Herbal extracts inhibit Maillard reaction, and reduce chronic diabetic complications risk in streptozotocin-induced diabetic rats.

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Abstract

Objectives: Accumulation of end-stage products of the Maillard reaction, also called advanced glycation end products (AGEs), is a hallmark of aging and the pathogenesis of chronic diabetic complications. The aim of this study was to determine whether safe and effective substances contained in four extracts of foodstuffs might slow the development of diabetic complications as well as slow the progression of aging.

Design: We evaluated the in-vitro activity of four extracts of dried herbs available in Japan (Anthemis nobilis, Crataegus oxyacantha, Houttuynia cordata, Vitis vinifera) and a mixture of these to inhibit the Maillard reaction. We also assessed whether a 12-week feeding of mixed herbal extract (MHE) admixed in MF chow to streptozotocin (STZ)-induced diabetic rats prevented the development of diabetic complications.

Results: Each of the four herbal extracts as well as the mixed extract dose-dependently inhibited the generation of Maillard reaction products in vitro with a potency similar to that of aminoguanidine (AG), a drug used for treating diabetic complications. Furthermore, after a 12-week, MHE supplemental feeding to STZ-induced diabetic rats, serum pentosidine and Nε-(carboxymethyl)lysine levels and wet weight of the kidney tended to decrease. MHE elicited AG-like actions and produced an inhibitory effect on pentosidine generation at lower concentrations than those observed for AG.

Conclusion: Through the inhibition of the Maillard reaction, MHE may slow the development of chronic diabetic complications as well as slow the progression of aging.

KEYWORDS: Herbal extracts, aminoguanidine, streptozotocin-induced diabetes mellitus, advanced glycation end products, diabetic complications

Introduction

Under hyperglycemic conditions, as is common in diabetes, the nonenzymatic reaction of glucose-derived aldehyde with the amino groups on proteins results in formation of a reversible Schiff base, which is converted to stable Amadori compounds by an Amadori rearrangement. The nonenzymatic reaction is called the Maillard reaction, through which advanced glycation end products (AGEs) are generated. 1 Accelerated endogenous generation of AGs is commonly associated with chronic diabetic complications and aging. It has been suggested that denaturation of collagen proteins by Maillard reaction is closely related to physical aging. 2,3 Thereafter, through a series of complex reactions like dehydration, condensation, oxidation, and rearrangement, the Amadori compounds are converted to highly reactive compounds such as 3-deoxyglucosone (3DG), glyoxal, and methylglyoxal, eventually leading to the formation of the final AGEs by way of the irreversible pathways of glucose metabolism. AGEs are comprised of Nε-(carboxymethyl)lysine (CML), pentosidine (Pent), pyrraline, crossline, and 2-(2-furyl)-3-(5)-2-(2-furyl)-1H-imidazole. 4 Miyata and Monnier 5) and Niwa et al. 6) have detected novel AGs such as pyrraline and imidazolone either in serum and the sereased tissues from diabetic rats or in erythrocytes, kidneys, and aortas from diabetic patients, by using monoclonal anti-pyrraline/imidazolone antibodies, respectively.

It has been demonstrated that the development of diabetic vascular complications is mediated by AGEs, which activate the AGE receptor (RAGE). Mice with genetically modified RAGE genes show dramatically altered phenotypes. 5) The Maillard reaction in skin collagen may accelerate the aging process in association with boosted protein bridging and a reduction of elasticity. 7) The levels of the Maillard reaction products in skin collagen increases with age. 8) Levels of glycosylated protein are also higher in the skin, nails, and hair of patients with diabetes as compared with levels in nondiabetic subjects. 9)

Aminoguanidine (AG), a Maillard reaction inhibitor, has been used to inhibit excess AGE formation and accumulation in hyperglycemic conditions. 10) AG, by interfering with the reaction of 3DG (an intermediate in the advanced Maillard reaction) with amino donors, has been shown to be effective in preventing retinopathy 11) and nephropathy 12) in diabetic patients, and atherosclerosis in diabetic rats. 13) AG has also been shown to prevent AG1-collagen cross-linking due to volume overload leading to cardiac hypertrophy. 14) However, it has been reported that AG causes anemia, impairs hepatic function, and is related to the appearance of autoantigens. 15) These undesirable side effects due to AG prompted our exploration of safer and more effective AGE inhibitors as compared with AG.

