Effect of Astaxanthin in Combination with α-Tocopherol or Ascorbic Acid against Oxidative Damage in Diabetic ODS Rats

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Summary The present study was performed to investigate the effect of astaxanthin in combination with other antioxidants against oxidative damage in streptozotocin (STZ)-induced diabetic Osteogenic Disorder Shionogi (ODS) rats. Diabetic-ODS rats were divided into five groups: control, astaxanthin, ascorbic acid, α-tocopherol, and tocotrienol. Each of the four experimental groups was administered a diet containing astaxanthin (0.1 g/kg), in combination with ascorbic acid (3.0 g/kg), α-tocopherol (0.1 g/kg), or tocotrienol (0.1 g/kg) for 20 wk. The effects of astaxanthin with other antioxidants on lipid peroxidation, urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) excretion, serum creatinine (Cr) level, creatinine clearance (Ccr), and urinary protein content were assessed. The serum lipid peroxide levels and chemiluminescent (CL) intensity in the liver of the α-tocopherol and tocotrienol groups were significantly reduced in comparison to that of the control group. In the α-tocopherol group, urinary 8-OHdG excretion, serum Cr level, Ccr, urinary albumin excretion, and urinary protein concentration were significantly decreased as compared with those in the control group. Additionally, the CL intensity in the kidney of the α-tocopherol group was significantly lower, but that of the ascorbic acid group was significantly higher than that in the control group. These results indicate that dietary astaxanthin in combination with α-tocopherol has an inhibitory effect on oxidative stress. On the other hand, our study suggests that excessive ascorbic acid intake increases lipid peroxidation in diabetic rats.

Key Words astaxanthin, α-tocopherol, ascorbic acid, oxidative stress, diabetes

Diabetes mellitus, which is characterized by hyperglycemia, is considered to induce oxidative stress via various mechanisms. These include glucose auto-oxidation, the formation of advanced glycation end-products (AGEs), and activation of the polyol pathway (1, 2). Enhanced oxidative stress generates lipid peroxidation and oxidized DNA, which causes damage to the tissue, and has been closely linked to diabetic complications such as retinopathy, neuropathy, and nephropathy (3, 4).

Antioxidants in fruits and vegetables have been reported to reduce and remove free radicals and lipid peroxidation. The increase of antioxidant status, which is achieved through sufficient intake of antioxidants, has been hypothesized to play an important role in protection against chronic disease in humans (5, 6). Astaxanthin, a xanthophyll carotenoid, possesses a potent antioxidant activity, and has a variety of biological activities, including anti-diabetes and anti-obesity effects (7–9). α-Tocopherol is an important lipophilic antioxidant in plasma and tissue, and may protect cellular components against peroxidative damage via the free radical scavenging mechanism (10). Among the vitamin E group, the four tocopherols and the four tocotrienols are distinguished structurally by their side chains. On the other hand, ascorbic acid, which exerts powerful antioxidative properties on the hydrophilic compartment, can scavenge chain initiation by removing aqueous radicals (11). Moreover, ascorbic acid potentiates the antioxidant effects of vitamin E by reducing tocopheroxyl radicals (12). The beneficial effects of these vitamins are assumed to counteract the oxidative stress induced by diabetes as antioxidants, and can act synergistically in a combination of these two antioxidant agents (13, 14). Thus, the administration of two different antioxidants, such as carotenoid and α-tocopherol or ascorbic acid, may be more effective against oxidation than either used alone. Hitherto, there has been no report on the effect of astaxanthin in combination with other antioxidants in diabetic-induced Osteogenic Disorder Shionogi (ODS) rats.

In this study, we investigated the effects of dietary astaxanthin in combination with α-tocopherol, tocotrienol, or ascorbic acid on oxidative stress in diabetic-ODS rats, and additionally examined the effect of combining astaxanthin with a high dose of ascorbic acid.

MATERIALS AND METHODS

Animals and diets. Male, 6-wk-old inherently scorbptic (Osteogenic Disorder Shionogi ODS (od/od)) rats, weighing about 180-200 g, were purchased from CLEA
Japan, Inc. (Tokyo, Japan). The ODS rats were given a basal diet and water containing 1 g ascorbic acid/L to prevent a vitamin C deficiency, before being fed the experimental diet (15). The ODS rats, weighing about 260 g on average, were injected with a single dose of streptozotocin (STZ, 45 mg/kg body weight; Sigma Chemical Co., MO, USA) dissolved in saline. The increase of the glucose level in the urine of diabetic rats was checked with a commercial kit (AKRAL-120, Shibayagi, Gunma, Japan) and a method of Lowry et al. (16), and adjusted by urinary Cr values. The creatinine clearance (Ccr, milliliters per minute per kilogram) was calculated as urinary Cr urine volume serum Cr$^{-1}$ body weight$^{-1}$.

