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Research article

Interaction between ascorbic acid and gallic acid in a model of fructose-mediated protein glycation and oxidation

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ABSTRACT

Background: Dietary plant-based foods contain combinations of various bioactive compounds such as phytochemical compounds and vitamins. The combined effect of these vitamins and phytochemicals remains unknown, especially in the prevention of diabetes and its complications. The present study aimed to investigate the combined effect of ascorbic acid and gallic acid on fructose-induced protein glycation and oxidation.
Results: Ascorbic acid (15 μg/mL) and gallic acid (0.1 μg/mL) reduced fructose-induced formation of advanced glycation end products (AGEs) in bovine serum albumin (BSA; 10 mg/mL) by 15.06% and 37.83%, respectively. The combination of ascorbic acid and gallic acid demonstrated additive inhibition on the formation of AGEs after 2 weeks of incubation. In addition, synergistic inhibition on the formation of amyloid cross-β structure and protein carbonyl content in fructose-glycated BSA was observed. At the same concentration, the combination of ascorbic acid and gallic acid produced a significant additive effect on the 2,2-diphenyl-1-picrylhydrazyl radical scavenging activity.
Conclusion: Combining natural compounds such as ascorbic acid and gallic acid seems to be a promising strategy to prevent the formation of AGEs.

1. Introduction

Over-consumption of high-fructose diets contributes to the acceleration of obesity-related metabolic disorders such as insulin resistance and diabetes and complications that are apparently associated with increased production of advanced glycation end products (AGEs) [1,2]. Fructose, like other reducing sugars, can react with protein through nonenzymatic glycation and consequently results in the formation of Schiff bases and further synthesis of AGEs. The interaction of AGEs with receptor for AGEs (RAGEs) triggers signal transduction, resulting in reactive oxygen species (ROS) production and inflammation [3]. Previous studies revealed that fructose is a faster reducing agent than glucose to induce the formation and accumulation of protein-bound fluorescence, Amadori products, and cross-linking products at physiological temperature and equal concentration [4,5]. Furthermore, fructose has been shown to more rapidly produce reactive dicarbonyl compounds and hydroxyl radicals than glucose, which results in cellular oxidative damages [6]. Moreover, fructose-induced protein glycation causes the formation of protein aggregation. Prolonged incubation with fructose induces a transition in albumin to form the amyloid structure and protein oxidation [7,8,9] associated with a number of degenerative diseases, including Alzheimer's disease, rheumatoid arthritis, atherosclerosis, and diabetes [10].

Scientists have recently discovered that increasing fruit and vegetable consumption is associated with reduced risk of cardiovascular diseases, diabetes, Alzheimer's disease, and age-related functional decline [11,12,13]. Although dietary intake of bioactive constituents from fruits and vegetables has clearly shown health benefits, clinical trials of the purified bioactive compounds do not appear to have as consistent effects as a diet rich in fruit and vegetables [14,15]. When fruits and vegetables are consumed, the vitamins, phytochemicals, and minerals may interact in apparently additive or synergistic manner, leading

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to enhanced biological activities. Many studies have attempted to investigate the combined effects of vitamins and phytochemical compounds on different biological and pharmacological activities [16,17]. Studies on the interactions among these compounds are required to gain a better understanding, which can ultimately lead to the development of combined functional foods.

Vitamins play a vital role in maintaining normal metabolic processes and homeostasis within the body. Vitamin C (ascorbic acid) is an especially effective antioxidant that scavenges physiologically relevant reactive oxygen species and reactive nitrogen species [18]. Several studies have demonstrated the beneficial effects of the combination of ascorbic acid with other antioxidants in various models [19,20]. Gallic acid is a well-known phenolic acid found abundantly in tea, grapes and other fruits, and wine [21]. The pharmacological activities of gallic acid include antioxidant, anti-inflammatory, antimutagenic, and anticancer properties [21]. Moreover, gallic acid and ascorbic acid have recently been shown to inhibit AGE formation in physiological model systems [22,23]. In the present study, we hypothesized that their combination might produce an additive or synergistic effect on the inhibition of AGEs formation and the prevention of glycation-induced protein oxidation. Therefore, the combined effect of gallic acid and ascorbic acid was investigated in fructose-induced protein glycation and oxidation *in vitro*.

2. Materials and methods

2.1. Materials

Bovine serum albumin (BSA), 2,2-diphenyl-1-picrylhydrazyl (DPPH), thioflavin T, aminoguanidine (AG), ascorbic acid, 1-deoxy-1-morpholino-D-fructose (DMF), nitroblue tetrazolium (NBT), gallic acid and L-cysteine were purchased from Sigma-Aldrich Co. (St. Louis, MO, USA). 2,4-dinitrophenylhydrazine (DNPH) was purchased from Ajax Finechem (Taren Point, Australia). Trichloroacetic acid (TCA) and guanidine hydrochloride were purchased from Merck (Darmstadt, F.R., Germany). All other chemical reagents used in this study were of analytical grade.

