【Invited Lectures】

1. Dicarbonyl stress: Metabolic drivers, pathophysiological consequences and opportunities for therapeutics

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Objectives: Dicarbonyl stress is the abnormal accumulation of dicarbonyl metabolites leading to increased protein and DNA modification contributing to cell and tissue dysfunction in ageing and disease. It is a specific and particular type of carbonyl stress involving accumulation of α-oxoaldehydes such as methylglyoxal, glyoxal and 3-deoxyglucosone. Major adducts formed with proteins and DNA are also specific for this type of carbonyl stress – hydroimidazolone residues of protein and imidazopurinone residues of DNA. Enzymes metabolising dicarbonyls, glyoxalase 1 (Glo1) and aldoketo reductases, provide an efficient and stress-responsive enzyme defence against dicarbonyl stress. It contributes to ageing, disease and activity of cytotoxic chemotherapeutic agents.

Results: The metabolic drivers for dicarbonyl stress are increased formation and/or decreased metabolism of dicarbonyl metabolites, and by exposure to exogenous dicarbonyls. Examples of increased endogenous formation of dicarbonyls are increased formation of methylglyoxal in obesity and diabetes associated with glyceroneogenesis in triglyceride/fatty acid cycling and hyperglycaemia, respectively. Examples of decreased metabolism are: the kidney, retina and nerve in diabetes linked to the development of microvascular complications, and likely also tissues in renal failure. Examples of exposure to high levels of exogenous dicarbonyls are treatment with first generation glucose-based peritoneal dialysis fluids and some thermally processed foods and beverages.

Dicarbonyl stress contributes to pathology through inactivation, dysfunction and increased degradation of proteins. Key processes driven by dicarbonyl stress are: metabolic dysfunction, vascular inflammation and apoptosis – particularly anoikis. Glo1 transgenic models and clinical studies suggest this contributes to ageing, insulin resistance in obesity, cardiovascular disease, vascular complications of diabetes and renal failure, and neurological disorders.

Enhanced protection against dicarbonyl stress is usually considered to be beneficial. An exception is in cancer. Overexpression of Glo1 in tumours – in part, mediated clinically by GLO1 amplification – protects tumours from chemotherapy and thereby likely contributes to poor treatments outcomes, particularly in breast cancer and lung cancer. Development of Glo1 inhibitors may improve treatment outcomes. GLO1 duplication may occur via adaptive genomics to dicarbonyl stress.

Conclusion: Experimental therapeutic agents such as aminoguanidine were inadvertently developed as dicarbonyl scavengers but suffer from toxicity, instability and a poor pharmacokinetic profile. A better approach is to increase enzymatic metabolism of dicarbonyls through small molecule inducers of expression of Glo1 and aldoketo reductases through activation of transcription factor Nrf2. Anti-dicarbonyl stress functional foods and therapeutics are in development and clinical evaluation.

2. Application of glycation markers for clinical diagnostics

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Objectives: Glycation adducts of proteins have found diagnostic application in monitoring of blood glucose control in diabetes – particularly use of glycated haemoglobin A1C (measurement of early stage glycation adducts, N^{ε}-fructosyl-lysine and N^{α}-fructosyl-valine, of haemoglobin). Recently A1C has also been recommended for screening of people at increased risk of diabetes or pre-diabetes, impaired glucose tolerance and impaired fasting glucose, through a recommended range of A1C (5.7 – 6.4%). Measurement of advanced glycation endproducts (AGEs) has not yet found clinical translation. Other techniques that, in part, measure some AGEs – skin autofluorescence readers – have also been proposed.

Results: Since glycation is implicated as a causative mechanism in ageing and disease, it is expected that measurements of glycation adducts may contribute mechanism-based biomarkers for improved health screening and clinical diagnosis. Minimal and non-invasive sampling for direct biochemical measurement has been considered: blood, urine and saliva. Glycation adducts, free or residues of proteins, may be quantified. Diagnostic information thereby obtained depends on the analyte measured, its half-life, quantitative change and factors producing it. Particular glycated proteins may be detected and identified by high resolution Orbitrap mass spectrometry proteomics. For clinical translation techniques with moderate/high throughput and available to the non-specialist are likely to be most valuable.

Immunoassays have been in development for AGEs. They require careful validation and corroboration with stable isotopic dilution analysis liquid chromatography-tandem mass spectrometry (LC-MS/MS). Immunoassays find favour generally in clinical diagnostic measurements for their high throughput and established high level automation in the clinical setting. Where multiple glycation adduct measurements are required, immunoassays may be multiplexed. Concerns remain, however, on cross-reactivity to antigens and markedly increased cost of immunoassay multiplexing. To date, few multiplex immunoassays for any analyte have been validated for clinical application.

