Anti-Glycation Effects of Pomegranate (*Punica granatum* L.) Fruit Extract and Its Components *In Vivo* and *In Vitro*

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ABSTRACT

Accumulation of advanced glycation end products (AGEs) leads to various diseases such as diabetic complications and arteriosclerosis. In this study, we examined the effect of pomegranate fruit extract (PFE) and its constituent polyphenols on AGEs formation *in vivo* and *in vitro*. PFE, fed with a high-fat and high-sucrose (HFS) diet to KK-A^y mice, significantly reduced glycation products such as glycoalbumin (22.0 ± 2.4%), hemoglobin A1c (5.84 ± 0.23%), and serum AGEs (8.22 ± 0.17 μg/mL), as compared to a control HFS group (30.6 ± 2.6%, 7.45 ± 0.12% and 9.55 ± 0.17 μg/mL, respectively, $P < 0.05$). In anti-glycation assays, PFE, punicalin, punicalagin, ellagic acid, and gallic acid suppressed the formation of AGEs from bovine serum albumin and sugars. In this study, we discuss the mechanism of the anti-glycation effects of PFE and its components *in vivo* and *in vitro*.

Key words: advanced glycation end products (AGEs), pomegranate, ellagic acid, punicalagin, diabetes

INTRODUCTION

Glycation is a non-enzymatic multistage reaction starting with the binding of a protein and a reducing sugar such as glucose and fructose.[1–3] In the first stage of the reaction, a Schiff base is generated between an amino group of the protein and the carbonyl group of the sugar, producing an Amadori compound such as glycoalbumin and hemoglobin A1c (HbA1c). In the latter stages, the Amadori compound is oxidized, dehydrated, and condensed to finally generate advanced glycation end products (AGEs).[1,2] Some AGEs formed from a carbonyl compound such as glyceraldehyde and methylglyoxal are not generated through Amadori compounds.[4] AGEs are thought to be induced by aging and hyperglycemia. The resulting AGEs accumulation itself accelerates aging and causes diabetic complications in which protein denaturation, inflammation, and oxidative stress occur.[5] There are three ways to suppress the adverse effect of AGEs: (1) inhibition of AGEs generation, (2) degradation of the generated AGEs, and (3) inhibition of binding of AGEs to their receptors.[1,6,7]

Several studies have reported that polyphenolic compounds inhibit the formation of

AGEs, by inhibiting glycation reactions.[8,9] Pomegranate (*Punica granatum* L.) is a deciduous shrub of the Lythraceae family that contains various polyphenols such as ellagic acid (EA), punicalin (PC), and punicalagin (PG) within its fruit (**Figure 1**).[10] Some *in vitro* studies showed that pomegranate extract and EA inhibited AGEs formation in a glycation assay using bovine serum albumin (BSA) and glucose.[11–13] However, there are no *in vivo* studies designed to clarify whether the intake of pomegranate inhibits AGEs formation. In this study, we examined the effect of pomegranate fruit extract (PFE) intake on AGEs formation in the blood of type 2 diabetic model mice. Moreover, we compared the inhibitory effects of pomegranate-derived polyphenols on AGEs formation *in vitro*.

MATERIALS AND METHODS

Chemicals

PFE was prepared by drying the polyphenol fraction of Indian pomegranate fruits. Briefly, the juice from the fruits was passed through a synthetic resin to adsorb polyphenols. Then,

the resin was extracted by hydrous ethanol and the extract was dried to afford the PFE powder. The polyphenol contents of the PFE were determined by an HPLC method according to Zhang, Y. et al.[14] The contents of gallic acid (GA), EA, PC and PG were 1.0, 0.63, 2.7 and 9.1% (w/w), respectively. PC and PG were isolated from the PFE. Glucose, fructose, EA, and aminoguanidine (AG) were purchased from Wako Pure Chemical Ind., Ltd. (Osaka, Japan). Glyceraldehyde and GA were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). BSA was purchased from Jackson ImmunoResearch Inc. (West Grove, PA, USA).

