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Effects of α-Tocopherol and β-Carotene Supplementation on Oxidative Damage by Lipid Oxidation in Rat Liver

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Abstract

The effect of α-tocopherol and β-carotene supplementation on reducing the oxidative damage in the liver of rats were studied. Forty-five male Sprague Dawley aged 4 weeks were randomly assigned to 9 groups of five for the 12 weeks of the study. Nine groups, sardine oil, sardine oil + Vt E, sardine oil + β-carotene, soybean oil, soybean oil + Vt E, soybean oil + β-carotene, lard, lard + Vt E, lard + β-carotene group, were prepared. Sardine oil, soybean oil, or lard was used for dietary fat and 200% of α-tocopherol or 150% of β-carotene was supplemented to each diet. Each diet supplied 65% of total energy as carbohydrate, 15% as protein, and 20% as lipid. The MDA value and protein carbonyl contents of sardine oil group were significantly different ($p<0.05$) to those of other fat groups indicating that the most severe lipid oxidation occurred in the group fed diet containing highly polyunsaturated fatty acid. When α-tocopherol or β-carotene was supplemented to the sardine oil diet, MDA value (−35%, −15%, respectively) and protein carbonyl content (−44%, −32%, respectively) decreased significantly ($p<0.05$). Cu, Zn-superoxide dismutase (SOD) and catalase activities of three different sardine oil groups with or without antioxidants were lower than those of soybean oil or lard group. The reducing effect of α-tocopherol on oxidative damage in sardine oil group supplemented with α-tocopherol was noticeable ($p<0.05$). However the adverse effect of β-carotene was observed. SOD and catalase activities of β-carotene supplemented groups were the lowest among the same fat groups, but the differences were not statistically significant. The possible cause of decreased enzyme activity seemed to be related to the vitamin A (Vt A) toxicity in the liver where retinol converted from dietary β-carotene in the intestinal mucosa was stored.

Key words : α-tocopherol, β-carotene, oxidative damage, antioxidative enzymes

INTRODUCTION

Polyunsaturated fatty acid has been known to have effects on lowering triglyceride and cholesterol contents in the plasma (1) and also preventing the development of atherosclerosis by producing a certain kinds of prostagladins that favors in inhibiting the platelet aggregation and dialating the blood vesseles (2). These advancing effects of polyunsaturated fatty acids were observed under the circumstances when polyunsaturated fatty acid is not oxidized. Otherwise the peroxidized products of lipid oxidation such as hydroperoxides or aldehydes readily interact with proteins, amino acids, enzymes, and other biochemicals which give rise to the oxidative damage in the biological system (3). Accumulation of oxidative damage may lead to various status of diseases, aging and ultimately to death. Therefore prevention of the oxidative damage have been studied extensively and concerns about the antioxidants thus increased.

Many investigators reported that tocopherols, especially α-tocopherol is very potent lipid antioxidant that can prevent the oxidative damage in liver and plasma *in vivo*. But the data corncerning the effects of β-carotene in this aspects are extremely limited. β-carotene which has the provitamin A activity is largely converted to Vt A in the intestinal mucosa and the efficiency of conversion varies in different species (4). In human, most of dietary β-carotene is converted into retinol in gut but approximately 15% of unchanged β-carotene is also absorbed into the lymph. Similarly to humans, horses and certain breeds of cattle are able to absorb some intact β-carotene. In most other species, dietary β-carotene is largely con-

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verted to Vt A before absorption. Thus, very little or no intact β-carotene is taken up into the circulation by rats, pigs and chickens (4).

The goal of the present study is to examine the effects of supplementation of antioxidants, α-tocopherol or β-carotene, on reducing the oxidative damage in the liver of rats. Each diet was designed to differ both fats and antioxidants, while all other variables were constant. The levels of lipid oxidation, activities of Cu,Zn-SOD and catalase which act as the primary enzymatic antioxidants in the liver, and concentration of α-tocopherol in the liver and retinol in the plasma were determined.