Stable isotopic dilution analysis LC-MS/MS is the analytical method of choice for detection and quantitation of glycation adducts. It has robust, cross-reaction-free multiplexing for combinations of analytes which can be done at minimal additional cost. It operates at moderate sample throughputs – similar to methods for A1C. It is also being developed for multiplexed protein analysis by quantitation of tryptic peptides. There are few LC-MS/MS-based clinically-regulated and approved diagnostic procedures. A major barrier to clinical use is lack of high level automation for use by the non-specialist.

Combination of biomarkers and clinical features in a diagnostic algorithm is often the most powerful approach to clinical diagnostics. Algorithms are developed by the process of machine learning where the weighting of

different features is optimized or trained on experimental clinical data and then validated on independent subject/patient datasets.

Conclusion: We recently developed diagnostic algorithms for screening, detection and typing of early-stage arthritis. Similar techniques are in development for other health screening and disease diagnostics. We combine protein glycation, oxidation, nitration and other markers in a data-dependent process. Such approaches will likely produce valuable clinical diagnostic applications of carbonyl stress.

Reference: Ahmed U, *et al*. Nature Sci Rep. 2015; 5: 9259.

3. Formation and reactions of pent-4-en-1-amine the counterpart of acrylamide from lysine

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Objectives: In the presence of reducing sugars and simple carbonyl compounds asparagine and phenylalanine are known to undergo carbonyl-assisted decarboxylative deamination reaction to generate what could be defined as amino acid derived "vinylic compounds" such as acrylamide and styrene, generally considered toxic due to their ability to react with proteins. In this study the ability of lysine to undergo similar transformation to generate pent-4-en-1-amine was explored in addition to its chemical reactivity in the presence of 5-methylfurfural and hydroxymethylfurfural.

Methods: Isotope labelling, independent synthesis, Pyrolysis/GC/MS, ESI-TOF-MS techniques were employed to characterize the reaction pathways. Solutions of relevant model systems were diluted in 1 mL of water and then again 1/100 with 50% methanol and 0.1% formic acid. Each sample (5 μL injections) was directly analyzed by liquid chromatography−mass spectrometry (LC−MS), on a 1200 series Agilent rapid resolution LC system coupled to an Agilent 6210 time-of-flight (ESI-TOF) instrument. The mobile phase consisted of 50% methanol, 0.1% formic acid at a flow rate of 0.3 mL/min. For isotope labeling studies [^{15}Nα]lysine•2HCl, [^{15}Nε]lysine•2HCl, [U-$^{13}C_6$]lysine•2HCl, [^{13}C-6]lysine•2HCl, [U-^{13}C6]glucose, [^{13}C-1]glucose, [^{13}C-2]glucose, [^{13}C-3]glucose, [^{13}C-4]glucose, [^{13}C-5]glucose and [^{13}C-6]glucose were used.

Results: Isotope labeling studies performed using lysine/glucose model systems have indicated that lysine can generate piperidine, a reactive amine capable of undergoing Maillard type interactions. Two possible mechanisms were identified for the formation of piperidine: one arising through decarboxylation of lysine alone to generate cadaverine (1,5-diaminopentane) followed by deamination to form pent-4-en-1-amine which in turn can cyclize into piperidine where both Nε and Nα atoms of lysine can be equally involved in its generation due to the symmetrical nature of the precursor diamine. On the other hand, in the presence of sugars, lysine, similar to asparagine and phenylalanine, can undergo carbonyl-assisted decarboxylative deamination reaction to generate pent-4-en-1-amine only through the Nε atom of lysine. The formation of pent-4-en-1-amine in the lysine/glucose model system was confirmed through detection of its Schiff base adduct with HMF. The complete labeling studies along with structural analysis using synthetic and other available precursors have shown the presence N-(5-methylfuran-2-yl)methylidene]penta-1,3-dien-1-amine incorporating pent-4-en-1-amine in its structure.

Conclusions: Lysine similar to asparagine and phenylalanine can undergo carbonyl-assisted decarboxylative deamination reaction to generate pent-4-en-1-amine the counterpart of acrylamide from lysine exclusively through the Nε of lysine. In the absence of carbonyl compounds it can undergo intramolecular cyclization to generate piperidine.

