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The Role of Ascorbic Acid on the Redox Status and the Concentration of Malondialdehyde in Streptozotocin-Induced Diabetic Rats

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We investigated the role of ascorbic acid on the redox status in streptozotocin-induced diabetic rats. In the plasma of diabetic rats, the ratio of reduced/total ascorbic acid was significantly decreased as compared with normal control. Ascorbic acid supplementation increased the reduced and total ascorbic acid contents as compared with diabetic control. In the rutin-treatment group, reduced and total contents of ascorbic acid were significantly decreased, however, the ratio of reduced/total contents of ascorbic acid had no difference as compared with diabetic rats. In the insulin-treatment group, this ratio is not significantly different as compared with diabetic control. However, in the insulin plus ascorbic acid treatment group, reduced form and the ratio of reduced/total ascorbic acid were significantly increased as compared with diabetic control. In addition, we measured the contents of malondialdehyde (MDA) in the plasma of diabetic rats. The contents of MDA was increased as compared with normal control, however, in insulin-treatment group, the contents of MDA was decreased as compared with diabetic rats. Ascorbic acid had no effects on the increases of MDA in diabetic rats. In conclusion, plasma ascorbic acid level and its reduced/total ratio reflects the status of the oxidative stress in the diabetic rats. Supplement of ascorbic acid did not correct the ratio of the reduced/total ascorbic acid. However, supplement of insulin and ascorbic acid corrected the ratio of reduced/total ascorbic acid.

Key words: Diabetes mellitus, Malondialdehyde, Ascorbic acid, Redox status

INTRODUCTION

A free radical is a molecule that contains an unpaired electron in its outer orbit and that can exist independently. Because electrons must have opposite spin to occupy the same orbit, electrons added to molecular oxygen must be transferred one at a time during its reduction, resulting in several highly reactive intermediates of which unstable electron configurations allow for the attraction of electrons from other molecules, resulting in another free radical that is capable of reacting with yet another molecule. This reaction is thought to contribute to lipid peroxidation, DNA damage, and protein degradation during oxidatively stressful events. The most well described consequence of the generation of free radicals and reactive oxygen species is lipid peroxidation. Most investigations have examined byproducts of lipid peroxidation, which are conjugated dienes, lipid hydrocarbons, and thiobarbituric acid reactive substances (TBARS). The most widely used technique to evaluate lipid peroxidation is the use of TBARS that include malondialdehyde(MDA) formed in peroxidizing systems, with results generally expressed as millimoles of MDA equivalents. Antioxidant defenses in the cell can temper the negative influence of free radicals and associated reactions. Vitamn E protects against lipid peroxidation by acting directly with a variety of oxygen radicals to form a relatively innocuous tocopherol radical. Ascorbic acid can interact with the tocopherol radical to regenerate reduced tocopherol. Changes in blood amounts of ascorbic acid and vitamin E in the blood have been used to indicate increased oxidative reaction.

Diabetes mellitus is associated with a high oxidative stress level, resulting from an imbalance between free

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radicals or reactive oxygen species production and the antioxidant systems. Antioxidants such as ascorbic acid, vitamin E, uric acid, and glutathione are decreased in diabetic rats (Singal *et al.*, 2001).

Ascorbic acid (2,3-enediol-L-gulonic acid) plays important roles as a chemical reducing agent, an antioxidant, and an enzymatic cofactor and is an essential dietary factor for many insects, invertebrates, and some avian and mammalian species, including humans (Cay *et al.*, 2001) existing as a reduced form, ascorbic acid, and an oxidized form, dehydroascorbic acid, *in vivo*. Disturbed ascorbic acid metabolisms with reduced ascorbic acid concentrations have been reported both in experimentally induced diabetes mellitus (DM) and in diabetic patients (McLennan *et al.*, 1988; Palmer *et al.*, 1998). An oxidative stress is thought to be responsible for ascorbic acid loss and the resulting increase in dehydroascorbic acid concentration in plasma (Abou-Seif *et al.*, 2000). The relative content of dehydroascorbic acid and ascorbic acid has been suggested to be a useful biomarker reflecting the oxidative stress and overall radical level on DM. Above mention corresponds to the fact that injection of dehydroascorbic acid into animals affects insulin secretion by the pancreas and induce DM (Pence and Mennear, 1979). Therefore, the ratio of ascorbic acid to total ascorbic acid as well as the level of total ascorbic acid is important.