Tea leaf, 16) herbs, 17,18) and oriental medicines 19) have been reported to inhibit the Maillard reaction. We studied foodstuffs marketed in Japan that might inhibit the Maillard reaction and possibly prevent the development of chronic diabetic complications. We selected four dried herbs after confirming their safety by toxicity testing (acute toxicity, genotoxicity, pesticide residue).
Materials and Methods

Herbal extracts (HEs) of Anthemis nobilis (Chamomile: flower, AN), Creataegus oxyacantha (Hawthorn: berry, CO), Houttuynia cordata (Doku-dami: whole plants, IJC), and Vitis vinifera (Grape: leaf, VV) were prepared as follows. One kilogram of each of the dried herbs was soaked in 20 liters of hot (80°C) water for 3 hours, followed by filtration and concentration under reduced pressure. The test extracts were prepared by pulverizing the concentrated raw extracts after freezing and drying. The mixed herbal extract (MHE) was prepared by soaking 100 g of each of the dried herbs in 8 liters of hot water followed by the same procedures described earlier. Test substances were individual extracts AN, CO HC, VV, mixed herbal extract (MHE) of the four, and Aminoquinidine hydrochloride (AG) code 010-12912 from Wako Chemical Co., Ltd., (Osaka, Japan).

Streptozotocin (STZ) code S0130 from Sigma Chemical Company Ltd., (MO, USA) 55 mg/kg (dissolved in citrate buffer, pH 4.5) was used for inducing diabetes.

Animals and Diabetes Induction

Male SLC:Sprague-Dawley rats, 5 weeks of age, were purchased from SLC Co., Ltd. (Shizuoka, Japan). A maximum of four animals were housed in each individual aluminum-made stainless cage (width 300 mm, depth 430 mm, and height 190 mm). All study animals were kept in a breeding room (room temperature 20°C–26°C, humidity 40%–70%, artificial illumination of 12 hours bright and dark cycle). For seven days, the rats were put in quarantine, acclimatized, and fed MF powdered food (MF) for mice and rats (Oriental Yeast Co., Ltd., Tokyo, Japan). The acclimatized animals were randomly assigned to four groups (n = 7 in each group): normal control (G1), STZ-induced diabetic rat control group (G2), MHE-treated, STZ-induced diabetic rat group (G3), and AG-treated, STZ-induced diabetic rat group (G4). After twelve hours of fasting, diabetes was induced in all animals except for those in the normal control group by a single tail vein injection of STZ.

Preparation of Test Chow

Chow for G3 or G4 was prepared by admixing MHE or AG in MF 0.2% (w/w) while G1 and G2 were fed MF only. All groups were fed their appropriate chow over twelve weeks. The chow and chlorinated drinking water were given ad libitum.Consumption of chow and water was estimated twice a week from the measured amount of uneaten chow. Approximate amount of MHE intake was 27mg/100g body weight in G3 and that of AG was 30mg/100g body weight in G4.

In Vitro Evaluation of Inhibitory Activities Against the Maillard Reaction

One hundred microliters of each of HEs, MHE or AG aqueous solutions were incubated for 40 hours at 60°C (A) with the mixture of 0.1 mol/L phosphate buffer saline (PBS) (100 μL), 40 mg/mL of human serum albumin (HSA, Sigma Chemical Co., Ltd., MO, USA) (200 μL), and 2 mmol/L of glucose aqueous solution (100 μL). At the same time, the blank test was carried out by replacing glucose solution with distilled water (B). The herbal extracts-free or AG-free samples were prepared as positive controls (C). At the same time, the blank test of positive controls was carried out by replacing glucose solution with distilled water (D). Concentrations of the Maillard reaction products in each of four (A, B, C and D) reaction mixtures were determined, and the relative product ratio of the Maillard reaction products was estimated by using the following equation:

Relative Product Ratio (%) = (A-B) / (C-D) × 100

Urinalysis and Blood Chemistry

Twenty-four hour urine samples were collected from animals in each group before, 0, 4, 8, and 12 weeks. Determination of urine levels of glucose (Electrode method) and protein (pyrogallol method) were performed by Bio Medical Laboratories (BML), Inc. (Tokyo, Japan).