MATERIALS AND METHODS

Diets and study design

Forty-five male Sprague Dawley aged 4 weeks were purchased from the National Institute of Health (Seoul, Korea) and were acclimatized for 3 weeks with a commercial basal diet to eliminate any carry-over effects of diets that might be observed in the studies of shorter duration. Animals were randomly assigned to 9 groups of five for the 12 weeks of the study (Table 1). Nine groups, sardine oil, sardine oil+Vt E, sardine oil+β-carotene, soybean oil, soybean oil+Vt E, soybean oil+β-carotene, lard, lard+Vt E, lard+β-carotene group, were prepared. Each diet supplied 65% of total energy as carbohydrate, 15% as protein, and 20 % as lipid. Sardine oil (Korea Co., Pusan), soy-bean oil (Dong-Bang Oil Co., Jinju), or lard (Dong-Kwang Co., Pusan) was used as the fat sources and α-tocopherol or β-carotene was used as the antioxidant sources. α-tocopherol and β-carotene were supplemented at the level of 200 and 150% to the amounts used in the AIN-76 diet preparation guidelines (5), respectively. Nine different diets were prepared every week and each diet was divided into 7 parts before they were kept at −20°C to minimize the lipid oxidation. Diets were given based on the RDA for the rats (6,13) and free access to water. Lights were used between 08 : 00~20 : 00h. The temperature and humidity of the animal facility were controlled at 22 °C and 50 to 60%, respectively.

MDA, protein carbonyl content, catalase activity, Cu, Zn-SOD activity, α-tocopherol in the liver and retinol concentration in the plasma were assessed after 12 weeks of feeding.

Table 1. The composition of experimental diets

(g/100g diet)

Ingredients	Group[1]								
	FF	FC	FT	SF	SC	ST	LF	LC	LT
Corn starch	69	69	69	69	69	69	69	69	69
Casein	15	15	15	15	15	15	15	15	15
Lipid									
Fish oil (sardine oil)	10	10	10	–	–	–	–	–	–
Soybean oil	–	–	–	10	10	10	–	–	–
Lard	–	–	–	–	–	–	10	10	10
Mineral mixture[2]	4	4	4	4	4	4	4	4	4
Vitamin mixture[3]	1	1	1	1	1	1	1	1	1
Antioxidant									
β-carotene	–	0.0096	–	–	0.0096	–	–	0.0096	–
α-tocopherol	–	–	0.015	–	–	0.015	–	–	0.015
Cellulose (CMC)	1	1	1	1	1	1	1	1	1

[1] FF : Fish oil(sardine oil), FC : Fish oil supplemented with β-carotene, FT : Fish oil supplemented with α-tocopherol, SF : Soybean oil, SC : Soybean oil supplemented with β-carotene, ST : Soybean oil supplemented with α-tocopherol, LF : Lard, LC : Lard supplemented with β-carotene, LT : Lard supplemented with α-tocopherol

[2] Mineral mixture (g per 1kg of mineral mixture) : $CaHPO_4$ 500.0g, NaCl 74.0g, K citrate H_2O 220.0g, K_2SO 52.0g, MgO 24.0g, MnCO 3.5g, Fe-Citrate 6.0g, $ZnCO_3$ 1.6g, $CuCO_3$ 0.3g, KIO_3 0.01g, $Na_2SeO_3 \cdot 5H_2O$ 0.01g, CrK$(SO_4) \cdot 12H_2O$ 0.55g, sucrose 118.03g

[3] Vitamin mixture (g per 1kg of vitamin mixture) : Thiamin HCl 0.5g, Riboflavin 0.5g, Pyridoxine HCl 0.583g, Niacin 2.5g, Ca pantothenate 1.33g, Folic acid 0.167g, Biotin 0.017g, Vitamin B_{12} 0.833g, Vit. A palmitate (500,000U/g) 0.667g, Vit. E acetate (500 U/g) 8.333g, Vit. D_2 trituration (400,000U/g) 0.208g, Menadione sodium bisulfite complex 0.125g, Choline 166.667g, Sucrose 817.567g

Liver dissection and blood sample preparation

Rats were killed in the dessiccator containing dry ice after 15 hrs of fasting. Blood sample was collected by direct cardiac puncture and transferred into EDTA K_2 bial immediately. Blood was centrifuged to get the plasma after shaking the bial several times. Liver was dissected and transferred onto a petridish containing the saline solution followed by washing several times. All samples were kept at −80°C for the further biochemical analyses.

