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Green Tea Extract Suppresses the Age-Related Increase in Collagen Crosslinking and Fluorescent Products in C57BL/6 Mice

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Abstract

Collagen crosslinking during aging in part results from Maillard reaction endproducts of glucose and oxoaldehydes. Because of the tight link between oxidative and carbonyl stress, we hypothesized that natural antioxidants and “nutriceuticals” could block collagen aging in C57BL/6 mice. Six groups of young and adult mice received vitamin C, vitamin E, vitamin C&E, blueberry, green tea extract (GTE), or no treatment for a period of 14 weeks. Body weights and collagen glycation were unaltered by the treatment. However, GTE or vitamin C&E combined blocked tendon crosslinking at 10 months of age ($p < 0.05$, adult group). GTE also blocked fluorescent products at 385 and 440 nm ($p = 0.052$ and < 0.05 , respectively) and tended to decrease skin pentosidine levels. These results suggest that green tea is able to delay collagen aging by an antioxidant mechanism that is in part duplicated by the combination of vitamin C and E.

Keywords

Glucose; polyphenols; oxidant stress; vitamin C; vitamin E

Introduction

Aging is characterized by the accumulation of protein crosslinks and modifications that participate in tissue stiffening and decreased protein turnover. Considerable evidence implicates the formation of advanced Maillard reaction products, also known as advanced glycation end products (AGEs), in these changes [1]. AGEs have also been associated with a wide variety of age-related diseases such as Alzheimer’s disease, atherosclerosis, osteoarthritis, cataract, diabetes, and end stage renal disease [2].

Of utmost interest is that the accumulation of AGE products and protein crosslinks reflects the existence of an aging clock. Kohn and Hamlin first observed the presence of an inverse

relationship between the age-related loss in collagen digestibility and maximum life span in mammalian species [3]. More recently, we found an inverse relationship between the accumulation rate of skin collagen pentosidine, a fluorescent AGE crosslink, and maximum life span in eight mammalian species [4]. Furthermore, in a longitudinal study in C57BL/6 mice we found that the accumulation rate of glycated and AGE products in skin collagen predicted accelerated death rate in both *ad libitum* and food-restricted mice [5].

In addition to AGE products, strong inverse correlations between food intake and longevity, and positive associations with various parameters of collagen crosslinking or protein markers of oxidant stress were reported [6–9]. With few exceptions, most anti-aging interventions in calorically restricted rodents have revealed a strong association between decreased food intake and delayed age-related increase in collagen crosslinking. Both in rodents and primates dietary restriction and lower body weight were accompanied by a decrease in glycemia [10–12]. Thus glycemia appears to modulate the aging rate of collagen, either directly by acting on glucose-derived crosslinks, or indirectly, by acting on cellular metabolism and release of crosslink precursors.

The growing evidence that carbonyl stress might be in part linked to oxidant stress [13] led us to test the hypothesis that nutraceuticals and antioxidant vitamins might be able to decrease collagen aging without impacting glycemia and body weight, since any decrease in these parameters was likely to also decrease collagen aging rate.

Below, we have tested the effects of blueberry and green tea extracts for their ability to prevent the age-related increase in collagen crosslinking and AGE product formation in aging C57BL/6 mice, and compared their effects with those of mice receiving vitamin C, vitamin E, or both. Blueberry extract was chosen based on reports that it is high in antioxidant content and can reverse neurological deficits in aging [14]. Green tea extract was tested for its widely promoted beneficial health effects that have been attributed to its antioxidant properties [15].