Numerous high-performance liquid chromatographic methods have been developed for the analysis of ascorbic acid (Iwata *et al.*, 1985; Speek *et al.*, 1984). The electrochemical detector is suitable for measuring ascorbic acid content in biological fluids and tissues. It has been developed on the basis of the oxidation-reduction properties of ascorbic acid. Many of the chromatographic systems are designed for the simultaneous measurement of ascorbic and dehydroascorbic acid as well as their isomers and derivatives. To measure dehydroascorbic acid, first reduce it to ascorbic acid by a reducing agent such as dithiothreitol before analysis (Lykkesfeldt, 2000). Both the ion exchange and reversed phase chromatographic columns are commonly used.

In this study, we investigated the absolute concentration and redox status of ascorbic acid and the effects of ascorbic acid, rutin, and insulin on the reduced/total contents of ascorbic acid in diabetic rats. The relation between ascorbic acid status and other parameters of oxidative stress such as malondialdehyde (MDA), a by-product of lipid peroxidation was investigated.

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 (6 weeks, 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). 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. Ascorbic acid or rutin was given at concentration of 1 g/L each in drinking water, 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 ascorbic acid solution was adjusted to 7.0 by adding sodium bicarbonate (2 g/L). After 8 week of induction of DM, some rats were randomly selected to inject insulin. Pork insulin was injected subcutaneously at a daily dose of 8-80 U/kg for 1-4 weeks. The dosage of insulin was adjusted by monitoring urinary glucose twice a day to prevent ketonuria or glucosuria (Wohaieb and Godin, 1987).

Plasma

Blood was collected from rat-tail artery or femoral artery. Blood samples were immediately centrifuged and an aliquot of the top plasma of each sample was directly mixed with an equal volume of precooled 10% PCA for ascorbic acid measurement. The precipitate was spun down by centrifugation and the samples were kept at -70 until analysis.

Determination of Malondialdehydes (MDA) in plasma

For preparation of samples to be analyzed, an aliquot of 10 μL plasma was mixed with 1.0 mL of 0.2% TBA in 2 M sodium acetate buffer (containing 1 mM DTPA, pH 3.5) and 10 μL of 5% BHT in 99% ethanol. The sample mixture was incubated at 95 for 45 min. After cooling with tap water, it was then passed through a 0.2-μm Millipore filter. The TBA-MDA complex was eluted at flow rate of 1 mL/

min and the complex was monitored by fluorescence detector with excitation at 515 nm and emission at 553 nm absorbance using octadecylsilyl reverse phase column and 0.1% ethanolamine/acetonitrile (2:1, v/v) as a mobile phase. The TBA-MDA complex was determined by fluorescence detection using a BAS model FL-45 fluorescence detector (excitation 515 nm, emission 553 nm) (Therasse and Lemonnier, 1987). The plasma MDA level was calculated from the calibration curve prepared using 1,1,3,3-tetraethoxypropane (Fukunaga *et al.*, 1995; Hultqvist *et al.*, 1997; Nielsen *et al.*, 1997).

Ascorbic acid measurements

Reduced ascorbic acid

The PCA-treated plasma samples were gently thawed and kept on ice at all times. For the ascorbic acid measurement, 100 μL of sample supernatant was diluted with 0.125 M phosphate buffer 250 μL (pH 9.0), resulting in a pH of 2.6 to 2.7. Twenty microlitres of this mixture was injected into the HPLC system.