Blood samples were collected from the tail vein before, 0, 4, 8, and 12 weeks. Blood glucose levels were determined by using Glucocard™ (ARKRAY, Kyoto, Japan). Serum levels of the Maillard reaction products were determined quantitatively after centrifugation of the venous blood at 3,000 rpm for 10 minutes. The amount of furoseine was measured by HPLC method. As first, 100 μL of serum added 50 μL trichloroacetic acid solution and centrifuged at 10,000 rpm for 5 minutes. The supernatant was reacted at 120°C for overnight, followed by evaporation to dryness. The dried extract was reconstituted distilled water and was later use for HPLC. HPLC assay applied isocratic system of phosphate-methanol solvent on reverse phase TSK-GEL ODS-80TM column (4.6 × 250 mm, TOSOH, JAPAN) at 280 nm detection. The amount of 3-Deoxyglucose (3DG) was measured by HPLC method. Briefly, 300 μL of serum or 3DG standard added to 30 μL of 60% perchloric acid solution and centrifuged at 3,000 rpm for 5 minutes. The supernatant neutralized using disodium carbonate added to 0.1% 2,3-diaminonaphthalene and 0.005% 2,3-pentandione, the reaction was performed at 4°C for overnight. The reaction mixture was extracted using ethyl acetate, followed by evaporation to dryness. The dried extract was reconstituted 50% methanol and was later use for HPLC. HPLC assay applied linear gradient system of 50 mmol/L phosphate-methanol-acetoniitrite solvent on reverse phase TSK-GEL ODS-80TM column (4.6 × 250 mm, TOSOH, JAPAN) at 268 nm detection. The amount of pentosidine (Pent) was measured by ELISA method. After adding 50 μL of serum or Pent standard to 20 μL pronase and 80 μL of Tris-HCl buffer, the mixture was incubated for 55°C for 90 minutes, and heated at in boiling water for 15 minutes to inactive on the enzyme. These pretreated sample were added to each well and incubated 37°C for 1 hour after washing. Then, 50 μL of pentosidine antibody and pentosidine standard solution or pretreated sample were added to each well and incubated at 37°C for 1 hour after washing. A color development regent containing 0.5 mg/mL of 3,3′5′-tetramethylbenzidine (TMB) was added to each well. The reaction was stopped 10 minutes later by adding 100 μL of TMB stop buffer. The absorbance was measured within 10 minutes at 450 nm and 630 nm. The standard curve was obtained by measuring standard pentosidine solutions at 0, 0.00005, 0.0005, 0.005, 0.5 and 5.0 μg/mL. Also the amount of N²-(carboxymethyl) lysine (CML) was measured by ELISA method, instead of CML antibody and CML standard solution. These were measured by Fushimi Pharmaceutical Co., Ltd. (Marugame, Japan).

Statistics

Results are expressed as means ± SE (standard error). Differences among means within levels of a factor were determined by using the Tukey’s test. Significant differences were assumed at the level of p < 0.05.
Results

During acclimation, the G1 animals showed no signs or symptoms of infection and gained weight up to 526.2 ± 15.9 g (mean ± SE) at 12 weeks. However, one of the G3 animals died after combat with other animals. At 12 weeks, Weight gains in G2, G3, and G4 rats were 219.8 ± 21.8 g, 224.0 ± 13.3 g, and 201.2 ± 13.0 g, respectively, with an average food consumption of 29.9 ± 2.9 g/day for each of the four groups with no between-group differences. Water consumption before STZ treatment of the G2, G3 and G4 groups and of the G1 control group amounted to 25.2 ± 0.7 mL/day; and after STZ treatment of G2, G3 and G4 groups, this increased to 188.8 ± 4.1 mL/day.

Blood glucose levels before STZ treatment and of the G1 control group were 104.7 ± 1.5 mg/dL; however the rats in groups G2, G3 and G4 treated with STZ became hyperglycemic (486.4 ± 9.1 mg/dL).

Glucose levels in urine and urine volume were 75.4 ± 7.9 mg/dL, and 8.4 ± 0.6 mL/24 hours, respectively, before STZ treatment of groups G2, G3 and G4 and of the G1 control group, with both increasing up to 6,775 ± 83.8 mg/dL and 152.4 ± 3.5 mL/24 hours, respectively, after STZ treatment of groups G2, G3, and G4. There was no difference in blood glucose, glucose in urine, and urine volume before or after treatment of groups G2, G3, and G4.

Protein levels in urine were 10.4 ± 1.4 mg/24 hours before STZ treatment of groups G2, G3 and G4, and of G1, but rose up to 18.9 ± 2.5 mg/24 hours, 16.1 ± 1.7 mg/24 hours, and 16.5 ± 1.6 mg/24 hours for G2, G3, and G4 groups, respectively. There was less of protein levels in urine of G3 (p=0.89) and G4 (p=0.76) rats compared with the G2 rats at 12 weeks.

Fig.1 shows the effects of HEs obtained from AN, CO, HC, and VV on the formation of furosine, 3DG, Pent, and CML. HEs from AN, CO, HC, and VV inhibited the production of AGEs such as 3DG, Pent, and CML in a concentration dependent manner at 0.1%–0.006%. Each of HEs reduced formation of 3DG and AGEs (Pent, CML) down to less than 21% at 0.1%, while all of them had essentially no inhibitory effects on furosine production.

![Figure 1](image_url)  
**Fig.1.** Inhibitory effects of herbal extracts (HEs) on the Maillard reaction products in vitro.
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*Fig. 2.* Effects of the mixed herbal extract (MHE), aminoguanidine (AG) and positive control (C) on the Maillard reaction products *in vitro.*

*Fig. 2* depicts the effects of MHE and AG on Maillard reaction products formation. They also elicited a concentration-dependent inhibition of 3DG and CML production. Both MHE and AG markedly (down to 20% to the control) inhibited Pent production at 0.2%, although MHE showed greater inhibitory activities with no concentration dependency as compared to AG, which showed Pent production accelerating activity at 0.1% and higher concentrations tested.

