



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.

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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 Victor³ 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 ($\epsilon = 22,000/\text{M cm}$). 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

Table 1

The percentage inhibition (%) of fluorescent advanced glycation end product (AGE) formation by gallic acid, ascorbic acid, and their combinations in fructose-induced protein glycation.

Experiments	%Inhibition	
	Week 1	Week 2
0.1GA	25.34 ± 2.91 ^a	15.06 ± 1.62 ^a
3.75AA	29.46 ± 2.54 ^{a,b}	16.83 ± 3.35 ^a
7.5AA	36.14 ± 2.89 ^{b,c}	20.93 ± 3.23 ^{a,b}
15AA	45.43 ± 0.23 ^c	37.83 ± 1.82 ^c
0.1GA + 3.75AA	37.97 ± 2.61 ^{b,c}	20.01 ± 2.19 ^{a,b}
0.1GA + 7.5AA	43.88 ± 1.83 ^c	25.27 ± 2.15 ^{a,b}
0.1GA + 15AA	55.34 ± 1.82 ^d	46.85 ± 0.61 ^d
AG	84.17 ± 0.64 ^e	80.54 ± 0.46 ^e

Data are expressed as mean ± S.E.M, n = 3. In each column, different letters for each mean indicate statistically significant differences at $p < 0.05$. 0.1GA: gallic acid (0.1 µg/mL = 0.59 µmol/L), 15AA: ascorbic acid (15 µg/mL = 63.94 µmol/L), 7.5AA: ascorbic acid (7.5 µg/mL = 42.62 µmol/L), 3.75AA: ascorbic acid (3.75 µg/mL = 21.31 µmol/L), AG: aminoguanidine (1 mg/mL).

acid with ascorbic acid (15 µg/mL) exhibited additive inhibition against fructose-induced AGEs formation at week 2. However, the percentage inhibition of AGEs formation did not increase with reduced concentrations of ascorbic acid (3.75 and 7.5 µg/mL) plus gallic acid, suggesting that they had no additive or synergistic interaction.

3.2. Effects of combined ascorbic acid and gallic acid on the level of fructosamine

The concentrations of fructosamine, the Amadori product, in glycated BSA are shown in Table 2. After 2 weeks of incubation, gallic acid at a concentration of 0.1 µg/mL markedly reduced the level of fructosamine (18.5%). A similar accompanying decrease in the level of fructosamine was also observed with ascorbic acid at concentrations of 7.5 µg/mL (23.5%) and 15 µg/mL (42.0%). There was a slight reduction in fructosamine production (10.6%) with ascorbic acid at a concentration of 3.75 µg/mL. However, there were no significant reductions of fructosamine with combinations of gallic acid (0.1 µg/mL) and ascorbic acid (3.75–15 µg/mL).

3.3. Effects of combined ascorbic acid and gallic acid on protein oxidation

Protein carbonyl content is generally considered a marker of protein oxidation in glycation reaction. After 2 weeks of incubation, gallic acid (0.1 µg/mL) significantly reduced the concentration of protein carbonyl by 48.0%, whereas ascorbic acid (3.5–15 µg/mL) suppressed protein carbonyl formation by 34.1–39.0%, as shown in Table 2. The combination of gallic acid (0.1 µg/mL) and ascorbic acid (15 µg/mL) resulted in a significant increase in the percentage reduction of protein carbonyl content (68.3%). The values obtained were significantly higher than those derived with individual compounds, indicating that an additive interaction occurred in the mixture. However, the percentage

reduction of protein carbonyl content did not increase with ascorbic acid (3.75 and 7.5 µg/mL) plus gallic acid (0.1 µg/mL), suggesting that at these concentrations, the combination could not exhibit an additive effect against glycation-induced protein oxidation.

3.4. Effects of combined ascorbic acid and gallic acid on the level of amyloid cross-β structure

Protein glycation generally induces the formation of amyloid cross-β structure in albumin. The level of amyloid cross-β structure in BSA was determined by thioflavin T assay (Table 2). The results showed that gallic acid (0.1 µg/mL) did not decrease the fluorescence intensity, whereas the presence of ascorbic acid (3.75–15 µg/mL) in the solution significantly reduced the fluorescence intensity by 1.6–15.7%. The addition of gallic acid (0.1 µg/mL) together with ascorbic acid (3.75–15 µg/mL) significantly increased the percentage inhibition of amyloid cross-β structure formation when compared with ascorbic acid or gallic acid alone. The percentage inhibition with the combination was greater than the summing effect of ascorbic acid (3.75–15 µg/mL) and gallic acid (0.1 µg/mL) by 25.9%, 27.0%, and 28.63%, respectively. These results suggest that gallic acid interacts with ascorbic acid in a synergistic manner for the inhibition of amyloid cross-β structure formation.