Total ascorbic acid

Total ascorbic acid was measured after reduction of the dehydroascorbic acid present in the sample. The reduction was carried out by adding 100 μL of the supernatant of the PCA-treated plasma sample to 0.5 M phosphate buffer 200 μL (pH 9.0), containing 10 mM dithiothreitol (DTT) resulting in a pH ranging from 6.1 to 6.2. After a reduction period (25°C, 5 min), the reaction was quenched by addition of 0.1 M H_2SO_4 50 μL. This resulted in a final pH of 2.6 to 2.7 and the sample was cooled to 0°C. Twenty microlitres of the mixture was used for the HPLC analysis (Lykkesfeldt *et al.*, 1995).

Chromatographic analysis

The plasma samples were analyzed on an automated HPLC system consisting of the following Pharmacia instruments units: autosampler (samples protected from light) and LKB 2248 pump. For peak detection, the chromatographic system was connected to an electrochemical detector equipped with a Shimazu L-ECD 85 mV. All units were connected to a personal computer for control and for collection and analysis of data (HPLC-manager, Nelson). The column was a Nova-pak c18 (particle size 4 μm, pore size 60 nm, 150×3.9 mm i.d., Waters) operated at 1.0 ml/min and fitted with a Nova-pak C18 precolumn (guard pak system, Waters). The precolumn was changed when the backpressure had increased significantly after injection of 400 to 600 samples. The mobile phase consisted of 0.1 M disodium hydrogen phosphate, 2.5 mM disodium-EDTA, and 2.0 mM tetrabutylammoniumhydroxide and the pH was adjusted to 3.0 with ortho-phosphoric acid. The mobile phase was filtered through a 0.45 μm filter under vacuum and degassed before use.

Glycosylated hemoglobin (Hb A_{1c}) assay

Hb A_{1c} representing long-term hyperglycemia was measured with Glyc-Affin GHb kit using affinity chromatography (Abraham *et al.*, 1983). The affinity gel (phenylboronic acid immobilized on agarose) and the microcolumns prepacked with this gel (Glyc-Affin System) were obtained from Isolab. For routine determination of glyco Hb, microcolumns were used according to the procedure supplied by this company. A 50-μL aliquot of the saline-washed red cells was lysed with 400 μL of the sample preparation reagent and 50 μL of this lysate was applied to a microcolumn. The nonglycosylated Hb fraction was eluted first in about 10 min with the first fraction elution agent. The glyco Hb fraction, which was bound to the gel, was then eluted with the second fraction elution buffer. The absorbance of each fraction was read at 415 nm in a spectrophotometer. The percentage glyco Hb value was calculated on the basis of these readings.

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

The role of ascorbic acid, rutin, and insulin on blood glucose and glycosylated hemoglobin in streptozotocin-induced diabetic rats

In diabetic rats, levels of plasma glucose and glycosylated hemoglobin were significantly increased as compared with those of normal control. Ascorbic acid or rutin had no effects on the increases of plasma glucose; however, insulin inhibited the increases of plasma glucose at 4 weeks-treatment groups (Table I). Ascorbic acid or rutin decreased the increases of glycosylated hemoglobin at 2 only weeks-treatment groups; however, insulin significantly inhibited the increases of glycosylated hemoglobin both at 2 weeks- and 4 weeks-treatment groups (Table I).

The role of ascorbic acid, rutin, and insulin on the body weight gain, pH, pCO_2, and pO_2 in streptozotocin-induced diabetic rats

In diabetic rats, body weight and pH of heparinized whole blood were decreased as compared with normal control. Ascorbic acid or rutin had no effects on the decreases of body weight and pH of heparinized whole blood. Insulin significantly protected from the decreases of body weight, but had no effects on the alteration of pH of heparinized

Table I. The effects of ascorbic acid, rutin, and insulin on blood glucose and glycosylated hemoglobin in streptozotocin-induced diabetic rats