***In vivo* experiments**

Six-week-old male type 2 diabetic mice (KK-Ay/TAJc1) were purchased from CLEA Japan, Inc. (Tokyo, Japan). A Normal group (n = 12) was fed a normal diet (AIN-93G) throughout the experimental period. The other mice (n = 39) were fed a high-fat (28%) and high-sucrose (30%) diet modified from AIN-93G (HFS) for 2 weeks to induce hyperglycemia. The hyperglycemic mice were further bred with HFS (control, n = 13), HFS containing 1.5% PFE

(n = 13), and HFS containing 0.3% AG (positive control, n = 13). The PFE concentration was set to the maximum concentration that had not affected to the food intake in a preliminary experiment. In the experimental diet, PFE and AG were replaced with the cornstarch of HFS. The mice were housed individually at 21 ± 1°C, 40–50% humidity, and a 12:12-h light-dark cycle (lights on at 07:00) with free access to water and limited access to the experiment feed only for 16 h (18:00–10:00). Mouse weight was measured once per week. The tail vain blood after 10 hours of starvation was collected to measure glucose levels by the mutarotase and glucose oxidase assay[15] (Glucose C II-Test Wako, Wako Pure Chemical Industries, Ltd.) and glycoalbumin concentration using the enzymatic assay[16] (Lucica GA-L, Asahi Kasei Pharma Co., Tokyo, Japan) once every two weeks. At the end of the feeding period, whole blood was collected from the postcaval vein to measure HbA1c using the enzymatic assay[17] (Norudia-HbA1c kit, Sekisui Medical Co., Ltd., Tokyo, Japan), serum AGEs using the ELISA assay (Rat/mouse Glyceraldehyde-derived AGEs ELISA Kit, Up Well Co. Ltd., Fukuoka, Japan) and lipid peroxide using the thiobarbituric acid reactive substances assay[18] (Oxiselect

TBARS Assay Kit, Cell Biolabs, Inc., San Diego, USA) according to the manufacturer's instructions. The present study was conducted in accordance with the Animal Experiment Guidelines of Josai University.

***In vitro* experiments**

BSA (50 mg/mL) and sugar (100 mM glucose, 100 mM fructose and 10 mM glyceraldehyde) were dissolved in 0.2 M phosphate buffer (pH 7.4). Each PFE-related sample was added to this solution to final concentrations of 10, 100, and 1000 μg/mL for PFE, and 10, 100, and 1000 μM for GA, EA, PC, and PG. The non-supplemented BSA + sugar solution was used as a control. The mixture was incubated at 37°C in total darkness: 15 days for the glucose-containing mixture, 5 days for the fructose-containing mixture, and 1 day for the glyceraldehyde-containing mixture. After incubation, the mixture was transferred to a 96-well black plate (Greiner Bio-One, Tokyo, Japan) to measure the fluorescence intensity (excitation = 370 nm, emission = 440 nm) using Spectra Max M2 (Molecular Devices, Sunnyvale, CA,

USA). The rate of AGES formation was calculated as follows:

$$AGE\ formation\ rate\ (\%) =$$

$$\frac{fluorescence\ intensity\ of\ sample\ (after\ incubation - before\ incubation)}{fluorescenec\ intensity\ of\ contorl\ (after\ incubation - before\ incubation)} * 100$$

Statistical analysis

Statistical analysis was performed with StatMate III software (ATMS Co., Ltd., Tokyo, Japan). The data were analyzed by Dunnett's test and Tukey's test. Differences were considered significant at $p < 0.05$. The data are expressed as means ± SE.

RESULTS

Food intake and body weight

To evaluate the anti-glycation effect of PFE *in vivo*, glycation markers such as glycoalbumin, HbA1c, and serum AGEs of KK-A^y mice fed the sample (PFE with HFS) diet for 8 weeks after induction of hyperglycemia were analyzed. **Table 1** shows total caloric intake and total food intake during the experimental diet, and body weight at the end of the experiment. Although the amount of food intake during the sample feeding was significantly lower in HFS-based groups (HFS, PFE, and AG) than the Normal group, the total calorie consumption of all groups was virtually identical. The net intake of PFE was calculated as 1.5 mg/ g/ day. Final body weight was significantly higher in the HFS based-groups than in the Normal group. There were no differences in caloric intake and weight gain between the HFS groups.