Measurement of lipid peroxidation and urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG). The lipid peroxide levels in the serum were measured as the thiobarbituric acid reactive substances (TBARS) using the method of Yagi (17) as described in a previous study (18). Chemiluminescence (CL), generated via free radicals, is related to lipid peroxidation (19), and was determined as described by a previous study (18). The urinary 8-hydroxy-2′-deoxyguanosine (8-OHdG) concentration was measured using the 8-OHdG enzyme-linked immunosorbent assay kit (Japan Institute for Control Aging, Shizuoka, Japan), and adjusted by urinary Cr values.

Determination of α-tocopherol and α-tocotrienol concentration in serum and liver. The α-tocopherol and tocotrienol concentration in the serum was determined using the modified method described by Kieu et al. (20). The levels of these compounds in the liver were measured using the method of Ueda and Igarashi (21) with a slight modification. Briefly, the liver (100 mg) was homogenized with 1% NaCl solution (0.1 mL). The homogenate was pipetted into 10 mL centrifuge tubes with caps and 6% ethanolic pyrogallol (1.0 mL), 2% NaCl solution (2.25 mL), hexane solution of 2,2,5,7,8-pentamethyl-6-chromanol (1 μg/mL) as an internal...
acetone containing 0.2 mL were added and mixed well. After centrifugation (3,000 rpm, 5 min), the supernatant was evaporated under N₂ (5.0 mL). After centrifugation (3,000 rpm, 5 min), the standard (0.1 mL), and 50% ether hexane solution (Sigma Chemical Co.) used as an internal standard of the HPLC system. The HPLC system was composed of a JASCO DU-980 chromophotograph (JASCO, Tokyo, Japan), a Shimadzu SPD-10A V UV-VIS detector at 474 nm, a Shimadzu spectrofluorometric detector (Ex. 297 nm, Em. 327 nm). The mobile phase was methanol/2-propanol (94:4 v/v) at a flow rate of 1.0 mL/min.

**Table 2.** α-Tocopherol and α-tocotrienol concentrations in serum and liver of ODS-diabetic rats.

| Rat groups | Control | Astaxanthin | Ascorbic acid | α-Tocopherol | Tocotrienol |
|------------|---------|-------------|---------------|--------------|------------|
| Serum      | 0.24±0.08 | 2.17±0.41** | 2.29±0.41**   | 18.65±2.27*** | 5.62±0.56*** |
| Liver      | —       | —           | —             | —            | 0.46±0.08  |

| Rat groups | Control | Astaxanthin | Ascorbic acid | α-Tocopherol | Tocotrienol |
|------------|---------|-------------|---------------|--------------|------------|
| Serum      | —       | 14.7±4.8    | 17.8±5.2      | 22.7±8.7     | 13.7±3.7   |
| Liver      | —       | 22.6±10.4   | 24.3±9.9      | 43.8±16.2    | 23.4±9.9   |

Values are means±SD (n=6–7).

Statistical significance: **p<0.01, ***p<0.001 vs. control group; ##p<0.01, ###p<0.001 vs. astaxanthin group.

**RESULTS**

α-Tocopherol and α-tocotrienol concentrations in serum and liver

The α-tocopherol and tocotrienol concentrations are indicated in Table 2. The accumulation of α-tocopherol in the serum and liver of the α-tocopherol or tocotrienol groups varied directly with either α-tocopherol or tocotrienol supplementation, respectively. The α-tocopherol levels in the serum and liver of the α-tocopherol group were 43 and 82 times higher than those in the control group (both p<0.001). Although the diet with a low dose of α-tocopherol was given to rats in the astaxanthin, ascorbic acid, and control groups, the α-tocopherol levels in the serum and liver of both the astaxanthin and ascorbic acid groups were about 10 times higher (p<0.01) than those in the control group. Moreover, in comparison with the astaxanthin group, the α-tocopherol levels in the serum and liver of the α-tocopherol and tocotrienol groups were significantly higher.

**Astaxanthin concentration in serum and liver**

The astaxanthin concentration in the serum and liver is shown in Table 3. All of the groups were fed astaxanthin, except the control group, and astaxanthin was detected in the serum and liver. In the α-tocopherol group, the astaxanthin concentrations in the serum and liver were 1.5 to 2.0 times higher than those in the astaxanthin group; however, there was no significant difference among the four groups.