4. AGEs, the sticky persistence of metabolic memories – implications for diabetic complications

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Objectives: Diabetes continues to cast a long shadow over the lives of many people. It is now clear that even transient hyper- or hypoglycaemia or increased glycaemic variability around healthy mean glucose levels can have long-lasting effects on the development and progression of diabetic complications including cardiovascular disease. Even after glycaemic control has been achieved and maintained for many years, the molecular legacy triggered by long periods of poor glucose control appear only slowly reversible, at best. This phenomenon has become known as "metabolic memory" or the "legacy effect". Metabolic memory has been used to explain many clinical observations surrounding diabetes and its management, including the lack of cardiovascular benefits in many short- and intermediate-term trials, and the potential utility of early intensive glycaemic control.
Results: Post-translational modifications induced following the generation of reactive dicarbonyls are one of the most important mediators of metabolic memory. Persistent modifications have the potential to shape structure and function. In addition, dicarbonyls trigger persistent epigenetic changes which alter the regulation of gene activation. Activation of the AGE-RAGE axis also triggers persistent pathogenic changes.
Conclusion: Taken together, targeting glycation appears to be an important means to both prevent sticky persistence of metabolic memories as well as erasing their clinical consequences.

5. Multiple detection of AGEs as markers for metabolic abnormalities

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Objectives: The quantification of advanced glycation end-products (AGEs) *in vivo* was originally performed using the fluorescent characteristics of AGEs. Subsequently, an anti-AGE antibody was developed as a more specific tool for detecting AGEs, and the involvement of AGEs in various diseases has been reported. However, since the epitope structures of anti-AGEs antibodies were not identified until the 1990s, little is known about the relationship between the structure and pathology of AGEs. However, the immunochemical measurement of AGEs in physiological samples is associated with potential artifacts due to pretreatment techniques, such as heating and alkaline treatment [1)]. Therefore, accurate measurement of AGEs is the most important issue to evaluate the pathophysiological significance of AGEs.

Results: We previously identified new AGE structure derived from glycolaldehyde (GA), termed GA-pyridine, in human atherosclerotic lesions by monoclonal antibody [2)]. Our recent study demonstrated that GA-pyridine was detected in human carotid artery and atherosclerotic plaque by iquid chromatography tandem mass spectrometry (LC-MS/MS). Furthermore, since N^{ω}-(carboxymethyl)arginine (CMA), a collagen-specific AGEs, and S-(2-Succinyl)cysteine (2SC), a reaction product between thiol group of proteins and fumarate of the Krebs cycle intermediate [3)], are also detected by LC-MS/MS, measurement of those structures will be a markers for metabolic abnormalities.

Conclusion: In this presentation, non-invasive measurement of AGEs in skin to evaluate diabetic complications will be also reported.

Reference: 1) Nakano M, *et al*. Amino Acids. 2013; 44: 1451-1456. 2) Nagai R, *et al*. J Biol Chem. 2002; 277: 48905-48912. 3) Nagai R, *et al*. J Biol Chem. 2007; 282: 34219-34228.

【Lunch Time Poster Presentation】

1. Glycation stress and anti-aging.

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Objectives: Glycation occurs in our body when a reducing sugar, such as glucose or fructose, or aldehydes (*i.e.* derived from alcohol) combines with a protein in a non-enzymatic reaction, finally forming advanced glycation end products (AGEs), which may accumulate in tissue. AGEs may also bind to a receptor for AGEs (RAGE) or other receptors, thus inducing inflammation in skin and other tissues. Glycation stress is a risk factor for aging and age-related diseases including osteoporosis, atherosclerosis, skin aging, and so on. Especially in persons with high glycative stress, it is important to reduce it. The purpose of this study is to elucidate the inhibitory effect of fruits, vegetables and herbs on AGEs generation.

Methods: Samples of fruits, vegetables and herbs were dried ground and extracted at 80°C for 1 hour; then added to two *in vitro* models of glycation between glucose and human serum albumin (HSA). The extract mixtures were incubated at 60°C and the AGEs fluorescence (excitation 370 nm/ detection 440 nm) measured after 40 hours using the ARVOTM MX 1420 ARVO series multilabel counter. The results are expressed as 50% inhibitory concentration (IC_{50}) for each sample.

Results: Primary prevention is achieved by encouraging maintenance of reasonable skeletal muscle mass, moderate exercise, and proper eating habits. These recommendations will help ensure even concentrations of blood sugar and reduce insulin resistance.

