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VITAMIN E, SELENIUM, TROLOX C, ASCORBIC ACID PALMITATE, ACETYLCYSTEINE, COENZYME Q, β-CAROTENE, CANTHAXANTHIN, AND (+)-CATECHIN PROTECT AGAINST OXIDATIVE DAMAGE TO KIDNEY, HEART, LUNG AND SPLEEN

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Male Sprague-Dawley rats were fed diets that varied qualitatively and quantitatively in antioxidants. Kidney, heart, lung, and spleen homogenates were incubated at 37°C with and without hydroperoxide or Fe^{+2}. Protection of antioxidants against oxidative damage to tissue was determined by measurement of oxidized heme proteins. Tissues from rats supplemented with dietary vitamin E and selenium showed protection compared to tissues from rats on the basal diet. Tissues from rats with diets containing larger quantities of antioxidants and both fat soluble antioxidants: vitamin E, β-carotene, coenzyme Q_{10}, ascorbic acid 6-palmitate and water soluble antioxidants: selenium, trolox C, acetylcysteine, coenzyme Q_0, (+)-catechin, showed the highest protection.

KEY WORDS: oxidized heme proteins, antioxidant nutrients, oxidative damage, tissue homogenate, rats.

INTRODUCTION

Oxidative damage of membrane lipids, proteins and DNA molecules initiated by reactive oxygen species is associated with various disease states.[1,2] Dietary antioxygenic nutrients, such as vitamin E, can effectively protect tissues against oxidative damage, helping to prevent the occurrence of various diseases.[3] Current clinical studies suggest that vitamin E supplements can significantly reduce the risk of coronary heart diseases.[4,5] Oxidized heme proteins (OHP) can be a measure of oxidative damage to tissues.[6,7] A computer aided heme proteins spectra analysis program (HPSAP) has recently been developed and used to study oxidative damage of heme proteins in both tissue slices and homogenate systems.[8–10] There is a strong correlation between the formation of OHP and TBARS, a widely used measurement of lipid peroxidation. Thus, measurement of OHP with HPSAP is an index of oxidative damage of tissues.[8,11]

We have demonstrated that dietary supplements of vitamin E, selenium, and β-carotene can inhibit oxidative damage of heme proteins as well as lipid peroxidation in liver, kidney, heart, lung, and spleen.[8,10] More importantly, supplementation

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by a combination of vitamin E, selenium, and β-carotene provides stronger protection against oxidative damage to tissues than supplementation by an individual antioxidant.

Trolox c, ascorbic acid palmitate, acetylcysteine, coenzyme Q, carotenoids, and flavonoids are potent antioxidants, and they can either prevent or intercept free radical reactions.[12-14] In this study, we investigated the augmentation of protection provided by vitamin E and selenium with the addition of relatively high doses of trolox c, ascorbic acid 6-palmitate, acetylcysteine, coenzyme Q, β-carotene, canthaxanthin, and (+)-catechin to diets. Rats were fed either an antioxidant deficient diet supplemented with vitamin E and selenium, or a diet supplemented by vitamin E, selenium and other antioxidants. Kidney, heart, lung, and spleen tissue homogenates were incubated at 37°C with or without prooxidants. Protection by antioxidants against oxidative damage to tissues was determined by measuring the formation of OHP.

MATERIALS AND METHODS

Chemicals

The antioxidants and chemicals used in this study were sodium selenite (Alfa Inorganics, Beverly, MA), trolox c (Aldrich Chemical Co., Milwaukee, WI), alpha-tocopherol acid succinate, coenzyme Q_0, coenzyme Q_{10}, (+)-catechin, dimethyl sulfoxide, trans-β-carotene, L-ascorbic acid 6-palmitate, acetylcysteine (Sigma Chemical, St. Louis, MO), canthaxanthin (Roche Vitamin and Fine Chemicals, Nutley, NJ), t-butyl hydroperoxide (TBHP) (Polysciences, Inc., Warrington, PA), and ferrous sulfate ($FeSO_4$) (Fisher Scientific, Fair Lawn, NJ 07410).