Material and Methods

Animal care and housing

Thirty C57BL/6 mice (Charles River, Raleigh, NC) 2.5 to 4 months old (“young mice»), and 30 mice, 8 to 10.5 months old (“adult mice”) were housed individually for 14 weeks according to the Guide for the Care and Use of Laboratory Animals [16]. Easy access to food and water along with adequate ventilation was provided. The relative humidity of the environment was between 40% and 70% and the temperature varied between 18° and 26°C. Lighting was controlled to support proper circadian rhythms. Mice were fed Mazuri Rodent Chow *ad libitum* (Purina Mills, St. Louis, MO; for composition see <http://www.mazuri.com/5663-5e09.htm>), and their food and water intake, and body weight were recorded daily. Mice were divided randomly into six groups of five mice each. Oral supplements of vitamin C, vitamin E (alphatocopherol, Sigma, St. Louis, MO), combination of vitamins C and E, blueberry extract (*Vaccinium pallidum*, “Blueberry Leaf”, organic alcohol extract, Nature’s Answer, Hauppauge, New York), and green tea extract (*Camellia sinensis*, “Green Tea Leaf”, alcohol-free extract, Nature’s Answer), were provided. The supplements were freshly mixed daily and added to their drinking water whereby dosage of the supplements was determined using the RDA for vitamins C and E, and the company-recommended dosages for blueberry and green tea extracts. Appropriate adjustments for body weight (i.e., 22 g and 28 g for young and adult mice, respectively) and daily consumption were made. On the average, it was estimated that mice drank 8 mL fluid per day and ate 3 g food. For young and adult mice, water bottles of 120 mL contained 28.2 and 36.3 mg vitamin C, and 4.2 and 5.4 mg vitamin E, 14.1 and 18.2 mL blueberry extract, and 21.2 and 27.2 mL of green tea

extract, respectively. Thus, young and adult mice received on the average 2.0 and 3.0 mg vitamin C, 0.28 and 0.36 mg vitamin E, 1.43 and 1.84 mg green tea extract, and 0.96 and 1.23 mg blueberry dry extracts per day, respectively.

After a 14-week period of supplementation, tail tendons and skin were prepared, stored at -20°C , and transported on dry ice for analysis at Case Western Reserve University.

In vitro effects of oxidant and carbonyl agents

In order to investigate the relative roles of oxidant versus carbonyl stress on tendon crosslinking, tendons (15 fibers per dish) from a 3-month-old Sprague-Dawley rat were incubated in Chelex-treated phosphate-buffered-saline (PBS) without or with 0.05 M xanthine and 50 units of xanthine oxidase to generate superoxide, 0.5 mM H_2O_2 with 100 μM Cu^{2+} , glucose (10 mM), ribose (5 mM), glyceraldehyde (0.5 mM), and methylglyoxal (0.5 mM) for 8 days at 37°C . Crosslinking was assayed by the tendon break time assay as described below.

Tendon break time

Tendon heat denaturation time (tendon breaking time, TBT) was determined according to Harrison [17] as modified by Sell [18]. Briefly, tail tendons were attached a 4–0 silk suturing thread on both ends and a 2 g lead weight on the distal end. The fiber and weight were attached to a switch linked to a timer and immersed in a 7 M urea borate buffer (pH 7.5) at the temperature of $40 \pm 0.5^{\circ}\text{C}$. Rupture of the tendon released the weight and stopped the timer. Five tendons from each mouse tail were assayed and an average break time was calculated.

Biochemical assays

Collagen-associated fluorescence was determined in solubilized tendon collagen as previously described. The tendons were defatted in chloroform/methanol (2:1). Ten mg (blotted dry weight) were digested with 280 units of Type VII collagenase (Sigma, St. Louis, MO) and centrifuged to yield a small pellet that was discarded. Sixty μL of the supernatant were diluted to 2.0 mL with water, and this solution was utilized for determination of collagen-bound fluorescence at 385 nm upon excitation at 335 nm, and at 440 nm upon excitation at 370 nm. Data were expressed in relative fluorescence units per mg collagen.

Glycated lysine (furosine), pentosidine, and carboxymethyl-lysine were assayed in acid hydrolysate of defatted skin collagen as previously described [5]. Salt-soluble and acetic acid-soluble fractions were removed and the resulting pellet was solubilized with collagenase in 0.5 M acetic acid incubated at 37°C for 12 hours. After centrifugation to remove a small pellet, 50 μL were used for determination of hydroxyproline content (see below). An equivalent of 500 μg hydroxyproline was dried by evaporation in a Savant evaporator (Savant, Holbrook, NY) and acid hydrolyzed for 18 hours at 110°C . The reconstituted hydrolysate was utilized for determination of glycated lysine as furosine by high-performance liquid chromatography (HPLC) as previously described [19]. Pentosidine was determined by a dual technique involving C-18 reverse phase and ion exchange chromatography [8]. Carboxymethyl-lysine (CML) was determined using the HPLC two-step procedure originally described by Glomb [20] and further detailed in Monnier *et al* [21]. Data were expressed in pmol per mg collagen assuming a 14% content of hydroxyproline, which was determined as previously described [5].

