Glycative Stress Research

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Review article

Glycative stress and anti-aging
4. The evaluation of glycative Stress: Evaluation for anti-glycative effect.

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Abstract

When measuring anti-glycative effects, test samples are added into the phosphate buffer solution including proteins and reducing sugars so that glycation occurs. Then, the amount of advanced glycation end products (AGEs), as well as the amount of intermediate glycative reaction products are measured. Regarding the amount of AGEs, it is easiest to measure the fluorescence (excitation wavelength: ex 370 nm/emission wavelength: em 440 nm), which is one of the chemical characteristics of AGEs. Other methods are to make measurements of pentosidine, and N^{ε}-carboxymethyl lysine (CML) as AGEs, along with 3-deoxyglucosone (3DG), glyoxal (GO) and methylglyoxal (MGO) as reaction intermediate products. Regarding anti-glycative effects, it is recommended to calculate 50% inhibitory concentration (IC_{50}), based on the inhibitory ratio of AGEs and reaction intermediate products in test samples. In order to grasp the positive control of AGEs inhibitory effects, aminoguanidine (an inhibiting agent of glycation) is utilized. The food materials proven to have an anti-glycative effect *in vitro* will be verified in the end by conducting a clinical trial for humans. It is the most important to keep trial food stuffs safe during the clinical trials. The glycative stress markers to be measured will be decided, based on the working point obtained from the *in vitro* measurement of anti-glycative effects for the intake materials. The results of the clinical trials are affected not only by the strength (weakness) of the anti-glycation of food stuffs, but also by the health status of subjects. Subjects with small amounts of AGEs accumulation or little glycative stresses are found to show a small change after the intake of materials with anti-glycative effects. In the case of healthy people, it was observed that those subjects who did not show a disorder of carbohydrate metabolism, who had high postprandial blood glucose levels and who showed a high accumulation of AGEs in the skin, were found to be affected by the anti-glycative effects.

In the case of anti-glycation clinical trials for cosmetic effects, not only inhibitory effects for production or accumulation of AGEs or the intermediate product of glycative reaction in blood and skin, but also the change of skin indexes including resilience, flexibility, brightness, and smoothness will be measured in order to assess the anti-aging effects.

***KEY WORDS*:** anti-glycative effect; evaluation method; clinical trial

1. Measurement of anti-glycative effects for natural products

To measure the anti-glycative effects for natural products, test samples as well as reducing sugars such as glucose and fructose will be added into the phosphate buffer (pH 7.4) including model proteins such as human serum albumin (HSA), collagen, and elastin, and will be kept at a temperature of 60 °C for 40 hours until a reaction occurs. Then, advanced glycation end products (AGEs) in the reaction liquid will be measured [1)]. The AGEs formation of the HSA-glucose reaction model (at a temperature of 60 °C, and for 40 hours) is equivalent to that which is obtained from the reaction time of about 60 days at a temperature of 37 °C (***Fig. 1***). To obtain the AGEs inhibitory rates (%) of test samples, AGEs and the intermediate products of the glycative reaction amount contained in the following four cases will be measured: (A) the HSA-glucose reaction liquid obtained when both test samples and the reducing sugar were added; (B) the amount obtained when only test samples were added; (C) the amount obtained when only the reducing sugar was added (D) the amount obtained when neither test samples nor the reducing sugar were added. Then, the measurement values will be put to the following equation.