Eating habits that reduce the glycation stress are to eat slowly, chew food well, choose foods that do not raise blood sugar rapidly (*i.e.* foods with a low glycemic index). High fructose syrups should be avoided.

AGE generation was inhibited by extracts from tea (*Camellia sinensis*), Japanese persimmon leaf (*Diospyros kaki*), banabá (*Lagerstroemia speciosa*), kuma bamboo (*Sasa veitchii*), Chinese blackberry (*Rubus suavissimus*), and mixed herb of Roman chamomile (*Anthemis nobilis*), hawthorn berry (*Crataegus laevigata*), dokudami (*Houttuynia cordata*), and grape leaf (*Vitis vinifera*).

To inhibit glycation stress is through AGE cleaving method as part of AGE breaking. Here, we evaluate pomegranate extract and its compound's cleaving activity and investigate its mechanism in inhibit glycation stress. Test samples including 4 pomegranate extracts, 12 pure pomegranate compounds, 2 pomegranate like compounds (gallic acid and pyrogallol) and N-phenacylthiazolium bromide (PTB), as positive control, were tested against 1-phenyl-1,2-propanegione (PPD), then PPD derivate, benzoic acid, was measured using HPLC by Vasan's method. We discovered that pomegranate extracts cleaving activity are stronger than PTB and 8 pomegranate compounds cleaving activity are twice stronger than PTB. The pyrogallol molecule structure play important role in pomegranate compounds cleaving activity.

Conclusions: Glycative stress can be reduced through an appropriate diet, lifestyle. Intake of anti-glycation materials also may be useful. In this presentation, I would like introduce the methods how to reduce glycative stress.

2. Glycation and bone diseases

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Objectives: Our body shape and structure changes with age, from childhood to youth our body develops and reach at pick and then started to loss muscles, joints and even bones and eventually loss height. People usually loss 1 cm of height every 10 years after age 40 and such a condition may lead to the severe diseases like osteoporosis, osteoarthritis, rheumatoid arthritis that can cause sudden fracture on back bone, bones of the forearm, hip and make the patients dysfunctional.

Results: Bone is an active live tissue composed of bone forming osteoblast and bone resorbing osteoclast and bone resorption regulator osteocyte cells. The mineralized bone matrix has an organic component mainly of collagen and an inorganic component of bone mineral made up of various calcium and phosphate salts. Our body always contains sugars like glucose, fructose and aldehydes as body fuel that readily reacts with the protein part of bone and inhibits calcification, and thus changes its structure and reduces strength. Moreover, osteoporosis patients have higher urine and serum pentosidine and N^{ε}-carboxymethyl-lysine (CML) level. This may be due to the glycation of bone collagen and then osteocytes and osteoblast activate osteoclast cells to cut down the glycated portion of bone and its regular work of the bone cells. But problem is somehow the signaling between osteoclast and osteoblast to reform bone is not working in osteoporotic patients. Higher concentration of advanced glycation endproducts (AGEs) may be responsible for that. Thus, AGEs reduce bone strength and quality by inhibiting mineralization along with excess bone resorption. High serum homocysteine and high urine pentosidine increase fracture risk 7.2 times more than usual.

AGEs also can activate our immune systems as foreign particles do and then initiate self-destructive diseases like inflammation, rheumatoid arthritis etc. AGE activated macrophages produce interleukin (IL)-1, IL-6, tumor necrosis factor (TNF)α which can trigger synovial fibroblast to stimulate osteoclast precursors to fuse together to form osteoclast and also activate osteoclast to resorb bone.

Conclusion: These changes cannot be avoided even in modern age, only our lifestyle can slow down this process. Regular calcium, vitamin D, colorful vegetables and fruits having higher amount of polyphenols and anti-oxidants, milk, yogurt uptake and regular physical exercise along with weight bearing exercise, avoiding smoking, alcohol, reducing the uptake of sugar, salt, coke altogether with adequate sleep can prevent age related bone diseases along with many more.

