Inhibitory Effect of 6-shogaol on Fructose-Induced Protein Glycation and Oxidation *in Vitro*

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Abstract

Advanced glycation end product (AGEs), the final products derived from the non enzymatic modification of proteins by reducing sugars, plays an important role in the development of diabetic complications. Therefore, the inhibition of AGE formation may be an important therapeutic strategy to prevent the development of AGEs related diseases. The aim of this study was to investigate the *in vitro* effect of 6-shogaol on fructose-induced the formation of AGEs and protein oxidation. Antioxidant activity of 6-shogaol was determined by 2, 2-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay. The protein glycation inhibitory potential was evaluated using *in vitro* BSA/ fructose model. 6-shogaol (0.1-10 mM) was incubated with BSA and fructose (0.1 M) at 37° C for 21 days. Antiglycation activity of 6-shogaol was investigated by measuring the formation of AGE-specific fluorescence and N^{ε}-(carboxymethyl) lysine (CML). In addition glycation induced protein oxidation was examined using the protein carbonyl assays. 6-shogaol exhibited an effective antioxidant activity, Where the results that 6-shogaol inhibited the formation of both fluorescent AGEs and non-fluorescent AGE (CML) in BSA/fructose solution. In addition an increase in protein carbonyl content of BSA incubated with fructose was attenuated by 6-shogaol (10 mM). In conclusion, These results suggest that 6-shogaol has a inhibitory effect on the formation of AGEs and protein oxidation *in vitro*, which may be mediated through its antioxidant activity.

Keywords: 6-shogaol, Advanced glycation end products, Protein glycation, Protein oxidation

Introduction

Protein glycation is the non-enzymatic reaction between sugars and protein, resulting in formation of the unstable and reversible products called Schiff bases. These products undergo rearrangements to form more stable structures called Amadori products. Oxidative degradation of Amadori products subsequently generates dicarbonyl compounds, which further react with amino groups of proteins to form stable and irreversible advanced glycation end products (AGEs) compounds. AGEs, a complex and heterogeneous molecules , can be divided into fluorescent crosslinking (such as pentosidine) and non-fluorescent, non-crosslinking compounds (such as N^{ε} -carboxymethyllysine (CML)) (Singh, Barden, Mori, & Beilin, 2001). There is also evidence that oxidative stress is involved in AGEs formation. Free radicals promote the oxidation of glucose and Amadori products, resulting in the accelerated formation of AGEs (Ott et al., 2014).

AGEs are well known as an important contributing factor in the pathogenesis of various diseases including diabetic complications, atherosclerosis, and aging (Ahmed, 2005; Goh & Cooper, 2008; Prasad, Bekker, & Tsimikas, 2012; Singh et al., 2001). Therefore, the inhibition of AGE formation may be an important therapeutic strategy to prevent tissue damage againt AGEs. Several pharmacological agents, such as aminoguanidine and metformin have been shown to inhibit the formation of AGEs and delay diabetic complications in animal models. However, these pharmaceutical agents have undesirable side effects (Singh et al., 2001; Tanaka et al., 1999). Therefore, there is interest in the search of

phytochemical compounds from natural products that may effectively and safely prevent protein glycation.