$$\text{AGEs inhibitory rate (\%)} = \{1 - (A - B) / (C - D)\} \times 100$$

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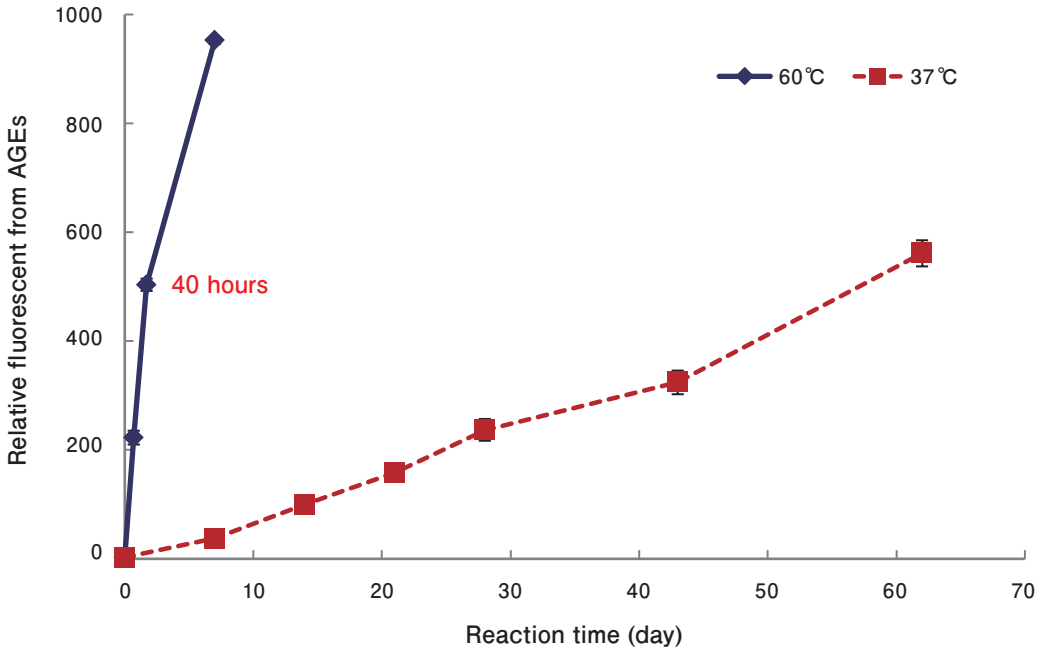

Fig. 1. **Amount of AGEs productions in HSA-glucose reaction model.**

Reaction was conducted between HSA (8 mg/mL) and glucose (0.2 mol/L) in 0.05 mol/L phosphate buffer (pH 7.4) at the temperature: 60°C (solid line) or 37°C (dotted line). AGEs measured the fluorescent (excitation wavelength 370 nm, detection wavelength 440 nm). Results are expressed as means ± SD (n = 3). AGEs, advanced glycation end products; HSA, human serum albumin; SD, standard deviation.

In order to measure anti-glycative effects, it is easiest to measure the fluorescence (excitation wavelength: ex 370 nm / emission wavelength: em 440 nm) radiated by AGEs. There are AGEs having a fluorescence such as pentosidine (ex 335 nm / em 385 nm) [2], crossline (ex 379 nm / em 463 nm) [3], and pyrropyridine (ex 370 nm / em 455 nm) [4]. There are AGEs that have a non fluorescence such as N^{ε}-carboxymethyl lysine (CML) and N^{ω}-carboxymethyl arginine (CMA). The intermediate product of glycative reactions includes 3-deoxyglucosone (3DG), glyoxal (GO), and methylglyoxal (MGO). In order to calculate the inhibitory ratio of test samples against AGEs as well as the intermediate product of glycative reactions, the formation of these materials in the reaction liquid will be measured by using high-performance liquid chromatography (HPLC) and enzyme-linked immunosorbent assay (ELISA) which are obtained from AGE antibodies. The anti-glycative effects will be calculated as follows: the concentration of test samples to measure will be set to more than 3 concentrations so that a reaction occurs. Then, 50% inhibitory concentration (IC_{50}) will be calculated, based on the inhibitory rate of AGEs or the intermediate product of glycative reaction in each reaction liquid.

Regarding the positive control of inhibitory effects of AGEs, aminoguanidine, which is an inhibitory agent for glycation reactions, will be used. Aminoguanidine is an AGE formation inhibitor developed with the aim of curing diabetic complications [5]. Aminoguanidine blocks carbonyl group in the molecule of the intermediate product of glycative reactions. Aminoguanidine is not approved as a medical product in the domestic market, because there was a side effect recognized in Japan. Regarding the *in vitro* evaluation system of anti-glycative effects, it is possible to presume a point of action of materials against a glycative reaction complicated with multiple routes, by measuring many kinds of AGEs [6]. Regarding this evaluation system, *Houttuynia cordata*, *Crataegus laevigata*, *Anthemis nobilis*, *Vitis vinifera*, mixed herb [7], purple flower of *chrysanthemum* [8], kuma bamboo grass (*Sasa veitchii*) [9], herb teas [10], and fruits [11] are reported. There are anti-glycative effects reported in a variety of plant-based organic materials such as tea leaves [12], herbs [13], spices [14], herbal medicines [15], though the evaluation system is a little different. It is presumed that the anti-glycative effects are mainly caused by polyphenols that are present in plants.