Animals and Diets

Male Sprague-Dawley rats (Bantin & Kingman, Fremont, CA) weighing 40–60 g were fed experimental diets. The basal diet was a vitamin E and selenium deficient diet with 10% tocopherol stripped corn oil (Teklad Test Diet #TD 77068, mineral mix #170911, Teklad Test Diets, Madison, WI). The composition of the basal diet (g/kg) was as follows: Torula yeast, 300; sucrose, 560; corn oil, 100; Williams-Briggs modified mineral mix, 35; L-methionine, 4; thiamin HC1, 0.0004; riboflavin, 0.0025; pyridoxine HC1, 0.002; calcium pantothenate, 0.02; niacin 0.1; biotin, 0.001; folic acid, 0.002; vitamin B-12 (0.1% triturated in mannitol), 0.1; choline chloride, 1; dry retinyl palmitate (500,000 U/g), 0.028; dry ergocalciferol (500,000 U/g), 0.0064; and menadione, 0.001. Mineral mix contained (g/kg) $CaCO_3$, 207; $CaHPO_4$, 323; $MgSO_4$, 65.7; KC1, 209; Na_2HPO_4, 186; $CuSO_4$, 0.37; ferric citrate (16.7% Fe), 4.314; $MnSO_4$, H_2O, 4.40; $K10_3$, 0.029 and $ZnCO_3$, 0.60. Animals were housed according to NIH guidelines and had free access to deionized water and food. Dietary treatment had no effect on weight gain of the animals. Diets supplemented with antioxidants were divided into four groups: diet supplemented with vitamin E and selenium (diet 2); diet supplemented with vitamin E, selenium, and the fat soluble antioxidants β-carotene, coenzyme Q_{10}, ascorbic acid 6-palmitate, and canthaxanthin (diet 3) ; diet supplemented with vitamin E, selenium, and the water soluble, less fat soluble antioxidants trolox c, acetylcysteine, coenzyme Q_0 (diet 4) ; and diet supplemented with vitamin E, selenium, fat soluble, and water soluble antioxidants

(diet 5). (+)-Catechin, which has cis hydroxyl groups on an aromatic ring, was added to antioxidant enriched diets as a representative flavonoid. Canthaxanthin has the same structure as β-carotene with the addition of a keto oxygen on each ionone residue. Trolox is a model compound of vitamin E which does not have a phytyl side chain but instead has a 2-carboxyl on the hydroxychromane ring. Ascorbic acid 6-palmitate is a fat soluble antioxidant that generally does not release ascorbic acid. Ascorbic acid was not included in the diet because rats synthesize it. The protocol for the dietary study is shown in Table 1. The rats were fed experimental diets and distilled water for six weeks. Three animals were used in each dietary group.

Preparation of Tissue Homogenate

The rats were decapitated, and kidney, heart, lung, and spleen were immediately dissected and immersed in ice-cold Krebs-Ringer phosphate (KRP) buffer (pH 7.4). Organs were dried with filter papers and stored at −22°C. Frozen organs were cut into 0.5 cm^3 cubes, and homogenates were prepared by homogenizing 1 g of tissue with 9 ml of oxygenated KRP buffer containing glucose (10 mM) (pH 7.4). A motor driven tissue homogenizer was used.

Oxidative Reactions in Tissue Homogenate

Tissue homogenate (1.5 ml) was transferred to a 10 ml serum bottle and covered with a layer of parafilm. The homogenate was incubated in a gyrotory water bath shaker (New Brunswick Scientific Co., Inc., New Brunswick, NJ) at 37°C with continuous shaking (180 cycle/min). Tertiary butyl hydroperoxide (TBHP) was dissolved in dimethylsulfoxide and $FeSO_4$ was dissolved in distilled water. The prooxidants were added to the serum bottles immediately before the incubation.

TABLE 1
Addition of antioxidant nutrients to basal diet[a]

	Amount of antioxidant (mg/kg)				
	Diet				
Antioxidant	1	2	3 Fat Soluble	4 Water Soluble	5
Vitamin E	0	25	25	25	25
Se	0	0.3	0.3	0.3	0.3
β-carotene	0	0	45	0	45
Co-Q_{10}	0	0	30	0	30
Ascorbic acid 6-palmitate	0	0	100	0	100
Canthaxanthin	0	0	45	0	45
Trolox c	0	0	0	50	50
Acetylcysteine	0	0	0	200	200
Co-Q_0	0	0	0	100	100
(+)-Catechin	0	0	100	100	100

[a] The average body weight of rats before dietary treatment was 50 g. After six weeks of dietary treatment, the average body weight of rats was 250 g. There was no significant weight difference among dietary groups of three animals in each group.