6-shogaol is one of the major biologically active compounds in the ginger rhizome (Moon et al., 2014). It has been reported that 6-shogaol is mainly present in the dried and thermally treated ginger but is found in fresh ginger at small quantities (about 2-5 mg/g in ethanolic extraction) (Jolad, Lantz, Chen, Bates, & Timmermann, 2005; Jolad et al., 2004; Ok & Jeong, 2012; Zick et al., 2008). 6-shogaol has been found to possess a wide variety of pharmacological activities including antioxidant, anti-inflammatory, and anticancer properties (Ghasemzadeh, Jaafar, & Rahmat, 2015; Li et al., 2012; Moon et al., 2014; Shim et al., 2011; Zick, et al., 2008). In addition, 6-shogaol exhibited scavenging activities including DPPH, superoxide and hydroxyl radicals *in vitro* studies (Dugasani et al., 2010). In another study, it provided cytoprotection against oxidative stress-induced cell damage in the neuron-like rat pheochromocytoma cell line (Peng et al., 2015). Interestingly, 6-shogaol has been reported to exhibited the most potent antioxidant activities in ginger (Dugasani et al., 2010; Mashhadi et al., 2013). Since reactive oxygen species (ROS) are involved in formation of AGEs, we hypothesized that antioxidant activity of 6-shogaol would inhibit glycation process. However, there were no reports in the scientific literature regarding the inhibitory effect of 6-shogaol on the formation of AGEs induced by high fructose. Therefore, the aim of the present study was to investigate the effects of 6-shogaol against fructose-induced protein glycation and oxidation *in vitro*.

Material and Methods

Chemicals

6-Shogaol, bovine serum albumin (BSA; fraction V, fatty acid free), 2, 2-diphenyl-1-picrylhydrazyl (DPPH), 2, 4-dinitrophenylhydrazzine (DNPH), fructose, and ascorbic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Anti carboxymethyllysine antibody was obtained from Abcam Co. (Cambridge, UK). 6-Shogaol was dissolved in methanol (10%) before use.

DPPH radical scavenging activity

The scavenging activity of the 6-shogaol on DPPH radicals was measured according to the previous method (Schlesier, Harwat, Bohm, & Bitsch, 2002). Briefly, various concentrations of 6-shogaol (0.01 - 10 mM) were mixed with 0.2 mM DPPH as the stable free radical source. The reaction mixture was shaken vigorously and incubated for 30 min in the dark at room temperature, and the absorbance was measured at 517 nm. Ascorbic acid was used as positive control. The inhibition percentage of the DPPH radical was calculated according to the formula: Scavenging activity (%) = {(absorbance of control - absorbance of samples)/ absorbance of control} x 100.

In vitro glycation of BSA

Glycated BSA was performed according to a previously published method (Ardestani & Yazdanparast, 2007). Briefly, BSA (10 mg/mL, fatty acid free) was incubated with 0.1 M fructose in 0.1 M phosphate buffered-saline (PBS), pH 7.4 containing 0.02% sodium azide. The reaction mixture was incubated at 37 °C for 21 days in the absence or the presence of 6-shogaol (0.1 -10 mM). Aminoguanidine (AG) was used as a positive control. The glycated BSA formation was determined spectrofluorometrically at excitation and emission wavelengths of 355 and 460 nm, respectively.

Determination of the formation of N^{ε}-CML

After 21 days of incubation, the formation of non-fluorescent AGEs (CML) was measured by enzyme linked immunosorbent assay (ELISA) according to a previous study with slight modification (Ahmad, Pischetsrieder, & Ahmed, 2007). Briefly,

the samples were diluted in coating buffer (50 mM sodium carbonate buffer, pH 9.6) to final concentration 1 ug/mL. Aliquots of 0.1 mL of diluted samples were added to 96 well polystyrene plates and incubated overnight at 4°C. After three washing steps, wells were blocked for 1 h with phosphate buffered saline (PBS) containing 1% BSA and then 0.1 mL of anti-CML antibody (diluted 1:5000) was added. After 2 h wells were washed three times and 0.1 mL of HRP conjugatedanti rabbit IgG antibody (diluted 1:5000) was added to each well. After incubation for 1 h, wells were washed and 0.1 mL of tetramethylbenzidinewas added. The absorbance of samples was measured at 460 nm. The percentage of inhibition of CML formation was calculated using the following equation: Inhibition of CML (%) = {(absorbance of BSA/Fructose$_{460nm}$ – absorbance of samples$_{460nm}$)/absorbance of BSA/Fructose $_{460nm}$} x 100.