Statistical methods

TBT, fluorescence, furosine, pentosidine, and CML data were analyzed by the Analysis of Variance (ANOVA) test. To simultaneously compare all means of the independent variable,

the Student-Newman-Keuls (S-N-K) multiple comparison test was used [22]. Comparisons between control and individual intervention groups were made using the two-tailed Student's t-test. Pearson correlation coefficients were determined using SPSS Inc. (Chicago, IL) software.

Results

The effects of age and a 14-week period of supplementation of vitamins and nutraceuticals on body weight of the mice are shown in Figure 1. As expected, body weights increased with age. Among treatment groups, mean body weights varied by less than 5% from each other, and these variations did not differ statistically ($p > 0.05$). Thus, none of the interventions led to a decrease in body weight compared to the control group.

Skin collagen glycation, a parameter of mean glycemia, significantly increased with age ($p < 0.0001$), as previously reported [19] (Fig. 2). However, the amount of glycated lysine residues (furosine assay) within each age group was essentially unaffected by treatment (ANOVA, $p > 0.05$), except for the blueberry group in which it increased compared to the control ($p < 0.05$). There was a nonsignificant 10% decrease ($p > 0.05$) in glycated collagen in young mice receiving green tea extract. This effect, however, was absent in the adult group in which supplementation was initiated beyond puberty.

In contrast to the data above, tail tendon break time, a parameter of crosslinking, varied in major ways among treatment groups (Fig. 3). As expected based on previous publications, mean levels increased in all groups at 4 months compared to the 1 month control ($p < 0.0001$), and significantly in the 10-month-old mice receiving either no treatment, vitamin C alone, vitamin E alone, or blueberry extract ($p < 0.0001$). With the exception of blueberry extract, all adult treatment groups had significantly shorter breaking times than the control ($p < 0.05$, ANOVA). However, the most dramatic effects were observed in mice receiving the combination of vitamin C and E or the green tea extract. In fact, some of the vitamin C&E values at 4 months were not significantly different at 10 months ($p > 0.05$) and both the green tea and combined vitamin groups were highly significantly lower than control by Student's t-test ($p < 0.0001$). The green tea group also showed a trend toward suppression of crosslinking at 4 months.

Age effects were also observed in tail tendon auto-fluorescence measured at 370/440 nm and 335/385 nm excitation/emission wavelengths. While the other treatment groups appeared to enhance fluorescence, possibly due to vitamin C itself, green tea had a suppressive effect in the adult group, and fluorescence values were not significantly different from young treatment and control groups at both wavelengths ($p > 0.05$) (Fig. 4). The suppressive effect in the green tea group was significant for the 370/440 nm fluorophores compared to the control group ($p < 0.05$). Fluorescence at 335/385 nm was also lower compared to control, and this effect was borderline significant when examined by Student's t-test ($p < 0.052$).

Pentosidine, a fluorescent AGE product, was also determined in the insoluble fraction of skin collagen from adult mice following acid hydrolysis. It was significantly increased in 10-month-old versus 4-month-old mice in most groups ($p < 0.0001$), and decreased in both the vitamin E and pentosidine groups, but significance was reached only in the former ($p = 0.030$ vs control) (Fig.5).

Pearson correlation analyses using 2-tailed test and all data in the 10-month-old group revealed significant associations between skin pentosidine and body weight ($r = 0.376$, $p < 0.05$), skin fluorescence at 385 nm ($r = 0.400$, $p < 0.05$), and at 440 nm ($r = 0.523$, $p < 0.01$), as well as between tendon break time and body weight ($r = 0.380$, $p < 0.05$). Both fluorescence measurements were highly correlated ($r = 0.825$, $p < 0.01$).