2.2. Assay of protein glycation inhibitory activity

Glycated BSA formation was performed in accordance with a previously described method [24]. Briefly, BSA (10 mg/mL) was incubated with 0.5 M fructose in 0.1 M phosphate-buffered saline (PBS; pH 7.4) containing 0.02% sodium azide in the presence or absence of gallic acid (0.1 μg/mL), ascorbic acid (3.75, 7.5, and 15 μg/mL), or gallic acid plus ascorbic acid. The reaction mixtures were incubated in darkness at 37°C for 2 weeks. PBS was added as the solvent for all chemicals. The fluorescence intensity of glycated BSA was measured using a spectrofluorometer (Wallac 1420 Victor^3 V, PerkinElmer, Walham, MA, USA) at an excitation wavelength of 355 nm and emission wavelength of 460 nm. The percentage inhibition of AGEs formation was calculated using the following formula. AG (1 mg/mL) was used as a positive control for this study.

$$\% \text{ Inhibition} = \frac{Abs_{Control} - Abs_{Sample}}{Abs_{Control}} \times 100$$

2.3. Fructosamine measurement

The concentration of the Amadori product fructosamine was determined by NBT assay [24]. Briefly, glycated BSA (10 μL) was incubated with 90 μL of 0.5 mM NBT in 0.1 M carbonate buffer, pH 10.4, at 37°C. The absorbance was measured at 530 nm at 10 and 15 min using a spectrophotometer (PowerWave XS2, BioTek, Winooski, VT, USA). The concentration of fructosamine was calculated by using the different absorption at 10 and 15 min time points compared with the standard curve of DMF.

2.4. Determination of protein carbonyl content

The carbonyl group in glycated BSA was determined following a previously described method [24]. Briefly, 400 μL of 10 mM DNPH in 2.5 M HCl was added to 100 μL glycated samples. After 1 h of incubation in the dark, 500 μL of 20% (w/v) TCA was used for protein precipitation (5 min on ice) and then centrifuged at 10,000 × g for 10 min at 4°C. The protein pellet was washed with 1 mL ethanol/ethyl acetate (1:1) mixture three times and resuspended in 250 μL of 6 M guanidine hydrochloride. The absorbance was measured at 370 nm. The carbonyl content of each sample was calculated using the extinction coefficient for DNPH (ε = 22,000/M cm). The results were expressed as nmol carbonyl/mg protein.

2.5. Determination of amyloid cross-β structures

The concentration of amyloid cross-β structures was measured using thioflavin T according to a previously described method [24]. Briefly, 50 μL of 64 μmol/L thioflavin T in 0.1 M PBS, pH 7.4, was added to the glycated samples (50 μL) and incubated at room temperature for 60 min. The fluorescence intensity was measured using a Synergy 2 Multi-Mode Reader (BioTek, Winooski, VT, USA) at an excitation wavelength of 435 nm and an emission wavelength of 485 nm.

2.6. DPPH radical scavenging activity

Antioxidant capacity was measured using the DPPH assay according to a previously described method [25]. Briefly, various concentrations of gallic acid, ascorbic acid, and their combination (final volume: 100 μL) were added to 100 μL DPPH solution (0.2 mM in ethanol) and incubated for 30 min at room temperature. The decrease in the solution's absorbance was measured using a spectrophotometer (PowerWave XS2, BioTek, Winooski, VT, USA) at 515 nm. Percent DPPH radical scavenging activity was calculated according to the formula

$$\%\text{DPPH radical scavenging activity} = (A - B)/A \times 100$$

where A = absorbance of control without test compound and B = absorbance of test compound.

2.7. Statistical analysis

All data are presented as means ± S.E.M for each treatment group (n = 3). Statistical significance was evaluated by one-way ANOVA. Duncan multiple range test was used to analyze sources of significant differences. A p-value of <0.05 was considered statistically significant.

3. Results

3.1. The effects of combined ascorbic acid and gallic acid on the formation of AGEs

The BSA/fructose solution containing gallic acid (0.1 μg/mL) showed significantly less fluorescence intensity, corresponding to AGEs formation, at week 1 and 2 by 25.34% and 15.06%, respectively (Table 1). Moreover, ascorbic acid markedly decreased the fluorescence intensity in a concentration-dependent manner. The percentage inhibition of various concentrations of ascorbic acid (3.5–15 μg/mL) ranged from 29.46 to 45.43% at week 1 and 16.83 to 37.83% at week 2. It was interesting to establish whether gallic acid (0.1 μg/mL) and various concentrations of ascorbic acid interact synergistically or additively on the inhibition of protein glycation. The results showed that the combination of gallic