Biochemical analyses

The extent of lipid peroxidation in the liver was expressed as a malondialdehyde value (7) and protein carbonyl contents (8). The activities of SOD (9) and catalase (10) were measured as an indicator to the oxidative damage occurred by dietary fats. The effect of antioxidants supplementation on reducing the oxidative damage was also observed with the activities of SOD and catalase. The concentration of α-tocopherol in the liver was determined using the method of Taylor (11) and that of retinol in the plasma was measured by HPLC method of Bieri *et al.* (12). We first planned to determine the concentration of β-carotene in the liver which was not possible in the rats since β-carotene was converted to retinol before absorption so that the contents of β-carotene in the liver was not enough to be detected by HPLC. The liver sample preparation and the detailed analytical processes used for this experiment were reported in the previous paper (13) except retinol determination.

Determination of retinol content (12)

One hundred micro liter of retinyl acetate (2.0μg retinyl acetate dissolved in 1ml) was added to 100μl of plasma and vortexed for 10 sec followed by another 45 sec votexing after addition of 600μl of hexane. The hexane layer was separated after centrifugation at 6500rpm for 5min and then dried up with vacuum evaporator at 32°C. The powder was ressolved in 100 μl ethanol and 20μl of sample solution was injected into HPLC. Reagents used for this analysis were the special grade for HPLC. The chromatographic conditions of HPLC were listed in Table 2.

Statistical analysis

The results were analyzed with two-way ANOVA and differences were considered significant at $p<0.05$. The significant factors by two-way ANOVA were the fats and antioxidant sources. If significance were present, Duncan's multiple range test was employed to find out the differences between groups. Statistical procedures were performed using the SPSS package program (14).

RESULTS AND DISCUSSION

Effect of dietary fats on the liver weight of rat

The peroxide value and MDA contents of experimental diet fed to the animals were determined twice a week right after the diet was given. The mean values of 144 measurements were comparable to the

Table 2. Chromatographic conditions of HPLC for determination of retinol

Instrument	Spectra Physics HPLC system
Column	Spherisorb ODS − 1 (4.6nm × 25cm i.d.)
Detector	UV 318nm
Flow rate	1.5ml/min
Eluents	Methanol : Water=95 : 5 (v/v)

Table 3. The peroxide value (POV) and malonaldehyde (MDA) content of experimental diets[1]

Sources of lipid	POV (meq/kg diet)			MDA (meq/kg diet)		
	Control	β-carotene added	α-tocopherol added	Control	β-carotene added	α-tocopherol added
Sardine oil	$7.87^{c}\pm0.76^{c}$	$6.00^{c}\pm0.53^{B}$	$4.30^{c}\pm0.50^{A}$	$1.303^{c}\pm0.028^{C}$	$1.027^{c}\pm0.012^{B}$	$0.816^{c}\pm0.005^{A}$
Soybean oil	$2.40^{a}\pm0.20^{B}$	$1.30^{a}\pm0.46^{A}$	$1.07^{a}\pm0.31^{A}$	$0.109^{a}\pm0.008^{B}$	$0.096^{a}\pm0.005^{A}$	$0.099^{a}\pm0.005^{AB}$
Lard	$4.23^{b}\pm0.38^{B}$	$3.80^{b}\pm0.20^{AB}$	$3.57^{b}\pm0.21^{A}$	$0.177^{b}\pm0.005^{B}$	$0.169^{b}\pm0.005^{B}$	$0.143^{b}\pm0.005^{A}$

POV and MDA values are Means±S.D. of 144 replicates which were determinated twice a week for 12 weeks

[1] Experimental diet was prepared every week accoring to the AIN-76 guideline. α-Tocopherol and β-carotene were supplemented at level of 200 and 150% to the amount used in AIN-76 diet preparation, respectively

[abc] Different letters within a column (fat sources are varied) are significantly different ($p<0.05$)

[ABC] Different letters within a row (antioxidants are varied) are significantly different ($p<0.05$)

others (Table 3) (15). These values represents that peroxide and MDA in the diet were low enough not to carry over any errors by diet. Kwon *et al.* (13) reported that diet preparation should be controlled otherwise peroxides in the diet could be accumulated in the body which may give false data.