3. Polyphenols and anti-glycation activity

Parengkuan Lanny, Masayuki Yagi, Mari Ogura, Wakako Takabe, Yoshikazu Yonei
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Objectives: Polyphenols extracted from natural compound such as fruits, herbs or vegetables have powerful effects on preventing and even reversing the effects of oxidation, inflammation, and glycation. The purpose of this study is to examine the correlation between polyphenols and anti-glycation activity of various fruits, as well as to investigate several well-known polyphenol compounds for its effect on advanced glycation end product (AGE) inhibition.
Methods: Subjects were 105 samples of fruit, which were dried ground and extracted at 80°C for 1 hour. Total polyphenols were measured using Folin-Ciocalteu reagent with catechin as the standard. Polyphenol compounds were estimated individually using chromatographic technique.
Results: Mangosteen peel contains the highest polyphenol concentration (1.18 mg/mL) followed by pomegranate and sweetie peels. The polyphenols in fruit peel are 4 times higher than fruit flesh. There is correlation between polyphenol in fruit and its anti-glycation activity ($p = 0.001$). In apple varieties, “toki”, “sanfuji”, “mutsu” peels show higher polyphenol concentration compare to other apple varieties. Different apple varieties show different concentration of polyphenol compounds but, fisetin is more likely found in higher concentration in apple flesh than apple peel. Fruit peel show higher anti-glycation activity than fruit flesh. Polyphenols concentration in citrus fruit is higher than in rosaceae catagories but, the anti-glycation activity is higher in the rosaceae catagories.
Conclusion: There is a correlation between total polyphenols and anti-glycation activity in fruits. Approximately 50-100 g apple/day (= 9 mg polyphenols/day) is needed to obtain the desired anti-glycation activity benefits.
Acknowledgements: Supported by SIP Project ID 14533567.

4. Importance of protein selection during anti-glycation activity determination

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Objectives: Advanced glycation endproducts (AGEs) are the final products of the non-enzymatic reaction between reducing sugars and amino groups in proteins, lipids and nucleic acids and, recently, implicated as a major pathogenic process in diabetic complications or other degenerative diseases. Food components may play a role in therapeutic approach for the prevention of AGE formation. In our previous report, we described that the protein type used in glycation models primarily determines anti-glycation activity; type of sugars (glucose/fructose) has no marked effect, and extraction methods have case-dependent effect on anti-glycation activity. In previous study we only evaluated fluorescent AGEs in five *in vitro* glycation models. In the present study, it has been attempted to clarify the importance of protein selection during anti-glycation activity determination by investigating the effect of spice extracts against AGE formation.
Methods: We measured 3-deoxyglucosone (3-DG), glyoxal (GO), methylglyoxal (MGO), pentosidine by HPLC and N^{ϵ}-carboxymethyl-lysine (CML) by enzyme linked immunosorbent assay (ELISA) in the 3 models of human serum albumin (HSA)/glucose, bovine serum albumin (BSA)/glucose, and bovine skin collagen type I/glucose with both hot water and 50% ethanol extraction from test spice samples; oregano, ginger and cinnamon.
Results: The inhibition activity of a particular spice extract on 3-DG, GO, MGO, pentosidine, and CML significantly varied depending on the protein type used in glycation models with both water and 50% ethanol extraction. Therefore, the selection of protein is critical for the anti-glycation activity determination.
Conclusion: Model directed compound isolation from a particular spice against specific AGE, and study of the inhibition mechanism of that compound in each model should be taken into consideration and this aspect remains to be elucidated in further investigation.

5. Formulation of five curry making spice mixtures and investigate their effect on advanced glycation endproducts formation

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Objectives: Curry is a common food, containing a variety of spices, which have been demonstrated anti-oxidative or anti-glycative actions, preventing advanced glycation endproduct (AGE) formation. In order to develop an anti-glycative ready to serve curry, we attempted to formulate five combinations of curry making spice mixtures and investigate their effect on formation of fluorescent AGE, pentosidine, 3-deoxyglucoson (3-DG), glyoxal (GO) and methylglyoxal (MGO) in human serum albumin (HSA)/glucose model with both hot water and 50% ethanol extractions.
Methods: Fluorescent AGE was measured by fluorescent spectroscopy at excitation 370 nm and emission 440 nm. Pentosidine; and 3-DG, GO, and MGO were measured by HPLC. A system composed of glucose and HSA was employed as a model in our study.
Results: This is the first report of formulating curry making spice mixture with both water and 50% ethanol extraction on the anti-glycation point of view. The inhibitory activity of 50% ethanol extracted curry formulation against fluorescent AGE, pentosidine, and reactive carbonyl species (3-DG, GO and MGO) is better than that of water extracted formulation. The carbonyl trapping activity of curry mixtures is correlated with their polyphenol content.
Conclusion: This study provides information for a spice mixture formulation on the anti-glycation point of view. Further study remains to be conducted to measure the colour, taste, moisture and pH of curry prepared from the formulation to make it a ready to serve curry.
Acknowledgements: Supported by SIP Project ID 14533567.