Blood glucose, glycation products, and lipid peroxide levels

The fasting blood glucose concentration was consistently higher in the HFS-based groups

than in the Normal group (**Figure 2**). Although the glycoalbumin levels of the HFS group tended to be higher than those of Normal group, feeding of PFE and AG with HFS significantly decreased those levels after 4 and 6 weeks, respectively (**Figure 3A**). PFE also significantly reduced HbA1c levels at 8 weeks as well as AG (**Figure 3B**). Serum AGEs concentration showed a trend similar to that of glycoalbumin and HbA1c (**Figure 3C**). Among significant increases in serum lipid peroxide in all HFS-based groups, PFE slightly suppressed the level as compared to HFS ($p = 0.059$) (**Figure 4**).

***In vitro* experiments**

The AGEs formation derived from BSA with glucose, fructose, and glyceraldehyde *in vitro* was concentration-dependently suppressed by addition of PFE and its components (**Figure 5A–C**). An even lower concentration of PFE (10 μg/mL) significantly suppressed the AGEs formation from fructose and glyceraldehyde (**Figure 5A**). The anti-AGEs-formation potency of the PFE components was similar in the assays using different sugars. The potency of EA

and GA was comparable to that of AG. PC and PG showed higher potency than EA and GA. The lowest concentration of PC and PG (10 μM) remarkably inhibited the AGEs formation, especially with fructose (**Figure 5B**).

DISCUSSION

Most reports concerning the anti-glycation effect of PFE have been performed *in vitro*, in which the suppression of AGEs formation was via inhibition of the binding of proteins with sugars.[11–13] However, it has been unclear precisely what components of pomegranate contribute to this suppression. Only one *in vivo* study reported that PFE inhibited collagen-derived AGEs in humans.[19] However, there has been no investigation about the anti-glycation effects of pomegranate *in vivo*. Therefore, we have evaluated the anti-glycation effects of pomegranate in type 2 diabetes model mice, in which it is relatively easy to induce conditions that promote glycation. Additionally, the effect of the individual components of PFE was investigated by an anti-glycation *in vitro* assay. The results indicate that

pomegranate contains several components that have the ability to suppress AGEs formation in type 2 diabetic mice. Oral administration of PFE might not affect glucose absorption from meals in the intestine because there were no significant differences in caloric intake and body weight gain between HFS and HFS plus PFE. Higher levels of the fasting blood glucose concentration in the HFS plus PFE group indicate that PFE could not completely reverse the hyperglycemia. Therefore, the PFE-mediated reduction in HbA1c and glycoalbumin might be caused by inhibition of the reaction between the protein and glucose, that is, glycation. The accumulation of glycated albumin already started to decrease at 4 weeks after oral administration of PFE, also suggesting that PFE inhibited the early stages of glycation. The suppression of HbA1c by PFE also supports our hypothesis. The decreased accumulation of these glycation products probably resulted in the lower concentration of AGEs in PFE-fed mice. The additional benefit of PFE could be antioxidant effects that were observed as a slight decline in blood lipid peroxide levels. PFE and its constituent polyphenols have shown *in vitro* anti-glycation effects as evaluated by the formation of glucose- and fructose-derived

AGEs.[20,21] In this study, we revealed that PFE and its component polyphenols suppressed the generation of glyceraldehyde-derived AGEs *in vitro*, especially the ellagitannins PC and PG comparable to the anti-glycation therapeutic aminoguanidine. Glyceraldehyde more easily reacts with protein than fructose and glucose,[2,22] and the glyceraldehyde-derived AGEs are thought to be highly toxic.[4] The anti-AGEs-formation potency seemed to depend on the number of phenolic groups, suggesting that these polyphenols acted as an antioxidant in the latter stages of glycation *in vitro*. However, orally administered ellagitannins hardly transfer to the blood because of their relatively high molecular weight.[23–25] Ellagitannins like PC and PG are digested by gastric acid to EA some of which will appear in the blood. The rest of EA is metabolized by intestinal bacteria into urolithin which also will appear in the blood.[23,24,26,27]