Determination of protein carbonyl content

After 21 days of incubation, the carbonyl group in glycated BSA, a marker for protein oxidative damage, was assayed according to a previous method(Ardestani & Yazdanparast, 2007). Briefly, 0.2 mL of glycated samples was incubated with 0.8 mL of 10 mM DNPH in 2.5 M HCl for 60 min. Subsequently, After 1 ml of 20% (w/v) trichloroacetic acid was added to reaction mixture for protein precipitation and then centrifuged at 10,000 g for 10 min at 4 °C. The protein pellet was washed with 500 µl of ethanol/ethyl acetate (1:1) mixture and resuspended in 0.5 mL of 6 M guanidine hydrochloride. The absorbance was measured at 370 nm. The level of protein carbonyl contents was calculated based on the extinction coefficient for DNPH (ε = 22,000 M^{-1} cm^{-1}). The results were expressed as nmol carbonyl/mg protein.

Statistical analysis

The results were expressed as the mean ± standard error of the mean (S.E.M). Data were analyzed using one-way analysis of variance (ANOVA) and Dunnett's test (GraphPad Software Inc., San Diego, CA). $P < 0.05$ was considered to be statistically significant.

Results

Antioxidant activity

Antioxidant capacity was determined using DPPH assay. As shown in Figure 1, 6-shogaol had a scavenging activity on DPPH radicals in a concentration-dependent manner, similar to the positive control ascorbic acid.

Effect on the formation of fluorescent AGEs

In order to evaluate the formation of glycated BSA, the fluorescence intensity of the BSA-fructose solution was monitored at day 7, 14, and 21 of incubation. Compared with BSA alone, the fluorescence intensity of AGEs was gradually increased in the BSA-fructose system with increasing incubation time. However, treatment with 6-shogaol (10 mM) significantly decreased the formation of AGEs throughout the study period. At day 21 of incubation, the percentage inhibition of AGEs formation by 6-shogaol (10 mM) was 24 %, whereas that by the positive control AG (10 mM) was 68 % (Fig.2). The results indicated that 6-shogaol is less effective in the inhibition of AGEs formation than AG.

Effect on the formation of N^{ε}-CML

The formation of N^{ε}-CML, a major non-fluorescent AGE structure, was determined in BSA/fructose solution after 21 day of incubation. As shown in figure 3, the addition of 6-shogaol (10mM) into the solution significantly inhibited the formation of N^{ε}-CML by 67%, whereas AG (10mM) had the ability to prevent N^{ε}-CML formation approximately 50%.

Effect on protein oxidation

The protein carbonyl content in the BSA-fructose system was examined for determination of protein oxidation mediated by glycation process. 6-shogaol (10 mM) significantly reduced the carbonyl content in glycated BSA by 40%, as compared to BSA incubated with fructose (Figure4).

