to proteins is very fast and complete. The very small amount of free metal in the aqueous phase is enough for the increased production of the radicals enough to cause the damages to biomolecules (Hunt *et al.*, 1992). The aggravation of

the oxidative damage by vitamin C and prevention by metal chelator, however, strongly support the case of Fenton reaction (Kang *et al.*, 1998).

In the current experiment, this Fenton reaction apparently occurred in diabetic animals. The source of the free transition metal in the aqueous phase in DM may be the release from the binding proteins in the plasma. When it combines with other factors like the compromise of the microcirculation in cases of sciatic nerve, the oxidative damages become extensive enough to produce outright pathological changes and symptom. Vitamin C level might aggravate the Fenton reaction. This is further supported by the effect of rutin. Rutin, administered with vitamin C, prevented the aggravation of the oxidative damage. Because the main action of rutin *in vivo* is the metal chelation, it might chelate whatever amount of the transition metals released from the binding protein and sequester them from the aqueous phase, preventing the Fenton reaction (Afanas'ev *et al.*, 1989). Fenton reaction may contribute to the development of diabetic complications that can be inhibited by metal chelator, which cannot be corrected by vitamin C. Therefore, vitamin C in DM, especially in insulin-dependent DM, without correction of glucose metabolism or metal chelation is undesirable.

In conclusion, oxidative stress was increased in the sciatic nerve or lung of DM rats and vitamin C had no effects on oxidative parameter such as DNPH incorporation or conjugated dienes content. Vitamin C caused marked pathological changes in the lung of DM. The increases of parenchyma and the thickening of alveolar septa were observed and Rutin blocked such pathological changes.

REFERENCES

Afanas'ev, I. B., Dorozhko, A. I., Brodskii, A. V., Kostyuk, V. A., and Potapovitch, A. I., Chelating and free radical scavenging mechanisms of inhibitory action of rutin and quercetin in lipid peroxidation. *Biochem. Pharmacol.*, 38, 1763-1769 (1989).

Afanas'ev, I. B., Ostrachovich, E. A., and Korkina, L. G., Effect of rutin and its copper complex on superoxide formation and lipid peroxidation in rat liver microsomes. *FEBS Lett.*, 425, 256-258 (1998).

Afanas'eva, I. B., Ostrakhovitch, E. A., Mikhal'chik, E. V., Ibragimova, G. A., and Korkina, L. G., Enhancement of antioxidant and anti-inflammatory activities of bioflavonoid rutin by complexation with transition metals. *Biochem. Pharmacol.*, 61, 677-684 (2001).

Andersen, A. R., Christiansen, J. S., Andersen, J. K., Kreiner, S., and Deckert, T., Diabetic nephropathy in Type 1 (insulin-dependent) diabetes: an epidemiological study. *Diabetologia*, 25, 496-501 (1983).

Bors, W., Heller, W., Michel, C., and Saran, M., Flavonoids as antioxidants: determination of radical-scavenging efficiencies. *Methods Enzymol.*, 186, 343-355 (1990).

Cay, M., Naziroglu, M., Simsek, H., Aydilek, N., Aksakal, M., and Demirci, M., Effects of intraperitoneally administered vitamin C on antioxidative defense mechanism in rats with diabetes induced by streptozotocin. *Res. Exp. Med. (Berl),* 200(3), 205 -213 (2001).

Ceriello, A., Bortolotti, N., Crescentini, A., Motz, E., Lizzio, S., Russo, A., Ezsol, Z., Tonutti, L., and Taboga, C., Antioxidant defences are reduced during the oral glucose tolerance test in normal and non-insulin-dependent diabetic subjects. *Eur. J. Clin. Invest.*, 28, 329-333 (1998).

Christiansen, J. S., Gammelgaard, J., Tronier, B., Svendsen, P. A., and Parving, H. H., Kidney function and size in diabetics before and during initial insulin treatment. *Kidney Int.*, 21, 683-688 (1982).

Cross, C. E., Halliwell, B., Borish, E. T., Pryor, W. A., Ames, B. N., Saul, R. L., McCord, J. M., and Harman, D., Oxygen radicals and human disease. *Ann. Intern. Med.*, 107, 526-545 (1987).

Ellis, E. N., Steffes, M. W., Goetz, F. C., Sutherland, D. E., and Mauer, S. M., Relationship of renal size to nephropathy in type 1 (insulin-dependent) diabetes. *Diabetologia*, 28, 12-15 (1985).

Freeman, B. A. and Crapo, J. D., Hyperoxia increases oxygen radical production in rat lungs and lung mitochondria. *J. Biol. Chem.*, 256, 10986-10992 (1981).

Grinberg, L. N., Rachmilewitz, E. A., and Newmark, H., Protective effects of rutin against hemoglobin oxidation. *Biochem. Pharmacol.*, 48, 643-649 (1994).

Hunt, J. V., Bottoms, M. A., and Mitchinson, M. J., Ascorbic acid oxidation: a potential cause of the elevated severity of atherosclerosis in diabetes mellitus? *FEBS Lett.*, 311, 161-164 (1992).