**Body weight and biochemical parameters of serum, liver, kidney, and urine**

Table 4 shows the effects of astaxanthin and other

ANOVA followed by Dunnett’s test, and significant difference was determined among groups at p<0.05.
Table 4. Body weight and biochemical parameters in serum, liver, kidney, and urine of ODS-diabetic rats.

| Rat groups       | Control | Astaxanthin | Ascorbic acid | α-Tocopherol | Tocotrienol |
|------------------|---------|-------------|---------------|--------------|------------|
| Body weight (g)  | 333±1   | 343±27      | 338±19        | 342±6        | 342±10     |
| Serum glucose (mg/dL) | 320±40  | 335±41      | 361±65        | 313±40       | 324±14     |
| Hemoglobin A1c (%) | 7.6±0.8 | 7.2±0.7     | 7.4±0.5       | 7.3±0.4      | 7.1±0.4    |
| Urinary 8-OHdG (ng/mg creatinine) | 52±8    | 43±6        | 51±8          | 38±6*        | 39±7       |
| Serum CL intensity (counts/min)¹ | 245,784 | 45,784      | 28,712        | 38,770       | 44,303     |

¹ Chemiluminescent (CL) intensity assay.
Statistical significance: *p<0.05, **p<0.01 vs. control group; #p<0.05, ##p<0.01 vs. astaxanthin group.

Table 5. Kidney weight and renal function parameters in serum and urine of ODS-diabetic rats.

| Rat groups       | Control | Astaxanthin | Ascorbic acid | α-Tocopherol | Tocotrienol |
|------------------|---------|-------------|---------------|--------------|------------|
| Kidney weight (% body weight) | 1.29±0.1 | 1.14±0.17   | 1.19±0.18     | 1.05±0.12    | 1.17±0.04  |
| Urine volume (mL/24 h) | 179±41  | 146±21      | 176±29        | 139±32       | 146±39     |
| Serum creatinine (mg/dL) | 0.84±0.07 | 0.81±0.07  | 0.87±0.10     | 0.68±0.06*   | 0.78±0.07  |
| Urinary albumin (µg/mg creatinine) | 142±17  | 120±26      | 149±29        | 106±14*      | 118±16     |
| Urinary protein (mg/mg creatinine) | 12.0±2.1 | 9.2±2.2    | 11.1±1.9      | 8.0±1.3*     | 8.3±1.9    |
| Ccr (mL/min/kg body weight) | 4.57±0.56 | 4.71±0.84  | 4.44±0.46     | 5.91±0.59*   | 5.00±0.84  |

Values are means±SD (n=6–7). Ccr, creatinine clearance.
Statistical significance: *p<0.05 vs. control group.

antioxidants on body weight and biochemical parameters of the serum, liver, kidney, and urine. The dietary antioxidant supplementation did not affect the body weight, HbA1c, or serum glucose levels in the ODS-diabetic rats. However, there was a significant decrease (p<0.05) in urinary 8-OHdG content in the α-tocopherol group in comparison with the control group. The lipid peroxide levels in the serum, liver, and kidney were significantly decreased 88% (p<0.01), 80% (p<0.01), and 30% (p=0.01), respectively, by the administration of astaxanthin and α-tocopherol. The reductions of lipid peroxidation in the serum and liver of the tocotrienol group were 82% (p<0.05) and 38% (p<0.01), respectively. However, in the kidney, the lipid peroxide level of the ascorbic acid group was significantly higher (24%; p<0.05) than that of the control group. Furthermore, lipid peroxidation in the liver and kidney of the α-tocopherol group was significantly lower (p<0.01, p<0.05) than that of the astaxanthin group, while that of ascorbic acid group was significantly higher (both, p<0.05).

Kidney weight and renal function parameters of serum and urine

Kidney weight and renal function parameters of serum and urine are indicated in Table 5. The α-tocopherol group showed a decrease in the urine volume, but no significant differences were observed among the five groups. The kidney weight showed a tendency to decrease compared with the control group. The serum Cr level, urinary albumin excretion, urinary protein concentration, and Ccr as the renal function parameter, were significantly decreased (p<0.05) in the α-tocopherol group in comparison with that of the control group.

DISCUSSION

Oxidative stress resulting from increased production of reactive oxygen species (ROS) plays a key role in the pathogenesis of diabetic complications (3, 22). Recent research has focused on the potential role of antioxidant supplementation as a protection against the onset and progression of diabetic complications. In this study, we observed that the supplementation of astaxanthin and α-tocopherol is the most useful of the antioxidant combinations, indicating its ability to suppress oxidative stress.