3.5. Effects of combined ascorbic acid and gallic acid on DPPH radical scavenging activity

The combined effect of gallic acid and ascorbic acid on DPPH radical scavenging activity is shown in Table 3. Gallic acid (0.1 µg/mL) and ascorbic acid (15 µg/mL) had DPPH radical scavenging activities of 11.23% and 12.87%, respectively. The combination of gallic acid (0.1 µg/mL) and ascorbic acid (15 µg/mL) showed a significantly increased % DPPH radical scavenging activity (17.69%) when compared to the individual values, indicating that they produced additive interaction. However, the combination of gallic acid and ascorbic acid (3.75–7.5 µg/mL) did not show additive or synergistic effects against DPPH radical scavenging activity.

4. Discussion

The present study demonstrated the combined effect of ascorbic acid and gallic acid on fructose-induced protein glycation after 2 weeks of incubation. Interestingly, an additive interaction was observed with the combination of ascorbic acid and gallic acid. The combination of ascorbic acid and gallic acid produced a synergistic effect on the inhibition of protein oxidation. It was noteworthy that the antiglycation effect of ascorbic acid and gallic acid was also observed in other studies using different concentrations. Ascorbic acid (vitamin C) has been shown to reduce protein glycation both *in vitro* and *in vivo* [23,26]. Supplementation of ascorbic acid results in a significant decrease in serum protein glycation in middle-aged subjects [26]. Gallic acid, a naturally occurring compound, is a potent inhibitor of

Table 2

Effects of gallic acid, ascorbic acid, and their combinations on the level of fructosamine, amyloid cross β-structure, and protein carbonyl content in BSA incubated with fructose for 2 weeks.

Experiments	Fructosamine (mM)	Protein carbonyl content (nmol/mg protein)	Amyloid cross β-structure (435/485 nm) × 10 ³
BSA	0.21 ± 0.03 ^a	0.28 ± 0.01 ^a	2.23 ± 0.03 ^a
Control	1.19 ± 0.09 ^b	1.23 ± 0.08 ^b	4.40 ± 0.08 ^b
0.1GA	0.97 ± 0.03 ^{c,d}	0.64 ± 0.08 ^d	4.21 ± 0.09 ^b
3.75AA	1.06 ± 0.03 ^{b,c}	0.81 ± 0.03 ^{c,d}	3.71 ± 0.09 ^c
7.5AA	0.91 ± 0.06 ^d	0.77 ± 0.02 ^{c,d}	3.85 ± 0.10 ^c
15AA	0.69 ± 0.03 ^e	0.75 ± 0.07 ^{c,d}	4.33 ± 0.12 ^b
0.1GA + 3.75AA	1.03 ± 0.03 ^{c,d}	0.92 ± 0.09 ^c	3.14 ± 0.05 ^d
0.1GA + 7.5AA	0.94 ± 0.03 ^{c,d}	0.72 ± 0.02 ^d	3.21 ± 0.10 ^d
0.1GA + 15AA	0.65 ± 0.03 ^e	0.39 ± 0.03 ^e	3.26 ± 0.11 ^d
AG	0.94 ± 0.03 ^{c,d}	0.82 ± 0.06 ^{c,d}	3.05 ± 0.07 ^d

Data are expressed as mean ± S.E.M, n = 3. In each column, different letters for each mean indicate statistically significant differences at $p < 0.05$. 0.1GA: gallic acid (0.1 µg/mL = 0.59 µmol/L), 15AA: ascorbic acid (15 µg/mL = 63.94 µmol/L), 7.5AA: ascorbic acid (7.5 µg/mL = 42.62 µmol/L), 3.75AA: ascorbic acid (3.75 µg/mL = 21.31 µmol/L).

Table 3

The percentage of DPPH radical scavenging activity of gallic acid, ascorbic acid, and their combinations.