Liu, W. et al. Reported that urolithin possessed anti-glycation effect similar to aminoguanidhine.[13] Therefore, the anti-glycation effect of PFE shown in this *in vivo* study might be induced by these metabolites that had been degraded from ellagitannins in the

digestive tract. Additionally, ellagitannins might contribute to the inhibition of exogenous AGEs formation in the gastrointestinal tract. Further study is needed to clarify the mechanism of how the metabolites incorporated into the blood inhibit early stages of glycation.

Acknowledgements

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Figure Captions

Figure 1. Structures of pomegranate polyphenols.

Figure 2. Fasting serum glucose levels in diabetic model mice fed with Normal (n=11-12), HFS (n=12-13), HFS+PFE (n=12-13), and HFS+AG (n=11-13) diets. Data are shown as the means ± SE. (n = 10–13), Tukey's test $p < 0.05$.

Figure 3. Serum glycation markers of (A) glycoalbumin levels (n=11-13) at 4, 6 and 8 weeks, (B) hbA1c levels (n=11-13) at 8 weeks, and (C) serum AGEs (n=4) at 8 weeks in diabetic model mice fed normal, HFS, HFS+PFE, and HFS+AG diets. Data are shown as the means ± S.E., Tukey's test $p < 0.05$.

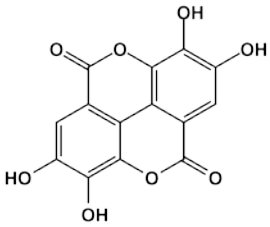
Figure 4. Serum lipid peroxide in diabetic model mice fed normal, HFS, HFS+PFE, and HFS+AG diets. Data are shown as the means ± SE. (n = 10), Tukey's test $p < 0.05$.

Figure 5. Anti-glycation effects of PFE and its polyphenols on (A) glucose-derived, (B) fructose-derived, and (C) glyceraldehyde-derived AGEs formation. Data are shown as the means ± SE. (n = 3), Dunnett's test, *$p < 0.05$, **$p < 0.01$.

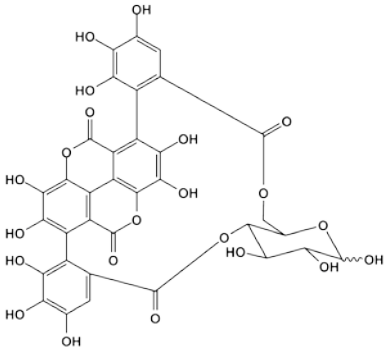
Table 1. Total food intake, total caloric intake and final body weight of diabetic model mice fed with normal (n=13), HFS (n=13), HFS+PFE (n=13), and HFS+AG (n=13) diets. Data are shown as the means ± SE. Tukey's test $p < 0.05$.

Parameter	Normal	HFS	HFS+PFE	HFS+AG
Total calorie intakes (kcal)	1262 ± 18 [a]	1240 ± 25 [a]	1220 ± 27 [a]	1266 ± 40 [a]
Total food intakes (g)	318 ± 4.5 [a]	241 ± 4.9 [b]	240 ± 5.2 [b]	247 ± 7.7 [b]
Body weight (g)	44.9 ± 0.76 [a]	48.6 ± 0.92 [b]	49.6 ± 1.1 [b]	49.3 ± 1.4 [b]

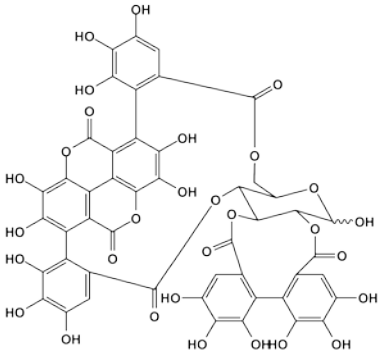
Figure 1.



ellagic acid



punicalin



punicalagin

Figure 2.