In our data (Table 2), in the astaxanthin group and ascorbic acid group, the dietary astaxanthin elevated the α-tocopherol concentrations in the serum and liver, even with the ingestion of low doses of α-tocopherol. In regard to antioxidant synergism between astaxanthin and α-tocopherol, a pronounced α-tocopherol-sparing action of astaxanthin was reported in the brain of Atlantic salmon (23). However, the mechanism by
which astaxanthin exerts its effect in these systems is not known. Several recent in vitro studies have reported that astaxanthin is an effective inhibitor of lipid peroxidation in the microsomal membrane, suggesting that its suppressing effect is more potent than that of \( \alpha \)-tocopherol (24, 25). Moreover, the presence of astaxanthin inhibited the loss of \( \alpha \)-tocopherol (24). These results suggest that astaxanthin may contribute to an interactive sparing of \( \alpha \)-tocopherol.

Enhanced oxidative stress has been shown in the kidney of STZ-induced diabetic rats (26). An increase of lipid peroxidation or urinary excretion of 8-OHdG in patients with diabetic nephropathy has also been reported, because the kidney is vulnerable to oxidative damage (22, 27). In our results, the increased levels of urinary 8-OHdG and lipid peroxidation in the serum, liver, and kidney were observed in the control group (Table 4). These results support the observation that enhanced oxidative stress is involved in the etiology of diabetic nephropathy. In contrast, in the \( \alpha \)-tocopherol group, the combined effects of astaxanthin and \( \alpha \)-tocopherol dramatically decreased both the urinary 8-OHdG concentration and lipid peroxide levels in the serum, liver, and kidney (Table 4). Additionally, astaxanthin in combination with \( \alpha \)-tocopherol positively reduced these biomarkers of oxidative stress more effectively than astaxanthin alone (Table 4). Recently, it was reported that urinary 8-OHdG is considered to be a parameter by which to estimate diabetic complications, such as nephropathy (4, 22). The inhibitory effect of urinary 8-OHdG content in the \( \alpha \)-tocopherol group might, at least in part, ameliorate the development of diabetic nephropathy.

Diabetic nephropathy is characterized by persistent proteinuria, hypertension, and deterioration of the renal function. The presence of excessive oxidative stress in the glomeruli of diabetic rats is implicated in the mesangial expression of the extracellular matrix and renal dysfunction, such as increased glomerular filtration, albuminuria, and glomerulosclerosis (14, 28, 29). Moreover, increased urinary albumin excretion and tissue 8-OHdG in the kidney were observed simultaneously in the STZ-diabetic rats (26). The diabetic db/db mice that were given 0.02% astaxanthin showed an improvement in the urinary 8-OHdG level, albuminuria and mesangial expansion, reflecting the reduction of 8-OHdG expression in the mesangial cells (8). In our experiments, the \( \alpha \)-tocopherol group was administered a low dose of astaxanthin (approximately 5 mg/d) in combination with \( \alpha \)-tocopherol. As a result, the serum Cr, urinary albumin and protein excretion, and Ccr were reduced significantly, in parallel with the decline of oxidative stress biomarkers in the kidney (Tables 4, 5). Moreover, in the \( \alpha \)-tocopherol group, we confirmed that the suppression of these renal function parameters were associated with maintaining a high antioxidant status of both astaxanthin and \( \alpha \)-tocopherol in the serum and liver (Tables 2, 3). There is accumulating evidence indicating that the higher serum antioxidant concentrations, such as carotenoid, \( \alpha \)-tocopherol, and ascorbic acid, are associated with a lower risk of development of diabetes and its nephropathy in patients with diabetes by inhibiting oxidative damage (5, 13). This may explain, in part, the reason that maintenance of endogenous antioxidant concentrations relates to the restored partial effects of renal dysfunction.

On the other hand, in contrast to the result of the reduction of lipid peroxidation in the kidney of the \( \alpha \)-tocopherol group, the ascorbic acid group showed markedly higher lipid peroxidation levels (Table 4). In particular, the Cl value in the kidney of the ascorbic acid group was higher than that of the control group. Ascorbic acid is known to promote the generation of reactive oxygen species by the presence of transition metals such as iron or copper in vitro (30). It has been reported that the plasma copper levels and lipid peroxidation via glycation resulting from hyperglycemia increase in diabetic patients (31). The increase of plasma copper level is caused by the release of copper ions from the enzyme by the glycation of Cu,Zn-SOD (32). Additionally, a large amount of ascorbic acid administration intravenously promotes lipid peroxidation in the serum of guinea pigs (33). Our data also imply that a high dose of ascorbic acid increased lipid peroxidation in the ascorbic acid group. Thus, this result suggests that the administration of a large amount of ascorbic acid may increase lipid peroxidation via enhancement of oxidative stress in diabetic-ODS rats.

In conclusion, the combination of astaxanthin and \( \alpha \)-tocopherol is capable of ameliorating oxidative injury through the suppression of oxidative stress induced by diabetes. Additionally, our results provide experimental evidence of increased lipid peroxidation from a high dose of ascorbic acid in vivo.

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