Treatment	2 weeks		4 weeks	
	Plasma glucose (mg/dl)	Hb A_{1c} (%)	Plasma glucose (mg/dl)	Hb A_{1c} (%)
Normal control	123.3 ± 24.7	4.0 ± 0.1	156.3 ± 5.1	5.9 ± 0.6
DM	380.7 ± 13.8#	13.4 ± 0.3#	482.0 ± 21.1#	15.7 ± 0.4#
DM+Ascorbic acid	403.8 ± 13.8#	12.8 ± 0.2#	468.0 ± 98.0#	15.0 ± 0.3#
DM + Rutin	350.8 ± 25.2#	12.0 ± 0.4**,#	504.0 ± 41.1#	15.4 ± 0.6#
DM + Insulin	388.0 ± 17.5#	10.2 ± 0.3**,#	390.5 ± 29.5*,#	10.6 ± 0.5**,#

The values are expressed as mean±S.E.M. for 8 animals. *P<0.05; **P<0.01 vs. diabetic rats. #P<0.05 vs. normal control. Hb A_{1c}; Glycosylated hemoglobin, DM; Diabetic rat.

Table II. The effects of ascorbic acid, rutin, and insulin on the body weight gain, pH, pCO_2, and pO_2 in streptozotocin-induced diabetic rats

Treatment	Body weight gain (%)	pH	pCO_2 (mmHg)	pO_2 (mmHg)
Normal control	42.9 ± 3.5	7.44 ± 0.01	35.3 ± 2.3	83.7 ± 5.6
DM	-11.7 ± 4.3#	7.38 ± 0.01#	49.5 ± 2.3	82.0 ± 2.1
DM+Ascorbic acid	-25.7 ± 5.2#	7.33 ± 0.02#	51.9 ± 2.6	80.5 ± 5.6
DM + Rutin	-11.7 ± 5.1#	7.30± 0.02#	48.0 ± 4.7	74.5 ± 4.2
DM + Insulin	13.5 ± 3.7**,#	7.38 ± 0.03#	39.4 ± 2.5	88.0 ± 4.9

The values are expressed as mean±S.E.M. for 8 animals. **P<0.01 vs. diabetic rats. #P<0.05 vs. normal control. DM; Diabetic rat.

Table III. The effects of ascorbic acid, rutin, and insulin on the concentration of malondialdehyde (nM) in the plasma of streptozotocin-induced diabetic rats

Treatment	2 weeks MDA (nM)	4 weeks MDA (nM)
Normal control	253 ± 20	251 ± 40
DM	373 ± 133	591 ± 66#
DM + Ascorbic acid	430 ± 34	616 ± 35#
DM + Rutin	289 ± 34	446 ± 57#
DM + Insulin	257 ± 39	328 ± 10*

The values are expressed as mean ± S.E.M. for 8 animals. *P<0.05 vs. diabetic rats. #P<0.05 vs. normal control. MDA; Malondialdehyde, DM; Diabetic rat.

whole blood as compared with those of diabetic rats (Table II).

The role of ascorbic acid, rutin, and insulin on the concentration of malondialdehyde in streptozotocin-induced diabetic rats

We measured the alteration of malondialdehyde (MDA) concentration in diabetic rats. In 2 weeks-treatment groups, there were no differences in groups of ascorbic acid, rutin, and insulin-treatment. However, in 4 weeks-treatment groups, MDA was significantly increased as compared with normal control and insulin inhibited the increases of MDA. Ascorbic acid or rutin had no effects on the increases of MDA (Table III).

The role of ascorbic acid, rutin, and insulin on the Redox status in streptozotocin-induced diabetic rats