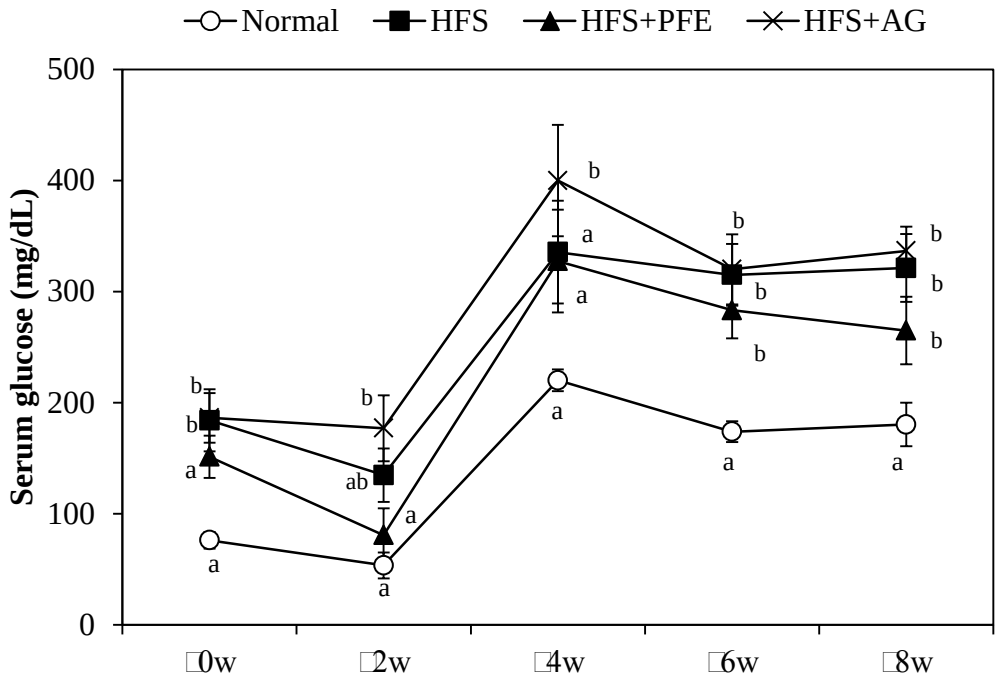

Normal
HFS
HFS+PFE
HFS+AG
Serum glucose (mg/dL)
500
400
300
200
100
0
0w
2w
4w
6w
8w

Figure 3.