Ihara, H., Shino, Y., Aoki, Y., Hashizume, N., and Minegishi, N., A simple and rapid method for the routine assay of total ascorbic acid in serum and plasma using ascorbate oxidase and o-phenylenediamine. *J. Nutr. Sci. Vitaminol. (Tokyo)*, 46, 321-324 (2000).

Kang, S. A., Jang, Y. J., and Park, H., In vivo dual effects of vitamin C on paraquat-induced lung damage: dependence on released metals from the damaged tissue. *Free Radic. Res.*, 28, 93-107 (1998).

Kinder, R., Ziegler, C., and Wessels, J. M., Gamma-irradiation and UV-C light-induced lipid peroxidation: a Fourier transform-infrared absorption spectroscopic study. *Int. J. Radiat. Biol.*, 71, 561-571 (1997).

Lanng, S., Thorsteinsson, B., Nerup, J., and Koch, C., Diabetes mellitus in cystic fibrosis: effect of insulin therapy on lung function and infections. *Acta Paediatr.*, 83, 849-853 (1994).

Low, P. A. and Nickander, K. K., Oxygen free radical effects in sciatic nerve in experimental diabetes. *Diabetes*, 40, 873-877 (1991).

Low, P. A., Nickander, K. K., and Tritschler, H. J., The roles of oxidative stress and antioxidant treatment in experimental

diabetic neuropathy. *Diabetes*, 46 Suppl 2, S38-S42 (1997).

Mekinova, D., Chorvathova, V., Volkovova, K., Staruchova, M., Grancicova, E., Klvanova, J., and Ondreicka, R., Effect of intake of exogenous vitamins C, E and beta-carotene on the antioxidative status in kidneys of rats with streptozotocin-induced diabetes. *Nahrung.*, 39, 257-261 (1995).

Palmer, A. M., Thomas, C. R., Gopaul, N., Dhir, S., Anggard, E. E., Poston, L., and Tribe, R. M., Dietary antioxidant supplementation reduces lipid peroxidation but impairs vascular function in small mesenteric arteries of the streptozotocin-diabetic rat. *Diabetologia*, 41, 148-156 (1998).

Piotrowski, P., Wierzbicka, K., and Smialek, M., Neuronal death in the rat hippocampus in experimental diabetes and cerebral ischaemia treated with antioxidants. *Folia Neuropathol.,* 39 (3),147-154 (2001).

Pryor, W. A. and Castle, L., Chemical methods for the detection of lipid hydroperoxides. *Methods Enzymol.*, 105, 293-299 (1984).

Pulido, R., Bravo, L., and Saura-Calixto, F., Antioxidant activity of dietary polyphenols as determined by a modified ferric reducing/antioxidant power assay. *J. Agric. Food Chem.*, 48, 3396-3402 (2000).

Raven, E. L., Peroxidase-catalyzed oxidation of ascorbate. Structural, spectroscopic and mechanistic correlations in ascorbate peroxidase. *Subcell. Biochem.*, 35, 317-349 (2000).

Robak, J. and Gryglewski, R. J., Flavonoids are scavengers of superoxide anions. *Biochem. Pharmacol.*, 37, 837-841 (1988).

Samuni, A., Aronovitch, J., Godinger, D., Chevion, M., and Czapski, G., On the cytotoxicity of vitamin C and metal ions. A site-specific Fenton mechanism. *Eur. J. Biochem.*, 137, 119-124 (1983).

Seglen, P. O., Preparation of isolated rat liver cells. *Methods Cell. Biol.*, 13, 29-83 (1976).

Starke, P. E., Oliver, C. N. and Stadtman, E. R., Modification of hepatic proteins in rats exposed to high oxygen concentration. *FASEB J.*, 1, 36-39 (1987).

Strojek, K., Ziora, D., Sroczynski, J. W., and Oklek, K., Pulmonary complications of type 1 (insulin-dependent) diabetic patients. *Diabetologia*, 35, 1173-1176 (1992).

Thrall, R. S., McCormick, J. R., Jack, R. M., McReynolds, R. A., and Ward, P. A., Bleomycin-induced pulmonary fibrosis in the rat: inhibition by indomethacin. *Am. J. Pathol.,* 95, 117-130 (1979).

Vinson, J., Hsu, C., Possanza, C., Drack, A., Pane, D., Davis, R., Klock, C., Graser, K., and Wang, X., Lipid peroxidation and diabetic complications: effect of antioxidant vitamins C and E. *Adv. Exp. Med. Biol.*, 366, 430-432 (1994).

Wright, A. and Nukada, H., Sciatic nerve morphology and morphometry in mature rats with streptozocin-induced diabetes. *Acta Neuropathol.*, 88, 571-578 (1994).

Zhang, P. and Omaye, S. T., Antioxidant and prooxidant roles for beta-carotene, alpha-tocopherol and ascorbic acid in human lung cells. *Toxicol. In Vitro*, 15, 13-24 (2001).