It is reported that the catechol-based compounds, such as flavonoids, including catechin and epicatechin contained in *Camellia sinensis*, and gallic acids contained a lot in galls of *Rhus javanica*, inhibit the forming of CML at concentrations below 10 μmol / L. On the other hand, the catechol compounds are reported to accelerate the formation of CML at concentrations of more than 1 mmol / L [16, 17]. It has not been clarified how the anti-glycative component contained in natural materials works. Therefore, it is necessary to be careful about the concentration when using natural materials.

2. Clinical evaluation of anti-glycative materials

The food materials recognized to have *in vitro* glycative effects will be verified by clinical trials. It is the most important to keep the test food samples safe during clinical trials. The materials for the test food samples must be examined regarding the borderline of pharmaceuticals to non-pharmaceuticals, the long history of safe use, single-dose toxicity, repeat-dose toxicity, Ames test, Rec-assay, and pesticide residue analysis, in order to confirm their safety. In addition, if possible, there should be investigations for relations between the ingredients and no observed adverse effect level (NOAEL), no observed effect level (NOEL), acceptable daily intake (ADI), as well as tolerable daily intake (TDI).

In the human clinical trial, the protocols will be created after the safety of food samples is confirmed. Moreover, practitioners of trials will recruit subjects based on the approval of the institutional review board (IRB), and conduct the trial after receiving the informed consent of the subjects. The result of the trial will be affected not only by levels of the anti-glycative stress of test food samples, but also by the health status of the subjects. Those subjects who showed a small accumulation of AGEs, and low level of glycative stress were found to show a small change after the intake of the anti-glycative materials. In the case of healthy subjects, those who did not have a carbohydrate metabolism disorder, who showed high levels of postprandial blood glucose level, and who showed much accumulation of AGEs in the skin, in other words, those who showed high levels of glycative stress, were found to be easily affected by the anti-glycative effects.

3. Measurement of food materials that contain anti-glycative effects

We detected the *in vitro* anti-glycative effects (inhibitory effects on fluorescent AGEs) against HSA in the hot water extract of *Houttuynia cordata*, fruits of *Crataegus laevigata,* flowers of *Anthemis nobilis*, and leaves of *Vitis vinifera*, which were herbs with a long history of safe use, and belonged to different groups in terms of the systemic botany. Additionally, it was proven that the extracts of each plant had different formation inhibitory effects such as those for 3DG, pentosidine, and CML. However, blending of these extracts made it possible to obtain inhibitory effects for various AGEs. The mixed herbal extracts (MHE) of these four herbs were commercialized as food materials "AG Herb MIX ™" by ARKRAY Inc. MHE was found to be significant, not only for anti-glycative effects to type I bovine hide collagen, but also for its inhibitory effects on CMA, which is a type of AGEs that form collagen specifically. Afterwards, we conducted a MHE trial using streptozotocin-diabetic rats and it was confirmed that MHE showed the same kind of anti-glycative effects as aminoguanidine [7].

Then, we conducted trials on the safety ofof MHE as food, regarding long history of safety use, single-dose toxicity, repeat-dose toxicity, Rec-assay, and pesticide residue analysis. In addition, we conducted a consecutive two week's trial of MHE at the rate of 3,000 mg/day (five times the adequate intake amounts) for ordinary adults, based on the approval of the human research ethics committee and the informed consent from subjects. As a result, it was clarified that there was little possibility that the intake of MHE has an effect on liver functions, renal functions, carbohydrate/lipid metabolism improving functions, as well as human drug metabolizing functions.