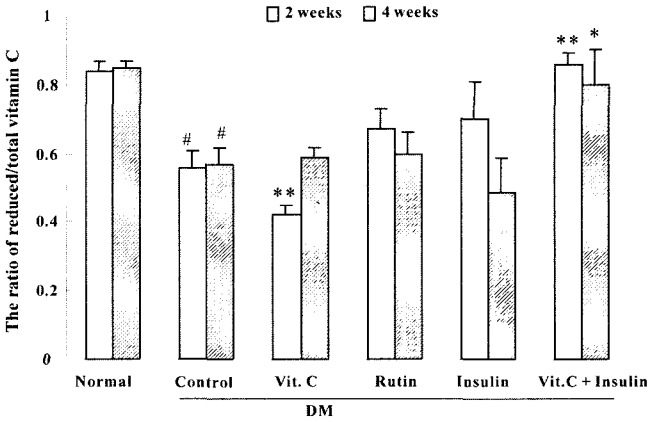

Fig. 1. The effects of ascorbic acid, rutin, and insulin on the ratio of reduced/total ascorbic acid at 2 weeks or 4 weeks after induction of diabetes. In diabetic rats, the ratio of reduced/total ascorbic acid was decreased as compared with normal at both 2 weeks and 4 weeks. However, ascorbic acid plus insulin inhibited the decreases of the ratio of reduced/total ascorbic acid. Rutin had no effect on the ratio of reduced/total ascorbic acid. Data are mean±S.E. for 8 animals. *P<0.05 vs. diabetic rats. #P<0.05 vs. normal control. DM; Diabetic rat.

We measured the alteration of redox status in diabetic rats. In diabetic rats, reduced form of ascorbic acid was significantly decreased as compared with those of normal control both 2 weeks and 4 weeks. The supplement of ascorbic acid increased the reduced and total ascorbic acid, which resulted in the decreases on the ratio of reduced/total form at 2 weeks (data not shown). Insulin had no effects in alteration of redox status in diabetic rats. However, supplement of insulin plus Vit. C significantly

improved the ratio of reduced/total form as compared with diabetic control (Fig. 1).

DISCUSSION

Ascorbic acid is widely regarded as an essential antioxidant in the human body and has even been called the most important antioxidant in human plasma (Cay *et al.*, 2001). The decreased level of ascorbic acid in diabetics has long been known to the medical society, and it has been considered to be due to the accelerated consumption of the vitamin. However, it has been unclear because of the lack of the accurate measurement of the ascorbic acid *in vivo*.

The rate of ascorbic acid degradation depends on ascorbic acid concentration, temperature, pH, oxygen, light, and presence of metal ions (Gibbons *et al.*, 2001; Khan and Martell 1967; Li *et al.*, 2001; Reynolds, 1965; Travacio *et al.*, 2000). When ascorbic acid is heated in an aqueous solution, the major products are dehydroascorbic acid, 2,3-diketogulonic acid, threonic acid, and oxalic acid (Simpson and Ortwerth, 2000). A group of volatile furan-type compounds and reductones are detected by the gas chromatography method. Some of these browning products have antioxidant activity, while others have destructive prooxidant effects, including lipid peroxidation, cytotoxicity, mutagenesis, and adduct formation with proteins and nucleic acids (Omura *et al.*, 1978).

The new improved measuring method of ascorbic acid would make the close monitoring of blood content and the redox status of ascorbic acid in diabetic rats (Lykkesfeldt *et al.*, 1995). For many years it has been possible to measure ascorbic acid in tissues by a variety of chemical procedures. Since ascorbic acid and dehydroascorbic acid are readily interconvertible by oxidation-reduction reactions, methods of analysis based on chemical reactions of either compound may be used. If a reaction of ascorbic acid is chosen, total ascorbic acid may be determined by first reducing any dehydroascorbic acid present.

A variety of agents have been used to extract ascorbic acid. Most are aqueous solutions of acids often including additives such as EDTA to restrict losses of the vitamin. The classical extracting solutions are metaphosphoric acids and trichloroacetic acids at concentrations of 3-6%. These reagents have the added advantage of precipitating proteins, a step which is always necessary before injecting samples onto HPLC columns.