In order to clarify the relative roles of oxidative versus carbonyl stress in the tendon break time increase, we have incubated intact tendons in Chelex-treated phosphate buffered saline with or without the superoxide generating system xanthine/xanthine oxidase, hydrogen peroxide (0.5 mM) and copper (100 μ M), glucose (10 mM), ribose (5 mM), or each 0.5 mM glyceraldehyde and methylglyoxal for 7 days at 37°C. This modeling experiment revealed that crosslinking could not be significantly catalyzed by reactive oxygen species only, and that the presence of reactive carbonyl compounds was necessary to mimic the *in vivo* tendon breaking time increase (Fig. 6). In these experiments, green tea catechins at 10 μ M (i.e., epicatechin, epicatechin gallate, epigallocatechin, epigallocatechin gallate) not only failed to block the crosslinking but tended to increase it (not shown). From these experiments we concluded that green tea or the combination of vitamins C and E must be acting indirectly on the crosslinking process.

Discussion

Since it was first introduced by Verzar [23], the tendon collagen heat denaturation/break time assay has been widely utilized in gerontological studies. With rare exceptions, most studies concur with the observation that the TBT is accelerated in shorter- versus longer-lived rodents, and decreased by caloric restriction. Examples include mouse (*Mus musculus*) versus white-footed mouse (*Peromyscus leucopus*) [24], DBA versus C57BL/6 mouse [8], and calorically restricted versus *ad libitum*-fed C57BL/6 mouse [8].

The association between longevity and tendon break time has recently taken a new twist with the discovery that mice homozygous for loss-of-function mutations at the Pit1 (Snell dwarf) locus had a > 40% increase in mean and maximal longevity [25]. These mutant dw(J)/dw animals showed delays in tendon break time increase. Surprisingly, male Snell dwarf mice, unlike calorically restricted mice, became obese, showing that their exceptional longevity is not simply due to alterations in adiposity *per se*. Yet, the decrease in tendon break time did reflect the increased lifespan. These data are reminiscent of earlier experiments by Delbridge and Everitt, which showed that hypophysectomy delayed the age-related increase in TBT in rats [26]. Since hypophysectomy also suppressed lysyl oxidase activity, a role for that enzyme is suggested [27].

The fact that vitamins C and E combined were able to block TBT increase clearly implicates an oxidative cross-linking mechanism. A similar involvement of oxidant stress in diabetes-mediated TBT increase was demonstrated earlier by us [28]. While lysyl oxidase (LOX) crosslinks play a major role in collagen maturation, it is difficult to conceive how they would explain the data in the older postpubertal group in whom the intervention was started at 8 months of age. In that regard, Reiser found LOX crosslinks were inconsistently decreased or unchanged by dietary restriction [29]. Thus, while actual measurements are needed to definitively exclude a role for LOX crosslinks, the ability of green tea to suppress crosslinking in collagen from postpubertal mice suggests the crosslinking mechanism that is inhibited by green tea does not depend on LOX.

While the ability of the combination of vitamins C and E to suppress TBT increase strongly suggests an antioxidant effect, reactive oxygen species by themselves cannot crosslink collagen as shown in Figure 6. Thus an element of carbonyl stress combined with an oxidant mechanism is needed. The latter could involve oxidation of pre-existing Amadori products on collagen fibers into crosslinks. In that regard, the lack of an effect of green tea on the Amadori product does not exclude its role as pentosidine precursor, since pentosidine is present in levels 100 times lower than the Amadori product. However, pentosidine can also originate from ascorbic acid and pentoses.

The paradoxical finding that green tea catalyzed cross-linking *in vitro* is not necessarily at odds with its *in vivo* effects. It is likely that the most reactive catechins are absorbed by gut proteins through a covalent binding (“tanning”) process, allowing those with less crosslinking activity but potent antioxidant activity to get into the circulation. Because neither the catechins nor the antioxidant vitamins have carbonyl scavenging capability, a plausible mechanism to explain their anti-crosslinking activity is action at the cellular level. Indeed, Nishikawa *et al* have shown that reactive oxygen species can inactivate glyceraldehyde phosphate dehydrogenase, resulting in increased methylglyoxal and AGE formation [30]. Determination of carboxyethyl-lysine (CEL) or argpyrimidine as markers of methylglyoxal modification will be needed to investigate this hypothesis.