Serum fructose levels were 14.1 ± 0.7 μg/mL, 16.3 ± 1.0 μg/mL, 18.7 ± 1.5 μg/mL, 14.6 ± 1.7 μg/mL in G1, G2, G3, and G4, respectively, at 12 weeks of acclimation. *Fig. 3* illustrates the effect of MHE and AG on serum levels of 3DG, Pent and CML in STZ-induced diabetic rats. Serum levels of 3DG, Pent and CML were significantly (*p*<0.05) lower in G1 as compared with those in G2, G3, and G4. There was less of Pent in the blood of G3 (*p*=0.18) and G4 (*p*=0.15) rats compared with the G2 rats at 12 weeks. Similarly there was less of CML in the blood of G3 (*p*=0.70) and G4 (*p*=0.05) rats compared with the G2 rats at 12 weeks. The aorta and tail tendon collagen isolated from the four groups were similar in appearance at 12 weeks. Hypertrophy in the kidneys and appendix was observed in G2, and hypertrophy in the appendix was noticed in G3 and G4. The wet weight of the left kidney in G2 (1.9 ± 0.1g) tended to be heavier than that found in G1 (1.5 ± 0.05 g, *p*<0.04), G3 (1.6 ± 0.1 g, *p*<0.28), and G4 (1.7 ± 0.1 g, *p*<0.59).
**Discussion**

**Do the Herbal Extracts Inhibit the Maillard Reaction?**

It has been reported that a variety of herbs including tea leaf\(^{14,15}\) and some kinds of vitamins\(^{16}\) inhibit the Maillard reaction. In the present study carried out using an in-vitro system, we assessed the inhibitory effects of selected herbal extracts (HEs) on Maillard reaction product formation by concomitantly determining the reaction intermediates and AGEs generated through interaction of glucose with HSA. All of the HEs, MHE, and AG lacked inhibitory effects on furosine formation, suggesting that these compounds may not have inhibitory activities against an early step in the Maillard reaction pathway. In contrast, the HEs of AN, CO, HC, and VV, as well as the MHE inhibited the production of 3DG, Pent, and CML in a concentration-dependent manner.

**Do the Mixed Herbal Extracts Slow the Development of Chronic Diabetic Complications?**

The Maillard reaction is characterized by nonenzymatic glycosylation of protein by glucose and the subsequent cross-linking through Amadori products leading to the formation of AGEs. By using pepsin to digest tail tendon collagen isolated from normal, STZ-induced diabetic, and AG-treated STZ-induced diabetic male Sprague Dawley (SD) rats, Kochkian et al.\(^{20}\) found that solubility was significantly increased with AG at 100 mg/kg (oral gavage for 10 weeks), suggesting that AG inhibits AGE formation and ameliorates cross-linking of tail tendon collagen. According to Nakamura et al.,\(^{21}\) the thiazolidine derivative OPB-9195 inhibits AGE formation and AGE-derived cross-linking, and attenuates AGE deposition in the glomeruli, resulting in prevention of the progression of diabetic glomerular sclerosis in
Otsuka-Long-Evans-Tokushima-Fatty (OLETF) rats. Bolton et al. provided the first clinical evidence suggesting that inhibition of AG formation may lead to clinically important attenuation of serious complications of type 1 diabetes mellitus, although they failed to demonstrate a statistically significant beneficial effect of AG on the progression of overt nephropathy due to type 1 diabetes.

It is well known that AGs activate the receptor for AG, leading to gene activation through NF-κB in the nuclear cascade of signaling pathways. Matsuura et al. demonstrated that only Pent, among the tested AGs activates NF-κB in PC-12 cells, suggesting that Pent is responsible for the tissue dysfunction. As shown in the present experiments, MHE showed a greater inhibitory activity against Pent formation as compared to that shown by AG. Therefore, MHE may be a more effective inhibitor of AG formation. Also, Dyer et al. reported that Maillard reaction products accumulate with age and are responsible for the chemical aging of collagen. Since much more Pent and CML are accumulate in the skin collagen of the type 1 diabetes patient, a link between aging and acceleration of the process in diabetes was made.

In the present study, we compared the inhibitory activities of 4 herbs and MHE, suppressing the Maillard reaction in vitro, with AG used as a reference compound, and also studied whether adding MHE supplement to the feed of STZ-treated rats for twelve weeks would inhibit the development of diabetic complications. The rats that were confirmed to have developed diabetes mellitus had increased serum levels of Maillard reaction products: 3DG, Pent and CML. Although MHE and AG did not show statistically significant, also decreased the left kidney weight, as evidenced by the absence of hypertrophy in the kidney. These results suggest that MHE may suppress chronic diabetic complications and one of the risks of aging.

References