6. Melatonin: Not a carbonyl scavenger

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Objectives: In our previous study of the human skin accumulation of AGEs and the lifestyle behaviors, as well as smoking and alcohol drinking, lack of sleep clearly promoted AGE accumulation. We hypothesized that melatonin may participate in this phenomenon. Here, we investigated carbonyl-scavenging capacity of melatonin in human serum albumin (HSA)/glucose model as part of revealing the undiscovered function of it.
Methods: Fluorescent AGE was measured by fluorescent spectroscopy at excitation 370 nm and emission 440 nm. Pentosidine; and 3-deoxyglucosone (3-DG), glyoxal (GO), and methylglyoxal (MGO) were measured by HPLC-FL and HPLC-UV/VIS respectively. A system composed of glucose and HSA was employed as a model in our study.
Results: Melatonin neither inhibited fluorescent AGE formation in both HSA-glucose and Collagen-glucose models nor inhibited generation of AGE intermediates such as 3-DG, GO, and MGO in HSA-glucose model.
Conclusion: Present study compelled us to conclude that melatonin has no direct action on AGE generation. Melatonin might act as cross-link breakers, which should take into consideration for further study.
Acknowledgements: Supported by JSPS KAKENHI Grant Number 26350917.

7. Anti-glycation activity of Japanese sake and its ingredients

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Objectives: Fermented foods, including beverage, are known to have a variety of health benefits. In this research, we examined the inhibitory effect of sake (rice wine) and its ingredients, organic acids, on advanced glycation endproduct (AGE) formation.
Methods: Subjects were 30 kinds of sake (20 Junmai ginjo-shu, 10 Honjozo-shu) and, as ingredients, ferulic acid and its metabolites (vanillin, vanillic acid and protocatechuic acid) and kojic acid (total 5 kinds). The anti-glycation activity was evaluated by measuring the AGE-derived fluorescence (Excitation 370 nm/Emission 440 nm) after incubating human serum albumin and glucose at 60°C for 40 hours with each sample. Reaction concentration was adjusted to be 10% volume content for sake samples and 0.1 mg/mL for ingredients. Results were expressed as percent inhibition of AGE formation (average ± standard deviation).
Results: Percent inhibition values of AGE formation were 27.4 ± 5.6% (maximum 39.5%, minimum 14.7%) for 30 sake samples. There was no significant difference in average values between Junmai ginjo-shu and Honjozo-shu. The individual difference was large in Junmai ginjo-shu. Regarding to ingredients, percent inhibition values were 38.3 ± 0.1% for vanillic acid and 41.6 ± 0.9% for protocatechuic acid.
Conclusion: Sake showed *in vitro* inhibitory effect on AGE formation, although individual difference was noted . As a next step, we have a plan to conduct the analysis of sake ingredients by a reverse-phase HPLC especially for 5 ingredients and to examine the relation between anti-glycation effect of sake and its ingredients.
Acknowledgements: Supported by SIP Project ID 14533567.

8. Effect of plants containing iridoids on anti-formation and degradation of AGEs

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Objectives: The reaction that proteins or lipids combine with reducing sugar non-enzymatically is called glycation. The reaction produces AGEs (advanced glycation endproducts). It was revealed that AGEs are involved in various chronic diseases. Therefore reports of anti-glycation increase in late years. It was also revealed that a plant containing iridoids provided anti-glycation activity by the report. However, there are few reports about degradation of AGEs. In this study we examined the effect of plants with iridoids (*Morinda citrifolia*, *Cornus officinalis* and *Olea europaea*) on anti-formation of AGEs by use of three model proteins (HSA, collagen and keratin). Furthermore, we examined activity of AGEs degradation to an index with degradation activity of α-diketone structure of 1-phenyl-1,2-propanedione (PPD).

Methods: Activity of anti- AGEs formation; Samples and glucose solution were added in each model proteins and reacted them at 60°C. Inhibitory activity of AGEs formation by each samples were calculated by formed fluorescent AGEs (excitation wavelength 370 nm, detection wavelength 440 nm).

Activity of AGEs degradation; PPD solution were mixed samples, reacted them at 37°C for 8 h. Benzoic acid which was produced when α-diketone structure broken was determined by HPLC. Activity of AGEs degradation by each samples were calculated by formed benzoic acid.

Results: We examined effects of plants containing iridoids on anti-AGEs. In Anti-formed AGEs, the highest activity was *Morinda citrifolia* seed extract in all models protein. The other samples also have inhibition action. But the inhibition intensity was difference by model proteins. In activity of AGEs degradation, *Cornus officinalis* fruit juice extract was most strongly (degradation rate; 29.8%), and in the next, *Olea europaea* leaf extract (26.5%), *Morinda citrifolia* seed extract (18.2%).