Moreover, using the same method, we conducted a double-blind parallel-group comparison test of MHE at the rate of 3,000 mg/day [19] against pre-diabetes (whose all times blood-sugar levels were 110～199 mg/dL, and HbA1c were 5.9～6.9 % based on the national glycohemoglobin standardization program: NGSP). As a result, it was confirmed there were two different tendencies which were as follows: subgroups with the blood-sugar levels of more than 110 mg/dL who took MHE showed a stronger suppressive tendency in blood 3DG ($p = 0.094$), compared to those who took placebo foods, while subgroups with the HbA1c of more than 6.3 % (NGSP) who took MHE showed a significant decrease in CML ($p = 0.048$, ***Fig. 2***).

Additionally, in the trials conducted in the same way as the type II diabetes trials (HbA1c: 6.1～9.6 % [NGSP]), subjects took MHE for 12 weeks consecutively at a rate of 600 mg/day (the adequate intake amounts). As a result, their blood 3DGs showed a significant decrease after 8 ～ 12 weeks ($p < 0.001$) [20]. During this trial, blood CML decreased significantly after twelve weeks ($p < 0.001$). Moreover, the resilience of the subjects skin significantly improved, as is shown in biological elasticity index R2 (Ua/Uf) which was measured by the Cutometer™ eight weeks ($p = 0.003$), and twelve weeks ($p < 0.001$) after the commencement of intake (***Fig. 3***).

We conducted a double-blind parallel-group study trial [21] of post-menopausal women with high glycative stress levels (showing high blood-sugar levels 60 minutes after intake of 200 g of rice, and skin AGE accumulation) in the same procedure. The subjects drank vinegar drinks containing indigestible dextrin (5 g/day) and MHE (100 mg/day) for eight weeks consecutively. As a result, regarding the group of subjects whose blood-sugar levels 60 minutes after the intake of rice were more than 150 mg/dL, the CML in stratum corneum (rate of change) collected by tape-stripping methods, significantly decreased, eight weeks after the commencement of intake of food samples ($p < 0.05$, ***Fig. 4***).

In the clinical trials, it is necessary that we select the glycative stress markers to be measured with reference to in vitro trial results or effects expected, after confirming the food samples with anti-glycative effects sufficiently safe. After all of these are completed, trial plans will be made. The plan will be executed upon the approval of the institutional review board (IRB).

4. How to show data of anti-glycative effects?

It is often the case that the *in vitro* anti-glycative effects are shown as IC_{50} of samples and are evaluated in comparison with aminoguanidine, which is the positive control. IC_{50} changes depending on the kinds of proteins or carbohydrates used in evaluation system of the in vitro anti-glycative effects, concentration, reaction temperature, time length, as well as the kinds of AGEs measured [22]. Therefore, it is necessary to integrate the measurement conditions when making a comparison with other data documents. In addition, as it often happens that the effective ingredient contained in natural

materials is not identified as a substance, IC_{50} is to be calculated based on the solid contents (residue on evaporation) or the total amount of polyphenol in the extract liquid. Moreover, the route of glycative reaction is diversified with a variety of kinds of AGEs formed [6]. Therefore, measurement data of each anti-glycative effect must be verified, checking which AGEs formation route there is to affect each material or substance.

The glycative stress markers used in the human clinical trials are selected based on the action point acquired by the in vitro anti-glycative effects measurement of the intake materials. The intake amount of measurement materials is set, based on the result of a large clinical trial of aminoguanidine conducted in the U.S. or the clinical evaluation data of the anti-glycative materials already commercialized. It is reported that a consecutive intake of aminoguanidine (150 mg/day, or 300 mg/day: for 6 to 36 months) improved diabetic nephropathy [5]. In the clinical trials focusing on the cosmetic effect of anti-glycative treatment, not only formation / accumulation inhibiting action of AGEs in blood or skin, but also changes of skin texture indexes including resilience, flexibility, brightness, as well as smoothness will be measured in order to evaluate their effects for anti-aging.

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Conflict of interest statement

There are no items deemed to be conflicts of interest in this research.