Another mechanism compatible with this proposition involves cellular lipid peroxidation products, such as 4-OH-nonenal [31] or malonyl dialdehyde [32]. Obviously, both the Maillard reaction as well as lipid peroxidation products can account for the *in vivo* formation of fluorescent collagen adducts [33].

What ingredients in green tea are likely to explain its anti-collagen-aging properties? The major ingredients of GTE fall into five major categories, the composition of which varies according to brand; i.e., the tannins, amino acids, caffeine, vitamins, and salts. A typical Sen-cha brew (first 1.0 minute brew at 90°C) from Japanese Kawanecha green tea contains about 13.0 g tannins (catechins, polyphenols), 2.3 g caffeine, 250 mg vitamin C, and 65.4 mg vitamin E per 100 g of tea leaves, according to manufacturer’s information. The latter translate into 33 ug Vit C and 8.6 ug Vit E per mL green tea extract consumed by our mice; i.e., five to eight times less than what has been supplemented in the experiments above. In the literature, however, much attention has been devoted to the biological properties of the catechins. These polyphenolic compounds have strong antioxidant properties and it was shown that several catechins are two to four times more potent than vitamin C and E [34]. Catechins are being widely investigated for their beneficial effects against cancer, inflammatory diseases, atherosclerosis, and cataracts [35, 36]. Yet, there are very limited studies on the effects of green tea and aging. Kumari *et al* [37] found green tea could prolong the mean life span of SAM mice by about 8 weeks, providing support for the free radical theory of aging. Most recently, while this study was in progress, Song *et al* reported that green tea suppressed collagen-linked fluorescence increase in aging rat aorta, but not skin [38].

In conclusion, our results show the ability of green tea and vitamins C and E to delay collagen cross-linking in adult mice. Green tea was also able to prevent the rise of fluorescent markers of collagen aging. Since the combination of vitamins C and E decreases crosslinking but not fluorescence, this suggests that the mechanism responsible for the TBT increase is different from that responsible for the fluorescence. The ability of green tea and the combination of vitamins C and E to block crosslinking without impact on glycemia suggests an antioxidant mechanism acting on cellular release of crosslinking agent(s). Finally, in view of the fact that unrelated processes, such as dietary restriction and mutation in the Pit gene, are associated with delayed collagen crosslinking and life span extension, it will be important to investigate whether chronic intake of green tea leads to a generalized delay in the aging process and prolongation of life span.

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Figure 1.

Effect of vitamin C, vitamin E, vitamin C & E, and blueberry and green tea extracts on body weight in C57BL/7 mice receiving the supplements in drinking water for a period of 14 weeks starting at 4 and 10 months of age. Data represent means $\pm$ S.D. Means which do not share a common superscript are statistically different from other means at $p < 0.05$ by Student-Newman-Keuls multiple comparison test. Effect of age: $p < 0.0001$ by analysis of variance (ANOVA). Effect of treatment: $p = \text{NS}$.

Figure 2. Effect of nutrient supplementation on skin collagen glycation as determined by the furosine assay. See Figure 1 for details and statistics. Treatment had no significant effect on tendon glycation (ANOVA, $p > 0.05$).

Figure 3.

Effect of nutrient supplementation on tail tendon break time (TBT) in urea. ANOVA shows the main effect due to age is significant ($p < 0.0001$). TBT is most markedly suppressed in the combined vitamin C & E and green tea groups.

Figure 4.

Effect of nutrient supplementation on tail tendon collagen-linked fluorescence. For details and statistics see legend to Figure1. Green tea suppresses fluorescence at 440 nm (excitation at 370 nm) ($p < 0.05$), at 385 nm upon excitation at 335 nm ($p = NS$).

Figure 5.
Effect of nutrient supplementation on skin collagen pentosidine levels. For details see Figure 1. There is a trend toward suppression of pentosidine ($p = \text{NS}$) by green tea and vitamin E.

Figure 6.

Comparison of the effects of superoxide (xanthine/xanthine oxidase), H_2O_2 with 100 μM Cu^{2+} , 10 mM D-glucose, 5 mM D-ribose, 0.5 mM glyceraldehyde, and 0.5 mM methylglyoxal on tendon break time increase upon incubation for 7 days at 37°C in Chelex-treated PBS.