Conclusion: These studies showed that plants containing iridoids had anti-AGE action such as anti-formation and degradation of AGEs.

9. Effect of choice of staple food and the addition of dietary fiber on changes in postprandial blood glucose level

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Objectives: Foods with a low glycemic index (GI) produce a slow elevation of postprandial blood glucose level, and postprandial hyperglycemia can be suppressed by eating foods in the appropriate order (*e.g.*, eating vegetables before steamed rice). This study was conducted to assess the effects of choice of staple food (carbohydrates) and 2 side dishes (soft boiled eggs, vegetable salad) on changes in postprandial blood glucose level, with the aim to develop a method of reducing glycation stress.
Methods: Nine staple foods [steamed rice, handmade udon (wheat noodles), boiled udon, bread, sekihan (steamed glutinous rice with red beans), buckwheat noodles, pasta, brown rice, rice cakes) and 2 side dishes (soft boiled eggs, vegetable salad) were used as test foods. Written consent to participate in the study was obtained from 19 healthy male or female subjects (age 30.3 ± 12.0 years). On the day of experimentation, fasting blood glucose levels were measured, and each test food was then eaten for breakfast over a period of 10 minutes. Blood glucose levels were measured using a glucose meter for self monitoring at 15, 30, 45, 60, 90, and 120 minutes after the start of food ingestion. Glycation stress was quantified in terms of blood glucose elevation and area under the curve of blood glucose elevation (AUC). Data obtained were statistically analyzed using Tukey's multiple comparison test.
Results: In the subjects exhibiting a blood glucose elevation of AUC ≥ 5,000 after eating steamed rice, the postprandial blood glucose levels observed after taking each of the 9 staple foods were compared. The maximum blood glucose level change tended to be lower for pasta than for brown rice ($p = 0.049$), lower for bread ($p = 0.060$) and pasta ($p = 0.060$) than for steamed rice, and lower for bread ($p = 0.050$) than for brown rice. The AUC was lower for bread and pasta than for steamed rice, and lower for pasta than for sekihan. Both the maximum blood glucose level change ($p = 0.029$) and AUC ($p = 0.029$) were lower for wheat-derived staple foods than for rice-derived staple foods. A comparison of postprandial blood glucose levels following ingestion of udon (thick Japanese wheat flour noodle) + side dish revealed that ingestion of udon + salad produced slower blood glucose level elevations and a smaller AUC than udon taken alone ($p = 0.029$). Udon + soft boiled egg produced a smaller AUC than udon taken alone ($p = 0.023$).
Conclusion: Changes in postprandial blood glucose level differed depending on the choice of staple food; the addition of dietary fiber may mitigate blood glucose elevation after meals and lessen glycation stress. In addition a combination of a staple food and a side dish may be more effective than low-GI foods in suppressing glycation stress.
Acknowledgements: Supported by Yoshinoya Holdings Co., Ltd**.** Approved by the Doshisha University Ethics Committee (#1228).

10. Grapefruit intake affect changes in postprandial blood glucose

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Objectives: Our recent research reports that grapefruit (GP) [*Citrus paradisi*] can repress the postprandial blood glucose level eating before and when we eat staple food. Also we verified that GP (yellow) have anti-glycation effect（IC_{50}: 0.490 mg/mL）in the *in vitro* glucose/human serum albumin (HSA) reaction model [1].We invested that difference of how to eat GP effects on postprandial blood glucose level.

Methods: Subjects were 17 male and female healthy, non-obese volunteers (27.5 ± 8.8 years) from whom informed consent was obtained. On the test day, fasting blood glucose was measured followed by test food ingestion for 10 min, then glucose levels were checked by a glucose meter for self-monitoring at 15, 30, 45, 60, 90, and 120 min. Postprandial blood glucose and the area under the curve (AUC) were analyzed and compared between 5 test food groups: “bred (170 g)”, “raw GF (180 g) + bred (132 g)”, “smoothie GF (200 g) + bred (132 g)”, “100% juice GF (200 mL)+ bred (132 g)”, “citric acid (10 mg/mL, 200 mL) + bred (170 g)”. Citrate contents were determined by F-Kit-citric acid (J.K. International, Japan).