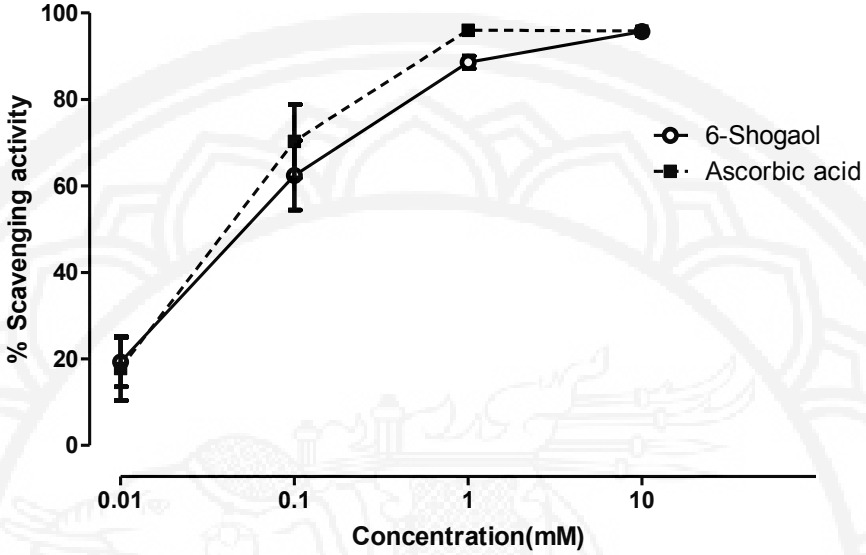

Figure 1 Scavenging activity of 6-shogaol (0.01 – 10 mM) and ascorbic acid (0.01-10 mM). Each value represents the mean ± SEM.

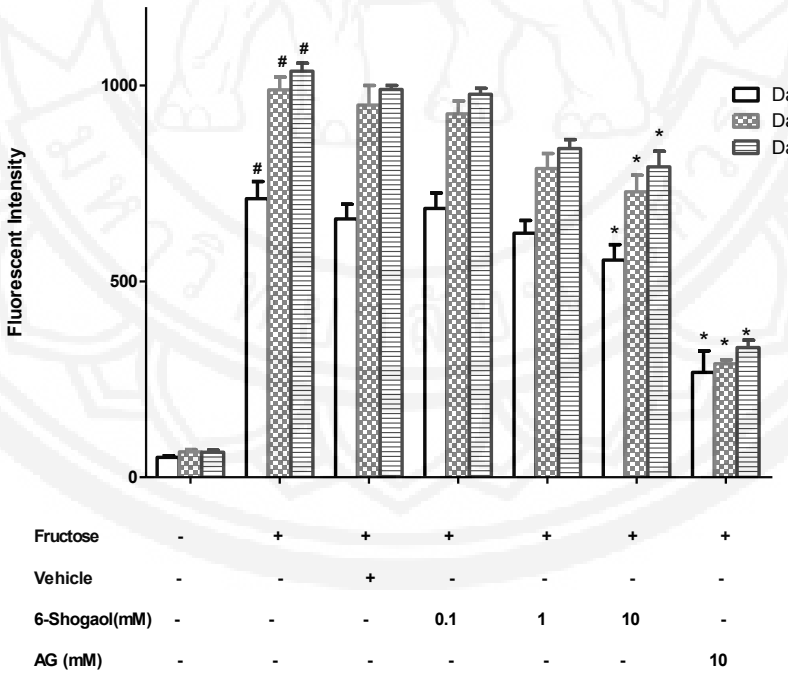

Figure 2 Effect of 6-shogaol (0.1-10 mM) and aminoguanidine (AG, 10mM) on the formation of fluorescent AGEs in BSA incubated with fructose for 21 days. Each value represents the mean ± SEM. #P<0.05 compared to BSA alone at the same time, *P<0.05 compared to BSA+Fructose at the same time.