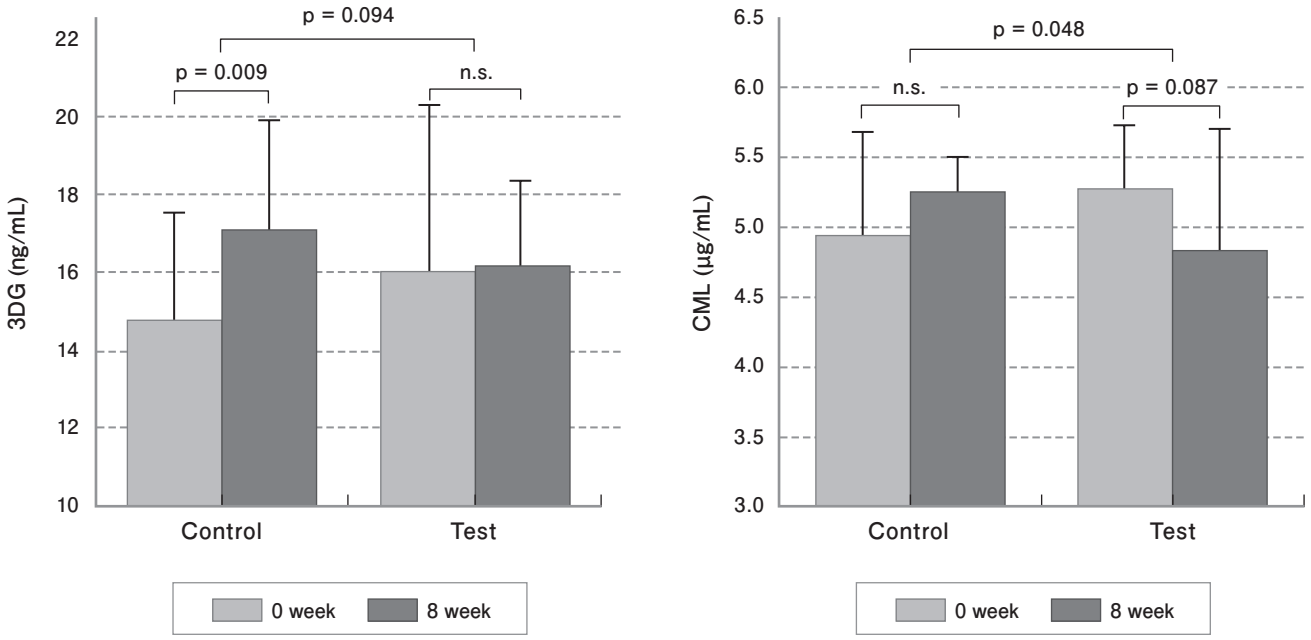

Fig. 2. **Subclass analysis of 3DG and CML in blood by mixed herbal extract intake.**

3DG: blood glucose reference values 110 mg/dL or higher. Subjects: n = 11 (Test Group), n = 8 (Control Group). CML: HbA1c values (NGSP) are 6.3% or higher. Subjects: n = 11 (Test Group), n = 7 (Control Group). Results are expressed as mean ± SD, the inter-group analysis by t-test, and the intra-group analysis by paired t-test. 3DG, 3-deoxyglucosone; CML, N^{ε}-(carboxymethyl) lysine; NGSP, National Glycohemoglobin Standardization Program; SD, standard deviation. Data are quoted from Reference 19.