Recently, the method of accurate measurement of plasma ascorbic acid and its redox status has been advanced (Iwata *et al.*, 1985; Speek *et al.*, 1984). With this, it has also been suggested that the level and the redox status of the plasma ascorbic acid represent the systemic redox status, or the presence of the oxidative stress *in vivo*. Actually, the plasma MDA was increased in diabetic rats, implying the generalized increase of oxidative stress (Young *et al.*, 1995). In this study, we measured the concentration of plsma MDA using HPLC. In diabetic rats, the concentration of MDA was significantly increased as compared with normal at 4 weeks groups. It is consistent with Young et al. (Young *et al.*, 1995). We measured the blood ascorbic acid using HPLC-ECD, which detected the reduced ascorbic acid (ascorbic acid). The content of oxidized ascorbic acid (dehydroascorbic acid) has been calculated by subtracting the content of ascorbic acid from the content of total ascorbic acid. The total ascorbic acid is the content of ascorbic acid measured after complete reduction with dithiothreitol (DTT). In diabetic rats, the level of ascorbic acid was significantly decreased and the ratio of reduced/total ascorbic acid or the ratio of ascorbate to total ascorbate also was decreased, suggesting that the more ascorbic acid exists in the oxidized form in the DM (Seghieri *et al.*, 1998). The supplementation of ascorbic acid in the drinking water (0.1% w/v) normalized the plasma level of total ascorbic acid. The supplementation of ascorbic acid, however, did not correct the decreased ratio of reduced/total ascorbic acid. Administration of rutin did not also correct the ratio of reduced/total ascorbic acid.

Insulin is a structurally and functionally well-characterized small globular protein containing A- and B-chains linked by three disulfides (one intrachain bond, A6-A11; two interchain bonds, A7-B7 and A20-B19). Its three-dimensional structure has been well studied by X-ray crystallography (Baker *et al.*, 1988) and NMR (Olsen *et al.*, 1996; Weiss *et al.*, 1991) since the 1970s; there are species differences in the amino acids of both chains. Within the β cell, insulin precursor is produced by DNA- or RNA-directed synthesis. Proinsulin, a long single-chain protein molecule, is processed within the Golgi apparatus and packaged into granules, where it is hydrolyzed into insulin and a residual connecting segment called the C-peptide by removal of four amino acids (Chevenne *et al.*, 1999). In this study, insulin therapy did not fully correct the ascorbic acid redox status. Insulin therapy plus ascorbic acid supplementation normalized the redox status or the total level of ascorbic acid.

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.*, 1998), 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).

Quenching of the oxidizing free radicals, repairing the oxidized macromolecules and regeneration of oxidized ascorbic acid all require the reducing power, that is the high energy electrons associated with the hydrogen. The source of this reducing equivalent of the energy is generated from the glucose metabolism as the form of NAD(P)H. The data suggest that the redox status of the plasma ascorbic acid can reflect the oxidative stress in diabetic rats, which did not appeared to be dependent on ketones. It is probable that the oxidative stress in the diabetic rats reflected by the level of plasma ascorbic acid might be from the impaired glucose metabolism, which might in turn reduce the production of NAD(P)H through the pentose-monophosphate shunt. There is a report that high-dose ascorbic acid supplementation has a beneficial effect in type II diabetics on both glycemic control and blood lipids (Eriksson and Kohvakka, 1995). In the case of type II DM, insulin dependent glucose metabolism might be still working at a certain level. The role of free transition metals *in vivo* has been suspected in various oxidative tissue damages, and the bleomycin-detectable iron has been introduced as the index of the free metal. On the other hand, there has been a report that ascorbic acid does not act as a pro-oxidant toward lipids in plasma containing bleomycin-detectable iron which is free iron or excess iron, and acts as an antioxidant (Berger *et al.*, 1997).

In summary, plasma ascorbic acid level and its reduced/total ratio reflects the status of the oxidative stress in the diabetic rats. Supplement of ascorbic acid or insulin did not correct the ratio of the reduced/total ascorbic acid. However, supplement of insulin plus ascorbic acid corrected the reduced/total ascorbic acid ratio. The contents of MDA was increased as compared with normal control, however, in insulin-treatment group, the contents of MDA was decreased as compared with diabetic rats. Ascorbic acid had no effects on the increases of MDA in diabetic rats.

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