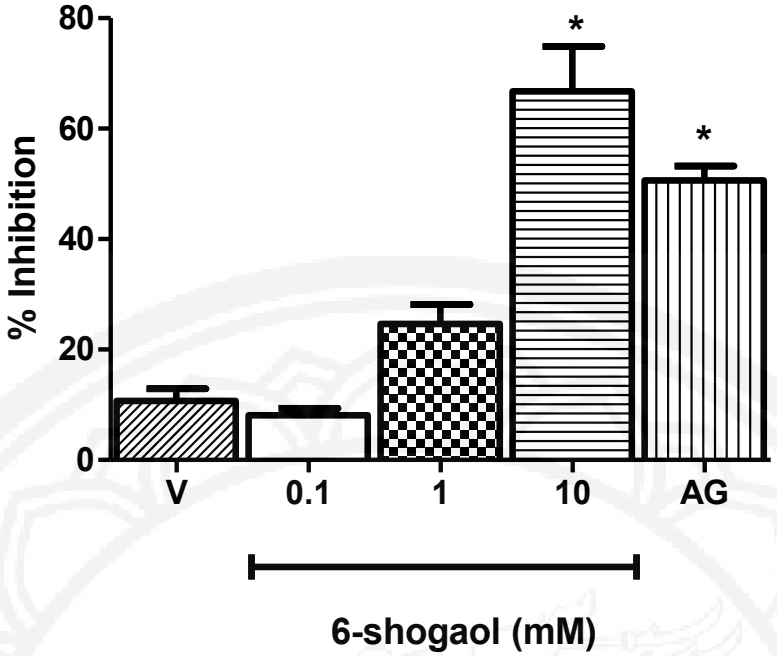

Figure 3 Effect of 6-shogaol (0.1-10 mM) and aminoguanidine (AG, 10mM) on the formation of CML in BSA incubated with fructose after 21 days of incubation. Each value represents the mean ± SEM. *P<0.05 when compared to vehicle.

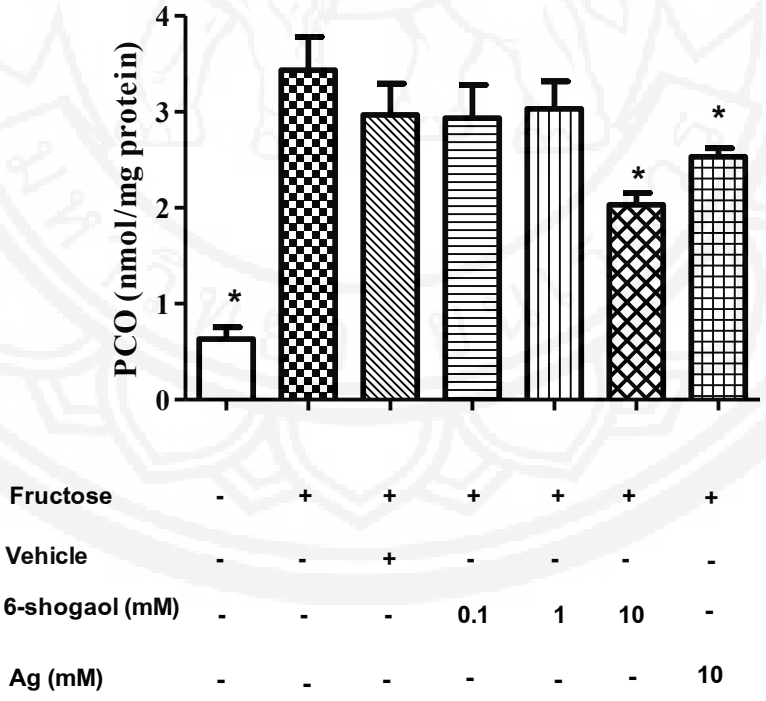

Figure 4 Effect of 6-shogaol (0.1-10 mM) and aminoguanidine (AG, 10mM) on protein carbonyl content in BSA incubated with fructose after 21 days of incubation. Each value represents the mean ±SEM. *P<0.05 when compared to vehicle.

Discussion

This study demonstrated that 6-shogaol decreased the formation of fluorescent and non-fluorescent AGEs (N^{ε}-CML) in BSA/fructose solution. In addition 6-shogaol inhibited fructose mediated protein oxidation *in vitro*.

Fructose has been excessively consumed in human diets over the last decades and considered to play an important role in the development of metabolic disorders and diabetic complications (Schalkwijk, Stehouwer, & van Hinsbergh, 2004; Tappy, Le, Tran, & Paquot, 2010). In diabetic patients, intracellular fructose can be converted from glucose via the polyol pathway, resulting in the formation of AGEs. Fructose, like glucose, is known to react with the free amino group of proteins to form reversible Schiff bases and Amadori products. Over the course of days to weeks, these products subsequently undergo conversion to dicarbonyl intermediates (such as 3- deoxyglucosone, methylglyoxal, and glyoxal) which react with other protein molecules to form fluorescent and non-fluorescent AGEs (Ott et al., 2014; Singh et al., 2001). *In vitro* studies showed that fructose, compared with glucose, is a much more potent initiator of the AGE formation (Singh et al., 2001; Sompong, Meeprom, Cheng, & Adisakwattana, 2013).