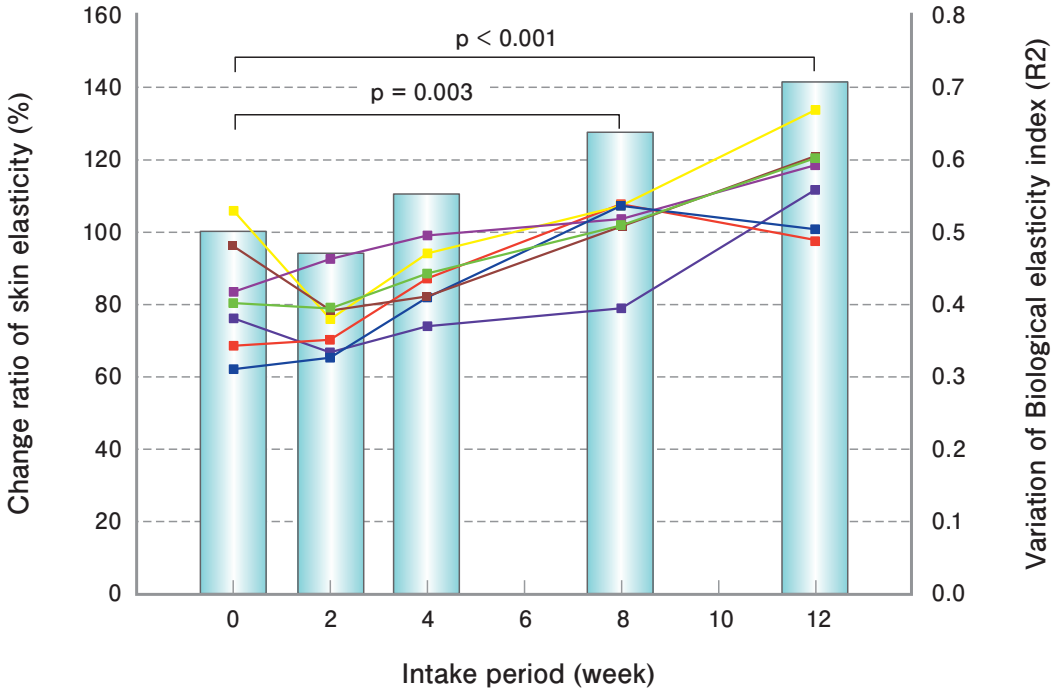

Fig. 3. **Variations of skin elasticity in type 2 diabetes.**

Subjects: n = 7 (Type 2 diabetes, HbA1c values [NGSP] : 6.1~9.5%)

Biological elasticity index (R2: Ua/Uf) was measured by Cutometer. Results are expressed as mean ± SE, statistical analysis by Dunnett's test. NGSP, National Glycohemoglobin Standardization Program; SE, standard error mean. Data are quoted from Reference 20.

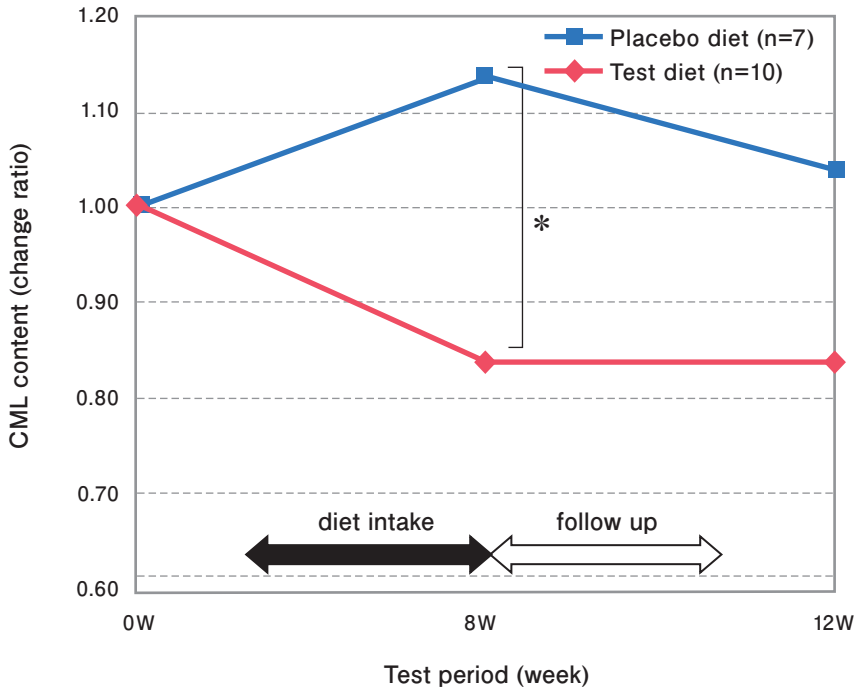

Fig. 4. **Subclass analysis of CML in skin corneum by intake of vinegar beverage containing mixed herbal extract.**

Subjects with high postprandial blood glucose (>150 mg/dL at 60 minute) are analysed. Subjects: n = 10 (Test diet), n = 7 (Placebo diet). CML content in the stratum corneum was measured by the tape stripping method. * $p < 0.05$, Mann-Whitney U test. CML, N^{ε}-(carboxymethyl)lysine. Data are quoted from Reference 21.

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