Experiments	Molar ratio (GA:AA)	(%) DPPH radical scavenging activity
0.1 GA	–	11.23 ± 0.48 ^a
3.75AA	–	4.39 ± 0.88 ^b
7.5AA	–	12.33 ± 0.66 ^a
15AA	–	12.87 ± 0.21 ^a
0.1GA + 3.75AA	1:36	9.15 ± 0.64 ^a
0.1GA + 7.5AA	1:72	10.13 ± 0.64 ^a
0.1GA + 15AA	1:108	17.69 ± 0.72 ^c

Data are expressed as mean ± S.E.M, n = 3. In each column, different letters for each mean indicate statistically significant differences at $p < 0.05$. 0.1GA: gallic acid (0.1 µg/mL = 0.59 µmol/L), 15AA: ascorbic acid (15 µg/mL = 63.94 µmol/L), 7.5AA: ascorbic acid (7.5 µg/mL = 42.62 µmol/L), 3.75AA: ascorbic acid (3.75 µg/mL = 21.31 µmol/L).

glycation [22]. Moreover, some studies have reported the inhibitory effect of ascorbic acid and gallic acid on the formation of amyloid cross β -structure [27,28]. Our present findings demonstrated synergistic inhibition by combined ascorbic acid and gallic acid on the formation of amyloid cross β -structure in BSA. The beneficial effect of the synergistic interaction may help reduce the risk of developing diabetes and its complications. A previous study has revealed the site specificity of AGE formation by fructose, which preferentially modifies lysine-524 of BSA [29]. Ascorbic acid and gallic acid are also capable of binding to human serum albumin [30,31]. The mechanisms of the observed interactions between ascorbic acid and gallic acid against fructose-induced glycation remain unknown. The binding site of ascorbic acid and gallic acid on albumin may be responsible for the molecular mechanisms. Their bindings possibly interact with the major site of protein modification by fructose, causing an increase in the ability to inhibit the formation of protein glycation and amyloid cross β -structure. The explanation for this hypothesis requires clarification through further investigation, and computer modeling may be used to evaluate the binding activity of these compounds on albumin.

Several major mechanisms by which antioxidants block the carbonyl group in reducing sugars and break the cross-linking structure in the formed AGEs have been proposed for the antiglycation activity [32]. The free radical scavenging ability of antioxidants may highlight other mechanisms for the reduction of AGE formation. The literature has described that superoxide anions from early glycation products are generated during protein glycation [33,34]. These products undergo fragmentation through reactive oxygen species-mediated reactions to generate short-chain carbohydrate intermediates that alter lysine and arginine residues to produce AGEs. Our findings have currently shown the free radical scavenging activity of ascorbic acid and gallic acid. When ascorbic acid was combined with gallic acid, it showed additive interaction on the free radical scavenging activity. According to the abovementioned antiglycation mechanisms, the combination of ascorbic acid with gallic acid inhibits AGE formation and protein carbonyl formation because of their ability to scavenge free radicals.

Ascorbic acid is an essential micronutrient that is involved in many biological and biochemical functions. The current recommended dietary allowance for vitamin C is 90 mg/d for men and 75 mg/d for women. In a physiological setting, for instance, with consumption of 5 to 9 servings of fruits and vegetables daily, ascorbic acid can achieve a steady-state concentration of approximately 80 µmol/L in the plasma [35]. Oral dosing of ascorbic acid (1.25 g) results in plasma concentrations that reach a plateau of 134.8 ± 20.6 µmol/L [35]. Gallic acid present in fruits and vegetables is considered to be a nontoxic compound that can be absorbed and metabolized by the body. The pharmacokinetic data of gallic acid in healthy humans are available. After a single oral administration of acidum gallicum tablets or black tea (each containing 0.3 mmol gallic acid) to the subjects, gallic acid was rapidly absorbed, and the maximum concentration of gallic acid observed in the plasma was 1.83 µmol/L with the tablets and 2.09

µmol/L with black tea at 1.3 h and 1.4 h, respectively [36]. In the present experiments, we used ascorbic acid and gallic acid at physiologically achievable concentrations on the basis of pharmacokinetic data. Considering the results presented here, the additive and synergistic interactions possibly occurred because the concentrations of ascorbic acid (15 µg/mL = 85 µmol/L) and gallic acid (0.1 µg/mL = 0.59 µmol/L) were in a physiologically achievable concentration range. The present findings may provide important *in vitro* evidence for clinical observations, showing that coadministration of ascorbic acid and gallic acid enhances the ability to inhibit protein glycation and oxidation. Further studies are required to ascertain the effect of ascorbic acid and gallic acid and their combination in diabetic patients.

5. Conclusion

The present results indicate that ascorbic acid and gallic acid exhibit additive inhibition of fructose-induced protein glycation. The combination also demonstrates synergistic inhibition of amyloid cross- β structure formation and protein carbonyl content in fructose-glycated BSA. Our findings in the *in vitro* experiments, although not directly applicable to humans, can help to better understand the *in vivo* interaction between vitamin C (ascorbic acid) and a phytochemical compound (gallic acid). The combination of ascorbic acid and gallic acid may be advantageous in designing new dietary supplements or nutraceuticals.

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Conflicts of interest

The authors declare no conflict of interest.

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