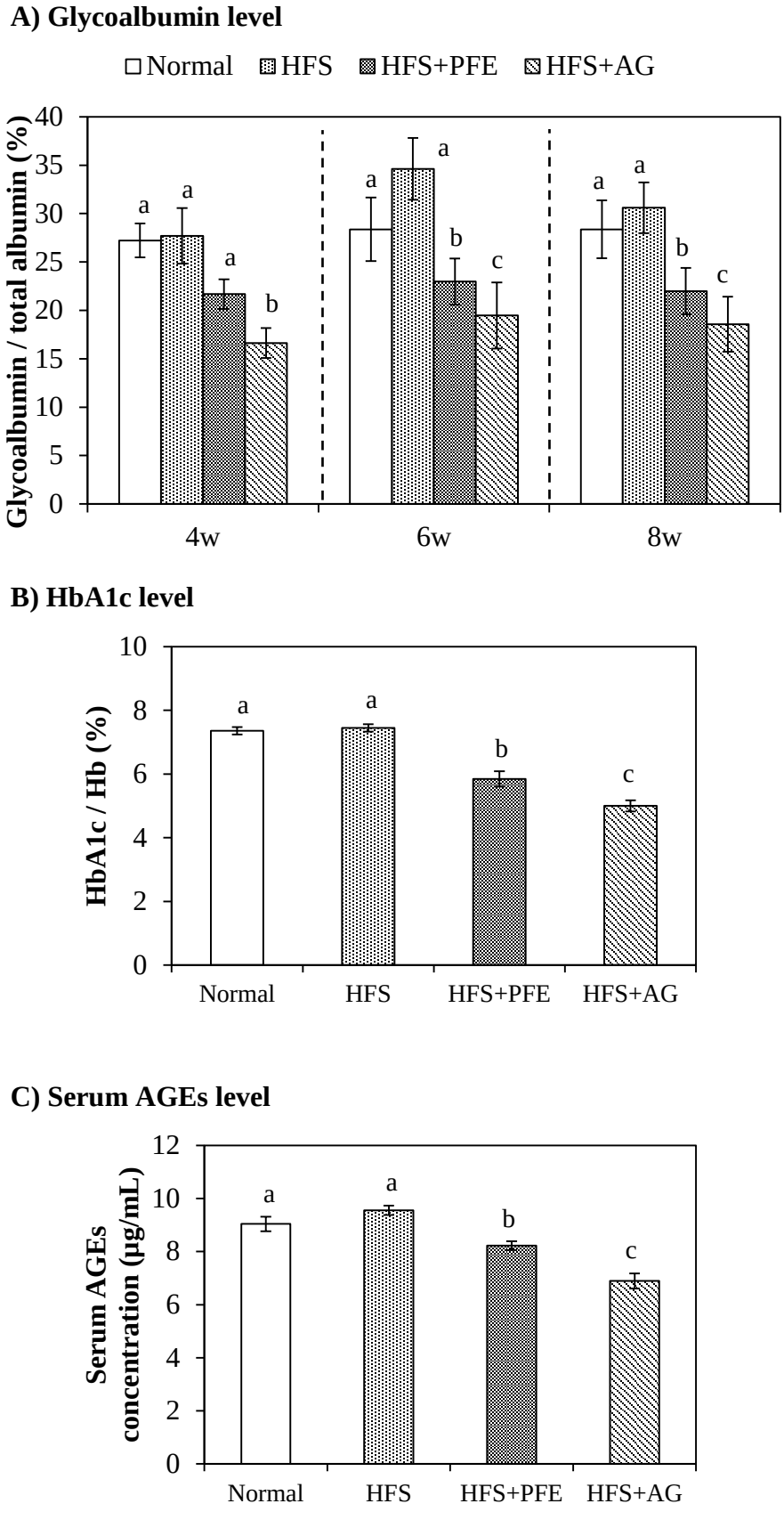

A) Glycoalbumin level
Normal
HFS
HFS+PFE
HFS+AG
Glycoalbumin / total albumin (%)
0
5
10
15
20
25
30
35
40
a
a
a
b
a
a
b
c
a
a
b
c
4w
6w
8w
B) HbA1c level
HbA1c / Hb (%)
0
2
4
6
8
10
a
a
b
c
Normal
HFS
HFS+PFE
HFS+AG
C) Serum AGEs level
Serum AGEs concentration (μg/mL)
0
2
4
6
8
10
12
a
a
b
c
Normal
HFS
HFS+PFE
HFS+AG

Figure 4.