Spectrophotometric Measurement of Tissue Homogenate

After incubation the absorbance spectra of tissue homogenates were obtained with a Beckman DU-50 spectrophotometer (Beckman Instruments, Inc., Fullerton, CA) Tissue homogenates (0.6 ml) were transferred to a 5 ml test tube and mixed well with 0.6 ml of glycerol. The homogenate glycerol mixture was measured in a microcuvette with a light path of 10 mm. Four layers of parafilm representing turbidity were used as background to subtract some of the absorbance due to turbidity inherent in tissue homogenates. The sample was scanned from 500 nm to 640 nm, and absorbance vs wavelength at 5 nm intervals was automatically recorded by a scan program in the spectrophotometer. The details and principles of spectrophotometric measurement of tissue homogenate are described in our previous study.[8]

Analysis of Absorbance Spectra of Heme Proteins of Liver Homogenate with Heme Protein Spectra Analysis Program

HPSAP is a spreadsheet program written with Lotus 123 (Lotus Development Corp., 55 Cambridge Parkway, Cambridge, MA) that contains visible spectra of individual heme proteins from 500 to 640 nm. The details of HPSAP and its operation and application are described in our previous studies.[8-11]

Statistical Analysis

The statistical package SAS (SAS Institute Inc., Cary, NC) was used to analyze all data. When significant F values were obtained using ANOVA, Duncans LSD procedure was used to determine significant difference ($P < 0.05$) between treatment means. Results were expressed as means ± standard deviation.

RESULTS

With HPSAP, oxidized heme proteins, such as methemoglobin, hemichrome, ferryl hemoglobin, oxidized mitochondria and microsomal cytochromes were determined. Figure 1 and Table 2 demonstrate the application of absorbance spectral measurement and HPSAP to the study of the effects of antioxidants against oxidative damage to heme proteins. Incubation of homogenates caused spectral changes mainly due to the formation of OHP in the tissue homogenates. For example, in heart or lung homogenates, absorptions at 540 and 575 nm decreased while absorptions at 500 and 630 nm rose as time progressed. The spectral changes were more marked in the homogenates from rats fed basal diet than in those from rats fed diet supplemented with antioxidants, indicating that the antioxidants effectively inhibited the production of OHP. HPSAP provided a quantitative interpretation of Figure 1 and Table 2. The analysis clearly showed that dietary antioxidants effectively protected tissues against oxidative damage.

Figure 2 shows the protection by antioxidant nutrients against heme protein oxidation in kidney homogenate during spontaneous oxidation. As expected, heme proteins in kidney from rats fed basal diet were most susceptible to oxidative damage. Approximately 40% heme proteins were oxidized during a moderate incubation (37°C for 0.5 h). By supplementing with vitamin E and selenium as in diet 2, the production of OHP was reduced to approximately 30%. The protection given by vitamin

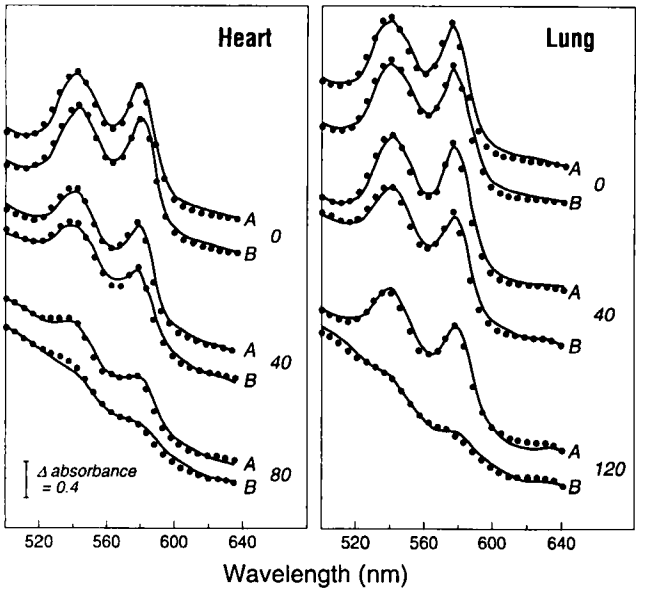

FIGURE 1 Visible spectra of heme proteins in heart and lung homogenates incubated at 37°C for 0, 40 80 and 120 min; . . ., experimental spectrum; ——, calculated spectrum. A, tissue homogenate from rats fed antioxidant enriched diets (heart, diet 3, lung, diet 4); B, tissue homogenate from rats fed basal diet.