The mean weight of liver for each dietary treatment is provided in Table 4. The liver weight of 9 groups were not significantly different among groups varied with the dietary fats and antioxidants except sardine oil supplemented with α-tocopherol group. Slight increase in liver weight was observed in the groups with β-carotene supplementation. This increase in liver weight fed β-carotene supplementation was also observed in 16 weeks of feeding (data were not shown). It seems that α-tocopherol supplementation tended to reduce liver weight while β-carotene treatment increases, however, is less conclusive.

Lipid oxidation in the liver

The MDA value and protein carbonyl contents were measured in the nine groups to see the effects of dietary fats on lipid oxidation and the preventing effect of antioxidants on it. The MDA values of sardine oil groups were significantly different from those of soybean oil or lard group either antioxidants were supplemented or not. The MDA value of sardine oil group was the highest among nine groups followed by sardine with β-carotene and sardine with α-tocopherol in order (Table 5). This results implies that highly polyunsaturated fatty acid diet (sardine oil) causes lipid oxidation more easily than polyunsaturated fatty acid (soybean oil) or saturated fatty acid (lard).

The effects of antioxidant supplementation on preventing lipid oxidation observed in sardine oil groups were noticeable. α-Tocopherol and β-carotene supplementation reduced MDA value significantly (−35 %, −15%, respectively, $p<0.05$). The effects of antioxidant supplimentation on preventing the liver oxidation by polyunsaturated fatty acid, is certain. α-Tocopherol supplimentation seemed to be more effective in reducing the degree of lipid oxidation than that of β-carotene.

Recently, it has been reported that protein carbonyl content can be used as a method for measuring the oxdiative damage by peroxidized lipid (13). Active oxygen species generated from the lipid radical or lipid hydroperoxide damage the proteins in the cell causing the modification of protein that usually occurs at γ-amino acid residues at or near that cation binding site (8). The carbonyl-bearing residues have not been completely indentified, but glutamyl semialdehyde appears to be the major residue (8). This reaction usually inactivates enzymes. The oxidative modification of protein occurrs far before lipid peroxidation proceeds (16,17) meaning that protein carbonyl contents rise before MDA value increases. Therefore protein carbony determination can be used as a sensitive method to measure the oxidative damage by peroxidized lipid. The protein carbonyl content in sardine oil group showed the highest value indicating that the most severe oxidative damage occurred. Oxi-

Table 4. The liver weight (wet base) of rats fed different diet for 12 weeks of which fats and antioxidants are varied

(g/100g body weight)

Sources of lipid	Antioxidant supplemented		
	None	β-carotene	α-tocopherol
Sardine oil	3.00±0.07[a]	3.14±0.23[a]	2.68±0.11[A]
Soybean oil	2.79±0.24	2.54±0.16	2.65±0.19
Lard	2.90±0.05	3.09±0.48	2.85±0.37

All values are Means±S.D. of three replicates
For the more detail, see the legend of Table 3

Table 5. The malondialdehyde (MDA) value and protein carbonyl contents of rat liver fed different fats for 12 weeks and the effects of antioxidants supplementation on lipid oxidation in the rat liver