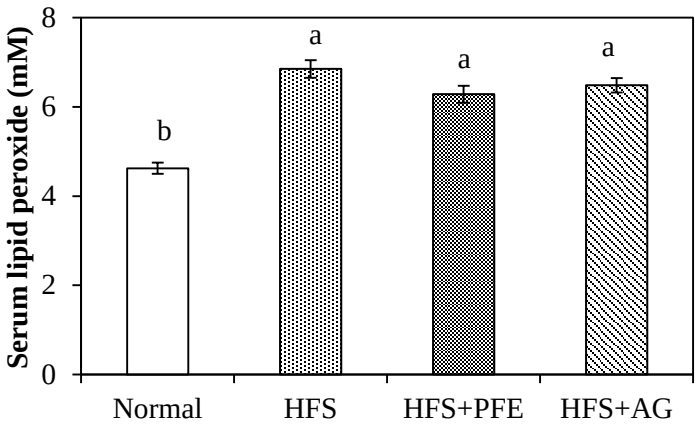
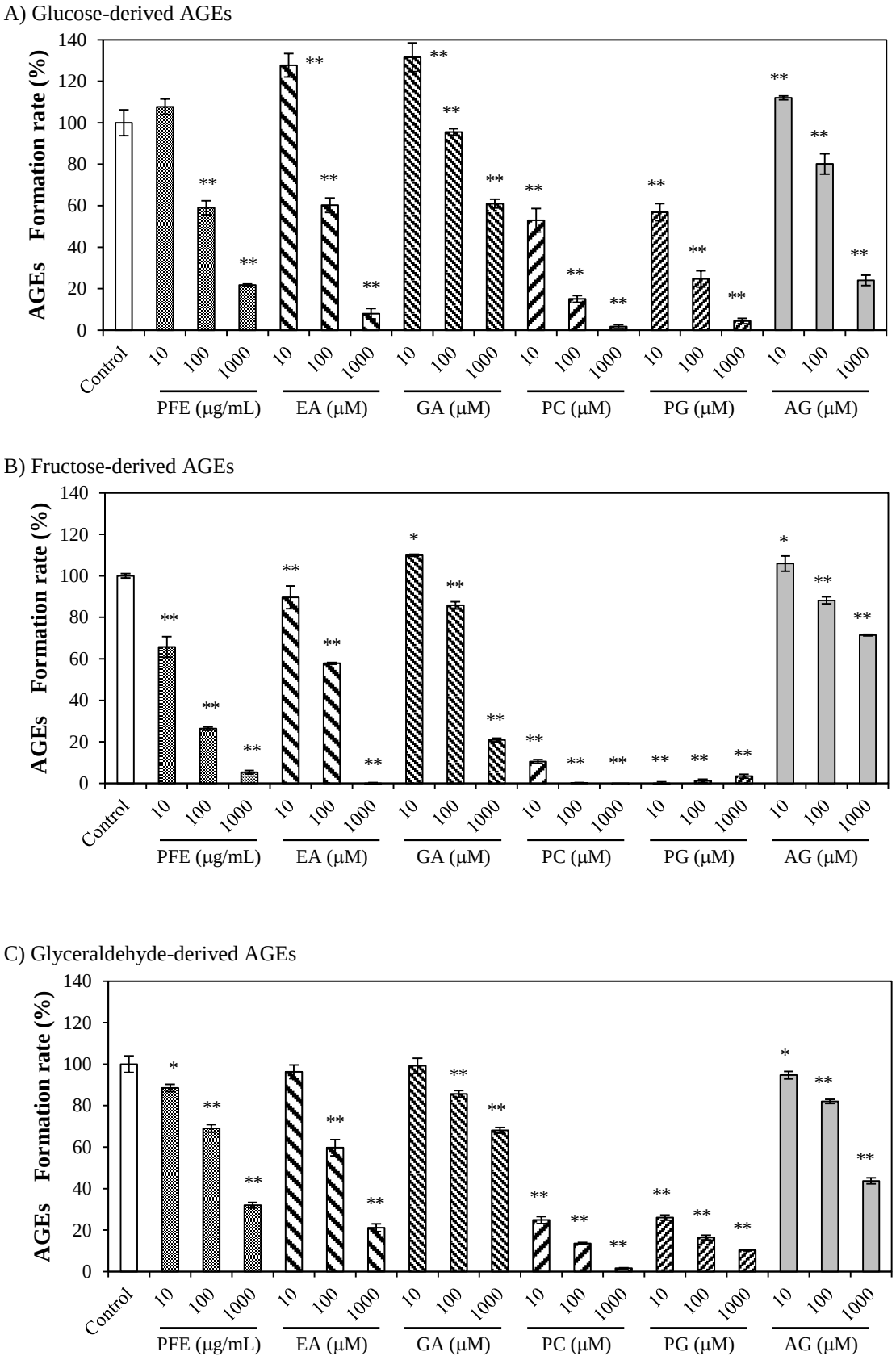


Figure 5.


A) Glucose-derived AGEs
B) Fructose-derived AGEs
C) Glyceraldehyde-derived AGEs
AGEs Formation rate (%)
0
20
40
60
80
100
120
140
Control
10
100
1000
PFE (μg/mL)
EA (μM)
GA (μM)
PC (μM)
PG (μM)
AG (μM)

Graphic for table of contents