TABLE 2
Determination of oxidized heme proteins (OHP) with heme protein spectra analysis program from spectra obtained from spontaneous oxidation of heart and lung homogenates incubated at 37°C

	Oxidized Heme Proteins (%).			
	Lung		Heart	
Time (min)	Basal Diet	Antioxidant Diet 4	Basal Diet	Antioxidant Diet 3
0	0.0	0.0	0.0	0.0
40	49.0	29.0	67.0	48.0
80			92.0	75.0
120	96.0	64.0		

E and selenium was significantly increased by adding various water soluble antioxidants or fat soluble and water soluble antioxidants as in diets 4 and 5. Only 15–20% heme proteins were converted to OHP in the kidney from rats fed diet 4 or diet 5 and incubated for 0.5 h. Diet enriched with fat soluble antioxidants (diet 3) did not show significant improvement in protection compared to diet 2. Diets 4 and 5 provided the strongest protection against heme protein oxidative damage among all diets supplemented with antioxidants in the kidney.

Figure 3 presents spontaneous heme protein oxidation in lung, spleen, and heart. Diets 3, 4, and 5 significantly lowered the production of OHP compared to that in tissues from rats fed antioxidant deficient diet and generally provided a stronger

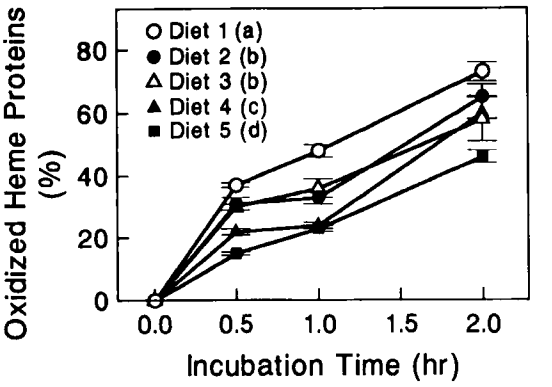

FIGURE 2 Production of oxidized heme proteins during the spontaneous oxidative reaction in kidney. For composition of each diet see Table 1. Tissue homogenates were incubated at 37°C. The values are expressed as mean ± SD for three rats. The diets labeled with different lower case letters are significantly different from each other at a 95% confidence level in protecting tissues.

protection than diet 2. Diet 4 was more effective than diets 3 and 5 in lung during the short period of incubation (40 min), while diet 3 was superior to either diet 4 and 5 in spleen.

Figures 4 and 5 show the protection of antioxidants against heme protein oxidation initiated by TBHP or Fe^{+2} in kidney and lung. In general, hydroperoxide and ferrous iron promoted the production of OHP in both tissues. Under oxidative stress, diets 3, 4, and 5 protected lung tissues more effectively than diet 2. In kidney tissues, however, only diet 4 or 5 was significantly more effective than diet 2 during the short period of incubation (30 min).

DISCUSSION

Studies have demonstrated that intracellular and plasma antioxidant concentrations are easily increased by dietary means or supplementation, thus dietary intake of antioxidant nutrients is an important and practical approach to protect humans against oxidative damage.[15-17] Studies conducted at this laboratory have also indicated that supplementation of vitamin E and selenium has a positive correlation with increase of vitamin E content and activity of selenium glutathione peroxidase at the tissue level.[18] In this study we planned to achieve maximum antioxidant protection and thus used multiple antioxidants. We used these antioxidants in amounts per kg of diet that may be suitable for human use. The rat diet was not high in fat compared to the average human diet but contained more polyunsaturated fatty acids than the average human diet. This study clearly showed that dietary supplements of vitamin E and selenium partially protected tissues against oxidative stress (Figures 2 and 3). With increased levels and kinds of antioxidant nutrients, the protection given by vitamin E and selenium was significantly augmented. The enhancement of protection against oxidative damage of heme proteins observed in diets 3, 4, and 5 can be attributed to: (1) relatively high levels of cellular antioxidants due to high dietary intake of antioxygenic nutrients; and (2) interactive effects of antioxidants.