Lipid	MDA (nmol/g liver)			Protein carbonyl content (μmol/mg prot)		
	Control	β-carotene added	α-tocopherol added	Control	β-carotene added	α-tocopherol added
Sardine oil	32.91[b]±0.64[B]	27.99[b]±6.38[B]	21.41[b]±3.57[A]	12.15[b]±2.80[B]	8.27±2.13[A]	6.87[a]±1.10[A]
Soybean oil	13.87[a]±0.82	14.20[a]±1.48	15.03[a]±0.96[a]	8.98[a]±2.25	8.12±1.65	8.67[ab]±1.85
Lard	14.84[a]±1.91	15.64[a]±1.32	16.66[a]±2.51	9.35[a]±0.50	9.27±0.92	9.85[b] ±3.70

See the legend of Table 3

dative damage was reduced by 44% and 32% when α-tocopherol and β-carotene was supplemented, respectively (Table 5) explaining that protein carbonyl content determination is more sensitive than MDA determination.

Effects of α-tocopherol and β-carotene on oxidative damage to superoxide dismutase and catalase in the liver by lipid peroxidation

The activities of Cu,Zn-SOD and catalase, which act as the primary enzymatic antioxdidants in the liver (18), present in cytosol where lipid peroxidation takes place dominantly (19) were determined to see the effects of dietary fats and the antioxidants supplementation on changes in enzyme activities (Table 6). The SOD and catalase activities in sardine oil group either supplemented with antioxidants or not were the lowest among different dietary fat groups where lipid peroxidation took place the most seriouly as shown in Table 5. This can be explained as an oxidative damage caused by lipid hydroperoxide or aldehyde which has free radicals produced during lipid peroxidation.

When the effect of Vt. E supplementation on enzyme activities were determined, SOD activity of sardine oil supplemented with Vt E group increased significantly ($p<0.05$) and catalase activity of that group also increased but it was not statistically significant compared to other groups. These results suggest that the effect of Vt. E on reducing the oxidative damage of antioxidative enzyme can be easily seen in the group which was fed with highly unsaturatted fatty acid diet or with large amount of dietary fat. This result is consistant with that of Vatassery (20) who reported that arachidonic acid is more potent oxidants than linoleic acid on Vt E oxidation. This tells that antioxidant effects of Vt E on fish oil oxidation is more noticeable than soybean or lard oil oxidation. Otherwise antioxidative enzyme productions would increase to compensate against oxidative damage (15).

Unexpected result obtained from the present study was that β-carotene supplemented groups showed the lowest SOD and catalase activities within the same fat groups, but the differences were not statistically significant. The cause of this loss is most likely related to the Vt A toxicity in the liver where retinol converted from dietary β-carotene was stored (4).

Unfortunately we did not measure retinol in the liver which were not detectable, but we can assume this result indirectly based on the plasma retinol contents. The concentrations of retinol in the plasma were higher in the β-carotene supplemented goups than other groups (Table 7). In rats, dietary β-carotene is readily converted to retinol before absorption,

Table 6. Cu,Zn-superoxide dismutase and catalase activity of rat liver fed different fats for 12 weeks and the effects of antioxidants supplementation on oxidative damage in rat liver

Lipid	SOD activity (Unit/min/mg prot)			Catalase activity (μmol/min/mg prot)		
	Control	β-carotene added	α-tocopherol added	Control	β-carotene added	α-tocopherol added
Sardine oil	1.51[a]±0.13[x]	1.38[a]±0.73[x]	2.01[x]±0.27[b]	0.90[a]±0.13[a]	0.63[a]±0.13[x]	0.94[b] ±0.14[a]
Soybean oil	2.65[b]±0.78	2.39[b]±0.21	2.59[b]±0.30	1.12[b]±0.23	1.07[b] ±0.06	1.07[ab]±0.26
Lard	2.69[b]±0.40	2.48[b]±0.36	2.69[b]±0.46	1.34[c] ±0.18[a]	1.00[b]±0.20[x]	1.23[b] ±0.17[a]

See the legend of Table 3

Table 7. The concentration of Vt. E in the liver and β-carotene in the plasma of rat fed antioxidants supplemented diets for 12 weeks