In the present study, the fluorescence intensity of the BSA-fructose solution was measured weekly to determine the formation of glycated BSA. The fluorescent AGEs was increased in the BSA/fructose solution compared to BSA alone. This is consistent with other studies (Jariyapamornkoon, Yibchok-anun, & Adisakwattana, 2013; Wu & Yen, 2005). However, 6-shogaol effectively inhibited the formation of fluorescent AGEs throughout the study period. CML is well-known example of non-fluorescent AGE generated from sequential glycation and oxidation reaction (Singh et al., 2001). The results also showed that 6-shogaol exhibited stronger inhibition of CML formation in BSA incubated with fructose in comparison to aminoguanidine.

It has been reported that many phytochemical compounds from natural products can trap dicarbonyl compounds generated during the glycation process, leading to the inhibition of AGE formation (Li, Zheng, Sang, & Lv, 2014; Lv, Shao, Chen, Ho, & Sang, 2011). Dicarbonyl intermediates such as methylglyoxal and glyoxal have received considerable attention as mediators of AGEs formation and are known to react with amino groups of proteins to form stable AGE compounds (Singh et al., 2001). Previous studies demonstrated that 6-shogaol was able to directly scavenge methyglyoxal, an intermediate reactive carbonyl of AGE formation (Zhu, Zhao, Wang, Ahmedna, & Sang, 2015). Therefore, the inhibitory mechanism of 6-shogaol against fructose induced glycation may be at least partly due to its carbonyl-trapping activity. In addition there is evidence to show that reactive oxygen species are involved in the formation of AGEs (Ott et al., 2014). 6-shogaol has been reported to have antioxidant activities both *in vitro* and *in vivo* studies (Li et al., 2012; Moon et al., 2014). In our study, 6-shogaol also exhibited a scavenging activity on DPPH radicals. Therefore, antioxidant properties of 6-shogaol may prevent fructose and Amadori products from self oxidation, leading to the inhibition of AGEs production. However, further studies of 6-shogaol are required to investigate the mechanisms of inhibition of AGEs formation.

AGEs are not only a major cause of protein modification, but it also induces oxidative damage to cellular macromolecules, leading to development of diabetic complications. In glycation process, free radicals can be produced by either sugar autoxidation or oxidative fragmentation of Amadori products. Free radicals such as superoxides, hydrogen peroxide and hydroxyl radical formed during the process of glycation can oxidize side chains of amino acid

residues in protein to form carbonyl derivatives (Ahmed, 2005; Goh & Cooper, 2008; Singh et al., 2001). Therefore, we measured the levels of protein carbonyl as an indication of oxidative damage to protein. In the present study protein carbonyl content in BSA/Fructose solution was significantly increased in comparison to BSA alone. Our findings are consistent with previous studies indicating that fructose-induced glycation increased protein carbonyl content (Adisakwattana, Thilavech, & Chusak, 2014; Jariyapamornkoon, Yibchok-anun, & Adisakwattana, 2013). The addition of 6-shogaol reduced the formation of protein carbonyl in BSA. Having demonstrated that 6-shogaol effectively decreased oxidative stress and free radicals, together with the ability to inhibit AGE formation suggested that it may reduce BSA oxidation by high fructose.

Conclusion and Suggestion

In conclusion, 6-shogaol prevents fructose-induced protein glycation and oxidation *in vitro*. The protective actions of 6-shogaol may involve its antioxidant activity. Therefore, antiglycation activity of 6-shogaol may be the potential usefulness in the prevention or improvement of AGEs-related disorders including diabetic complications. Howerver, further studies are required to investigate the antiglycation effect of 6-shogaol in animal models.

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