Oxidative damage is concurrent with the depletion of cellular antioxidants. In

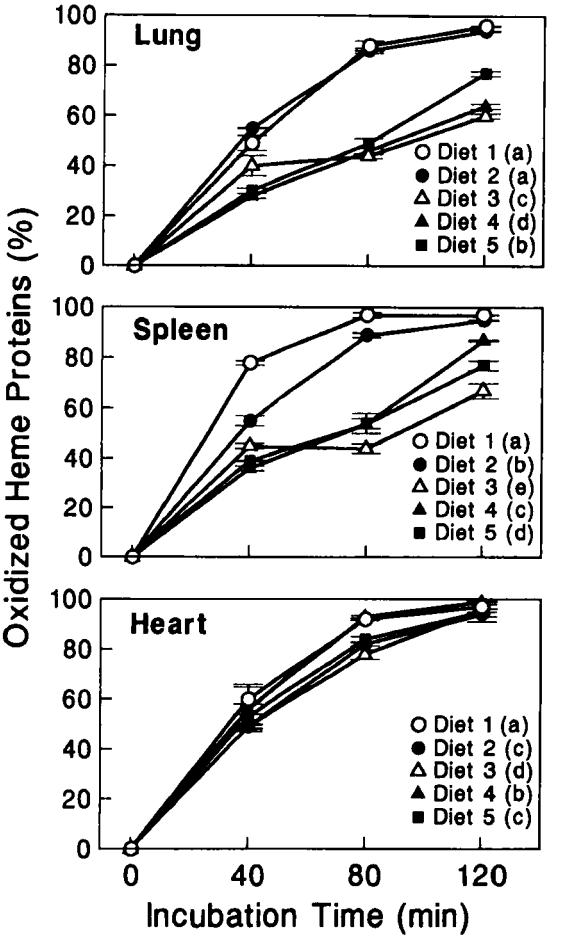

FIGURE 3 Production of OHP during spontaneous oxidative reaction in lung, spleen and heart. Tissue homogenates were incubated at 37°C. The values are expressed as mean ± SD for three rats. The diets labeled with different lower case letters are significantly different from each other at a 95% confidence level in protecting tissues against oxidative damage. At 0 time there was 0% OHP.

plasma, for example, lipid peroxidation was not observed until the concentration of antioxidants decreased to certain levels.[6,11] Thus, relatively high concentrations of cellular antioxidants could be crucial to protecting tissues against oxidative stress. Frei *et al.* (1989)[16] have demonstrated that by increasing the plasma ascorbate level from 1.0 mM to 5.0 mM, the lag phase of the formation of lipid hydroperoxides was extended from 3 h to 6 h. In the present study, cellular antioxidants were maintained at high levels by feeding rats high dose supplements of dietary antioxidants, thus improving protection against oxidative damage.

An interactive effect has been observed in multiple antioxidant systems. For instance, alpha-tocopherol, the primary antioxidant in cellular membranes, can be regenerated through the interaction between alpha-tocopheroxyl radicals and ascorbate, glutathione or acetylcysteine.[19-21] As ascorbate 6-palmitate or acetylcysteine or