Lipid	Vt E content (μg/g liver)			Retinol content (μg/ml plasma)		
	Control	β-carotene added	α-tocopherol added	Control	β-carotene added	α-tocopherol added
Sardine oil	59.26[x]± 5.54[x]	67.24[x]± 5.43[y]	64.47[x]± 4.76[x]	0.46[x] ±0.21	0.51±0.21	0.60±0.27
Soybean oil	71.15[y]± 7.74[x]	110.71[z] ±10.29[z]	94.86[y]±16.94[y]	0.68[y] ±0.06	0.77±0.31	0.53±0.18
Lard	70.92[y] ±10.81[x]	90.76[y]±14.06[y]	97.51[y] ± 8.69[y]	0.64[xy]±0.07	0.67±0.31	0.60±0.13

See the legend of Table 3

thus, very little or no intact β-carotene taken up into the circulation (4) is the reason why the groups supplemennted with β-carotene did not show any anti-oxidative activities against the oxidative damage. And also the concentrations of Vt E in the liver fed β-carotene supplemented diets were found to be higher than those of α-tocopherol supplemented groups (Table 7). In β-coretene supplemented groups, the oxidative damages on catalase and SOD were obseved in the presence of higher concentration of Vt E in the liver compared to those of α-tocopherol supplemented groups. This is interpreted that Vt A toxicity appeared before Vt E acted as antioxidants.

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식이지방의 산화에 따른 흰쥐 간의 산화적인 손상에 미치는 α-토코페롤과 β-카로틴의 강화 효과

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요 약

α-토코페롤과 β-카로틴을 강화한 식이가 흰쥐 간의 산화적인 손상을 줄이는 데 미치는 영향을 살펴보기 위하여 국립보건 연구원으로 부터 분양 받은 Sprague-Dawley계 숫쥐 45마리를 9군으로 나누어 식이지방과 항산화제 첨가 유무를 달리한 식이로 12주 동안 사육하였다. 식이지방원으로는 정어리유, 대두유, 그리고 라드를 이용하여 AIN-76 사료조제법에 따라 탄수화물이 총에너지의 65%, 단백질이 15%, 그리고 지방이 20% 되게 하였으며 여기에 α-토코페롤과 β-카로틴을 AIN-76에 사용한 양의 각 200%, 그리고 150% 만큼 첨가였다. 간의 MDA와 protein carrbonyl값은 정어리유군에서 가장 높아 고도의 불포화지방을 다량 함유한 식이군의 지방산화가 가장 현저히 일어남을 알 수 있었으며 이에 항산화제를 강화한 식이를 섭취시킨 경우 지방의 산화를 억제하는 효과도 정어리유군에서 가장 현저히 볼 수 있었다. α-토코페롤 강화한 정어리유군에서 MDA의 생성이 대조군에 비해 35%, β-카로틴 첨가군에서는 15%, 그리고 protein carbonyl의 생성은 각각 44%, 32% 감소되었음을 볼 수 있었고 그 감소의 정도는 모두 유의적이었다. 항산화제를 강화한 군의 지방산화 억제효과는 protein carbonyl값의 측정시 더욱 현저하게 볼 수 있어 지방의 산화정도를 측정하는 데 있어서 이 방법이 더 민감함을 알 수 있었다. Cu,Zn-SOD와 catalase의 활성을 측정하여 지방산화에 따른 효소의 손상정도를 살펴보았을 때, 역시 정어리유군에서 효소의 활성이 대두유군이나 라드군에 비해 현저하게 감소되었음을 볼 수 있었고, 여기에 항산화제를 강화시켰을 때 α-토코페롤 첨가군에서는 효소 활성이 높아지는 경향을 볼 수 있었으나 β-카로틴을 첨가한 군에서는 효소의 활성이 더욱 감소하는 반대의 결과가 나타났다. 이러한 현상은 식이에 첨가한 β-카로틴이 소장에서 Vt A로 전환되어 간에 축적되어 나타난 Vt A 독성 때문으로 추측된다. 쥐의 경우 식이로 섭취된 카로틴은 소장 점막에서 비타민 A로 거의 모두 전환된다는 보고(4)가 있다.