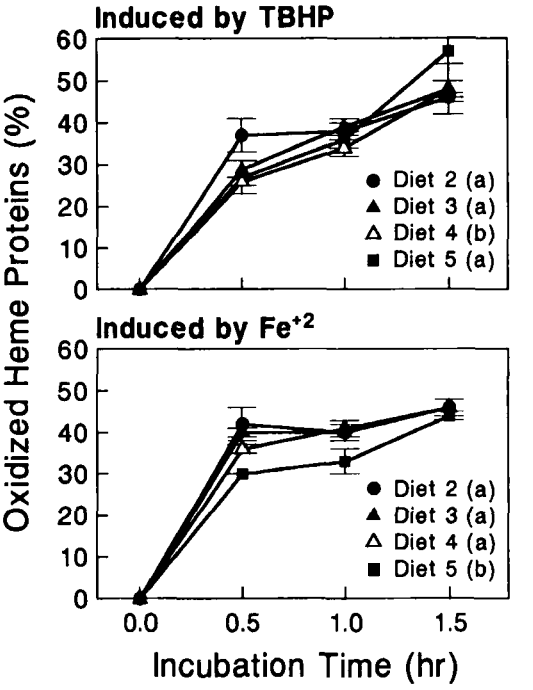

FIGURE 4 Heme protein oxidation induced by TBHP or Fe^{+2} in kidney. Tissue homogenates were incubated at 37°C and concentration of prooxidant was 0.05 mM. The values are expressed as mean ± SD for three rats. The diets labeled with different lowercase letters are significantly different from each other at a 95% confidence level in protecting tissues against oxidative damage. At 0 time there was 0% OHP.

both was added to diet 2, the depletion of alpha-tocopherol could be delayed, thus maintaining an adequate level of alpha-tocopherol in the membrane. As a result, the defenses in animals became more effective.

Cellular components are constantly attacked by free radicals and reactive oxygen species generated from both lipid and aqueous phases.[12] Both lipophilic and aqueous oxygen reactive species are responsible for oxidative damage of heme proteins even though the oxidative reaction is mainly carried out in the aqueous phase of the cell. Apparently, vitamin E protects heme proteins during the late stage of heme protein oxidation by breaking the chain reaction from lipid peroxidation.[8] Therefore, it is important to have both fat soluble and water soluble antioxidants in the cellular antioxidant defense systems. The interaction between lipid soluble and water soluble antioxidants may explain the generally superior protection provided by diets 4 and 5.

TBHP and Fe^{+2} are potent initiators of oxidative damage.[2,22] The reactive oxygen species generated from the decomposition of TBHP react with important cellular constituents, thus causing serious oxidative damage to tissues. Fe^{+2} acts as a catalyst in lipid peroxidation, thereby promoting oxidative damage in tissues. During the oxidation of heme proteins initiated by TBHP or Fe^{+2}, protection by antioxidant nutrients appeared to be less than that in spontaneous heme protein oxidation (Figure 4). The difference may be due to the strong oxidizing capacity of TBHP

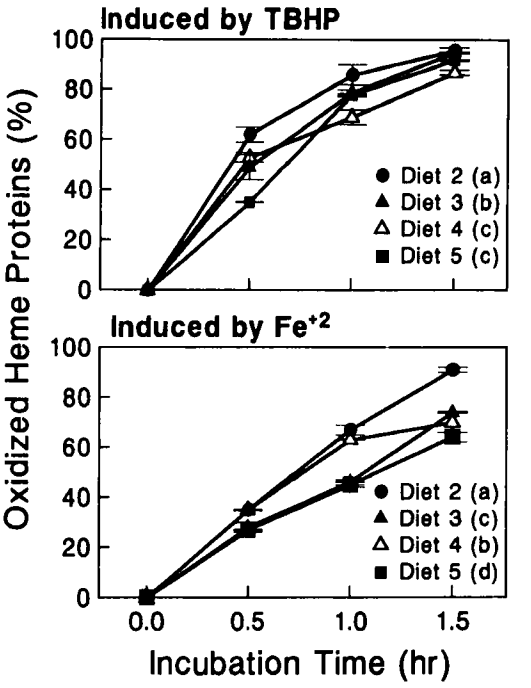

FIGURE 5 Heme protein oxidation induced by TBHP or Fe^{+2} in lung. Tissue homogenates were incubated at 37°C and concentration of prooxidant was 0.05 mM. The values are expressed as mean ± SD for three rats. The diets labeled with different lower case letters are significantly different from each other at a 95% confidence level in protecting tissues against oxidative damage. At 0 time there was 0% OHP.

and Fe^{+2} overwhelming the protective effect of antioxidants.

In this study we demonstrated that dietary supplementation by vitamin E and selenium provides strong protection against oxidative damage of home proteins in kidney, heart, lung, and spleen. Protection by vitamin E and selenium can be significantly improved by adding trolox c, ascorbic acid 6-palmitate, acetylcysteine, β-carotene, canthaxanthin, coenzyme Q_0, coenzyme Q_{10}, and (+)-catechin to the diets. These antioxidants have potential in stemming deteriorative processes in humans.

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