Results: Among the 5 groups, there is no significantly difference in AUC. Postprandial blood glucose levels, compared with bred, were significantly lower in “raw GF + bred” at 120 min ($p = 0.015$), “smoothie GF + bred” at 45 and 60 min ($p = 0.045$, $p = 0.032$), “100% juice GF + bred” at 60 and 120 min ($p = 0.009$, $p = 0.008$), and “citric acid + bred” at 120 min ($p=0.032$). Citric acid contents were 1.1 mg/mL in raw GP, 2.1 mg/mL in smoothie GP and 2.2 mg/mL in 100% juice GP.

Conclusion: It is suggested that citrate, as well as fibers in GP, may participate in reducing the postprandial blood glucose level, thus contributing repressing the glycation stress. Prior research revealed that anti-glycation activity is higher in peel than in fresh especially in the citrus fruits (*i.e.*, buntan [Citrus maxima], sudachi [*Citrus sudachi*]), it is suggested that GP, a citrus fruit, may raise anti-glycation effect by eating both fresh and peel.

Reference: 1) Parengkuan L, et al. Anti-Aging Medicine. 2013; 10: 70-76.

Acknowledgements: Supported by Dole Japan, Inc**.** Approved by the Doshisha University Ethics Committee (#1228).

11. Glycative stress and physical functional age.

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Objectives: Since exercise consumes sugar as energy, it is believed to reduce the glycative stress, thus contributing for our health promotion. In this study, we measured skin advanced glycation end product (AGE) accumulation as an index for glycative stress and examined its relation to walking exercise or Anti-Aging Medical Checkup data.
Methods: Subjects were 20 elderly (12 males, 8 females, 72.7 ± 4.6 years old) living independently in the Yurin district of Kyoto, registered in our pedometer-based physical activity program for three years from 2013 to 2015, to whom pedometers were provided and walking exercise encouraged. The Anti-Aging Medical Checkup was conducted three times every April. The functional ages (muscle age, bone age, hormone age, blood vessel age and neural age) were estimated by using the Age Management Check (Ginga Kobo, Japan). The skin AGE-derived Auto Fluorescence (AF) intensity was measured at the upper arms by AGE Reader^{TM} (DiagnOptics, Netherlands). Data analysis was conducted using SPSS (IBM Japan).
Results: The mean skin AF was decreased the year compared with last year from 2013 to 2015 (2013: 2.47 ± 0.37, 2014: 2.24 ± 0.28, 2015: 1.96 ± 0.42) (2012 vs 2013: $p < 0.001$, 2014 vs 2015: $p = 0.001$). The mean muscle age (2013: 56.5 ± 2.6, $p < 0.001$, 2014: 57.1 ± 2.6, $p < 0.001$, 2015: 57.6 ± 2.4, $p < 0.001$) and blood vessel age (2013: 62.0 ± 6.9, $p < 0.001$, 2014: 66.1 ± 9.5, $p = 0.001$, 2015: 69.4 ± 9.2, $p = 0.006$) were younger than the mean actual age for three years. The mean bone age (2013: 65.5 ± 13.4, $p = 0.04$) and hormone age (2015: 69.4 ± 8.7, $p = 0.001$) were younger than the mean actual age for one year. The mean muscle age (2013: $r = 0.443$, $p = 0.20$, 2015: $r = 0.448$, $p = 0.27$) and blood vessel age (2013: $r = 0.536$, $p = 0.08$) were positively correlated with skin AF.
Conclusion: Walking exercise seems to be effective in keeping subjects' youth of the physical functional age, especially for muscle age and blood vessel age, and to contribute to reduction of glycative stress.
Acknowledgements: Supported by SIP Project ID 14533567. Approved by the Doshisha University Ethics Committee (#14089).

12. Dietary diversity and skin autofluorescence in community-dwelling older adults: A cross-sectional study.

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Objectives: In accordance with progress of aging society in Japan, increase of the number of older adults with diabetes and frailty are predicted. Evidence has been recently accumulated where advanced glycation end products (AGEs) play an important role in the development of diabetic and cardiovascular complications, as well as the development of other chronic diseases. Dietary diversity is regarded as an important factor of instrumental activities of daily living, disability and mortality in adults. The aim of this study was to assess the correlation between dietary diversity and skin autofluorescence (SAF) in healthy older adults.

Methods: Design, Cross-sectional study; Setting, Yurin area, Shimogyo-ku, Kyoto City, Japan; Subjects, The study included 39 adults (19 males and 20 females). The inclusion criteria were: 1) age above 65 years, and 2) independently-living. Exclusion criteria were: requiring nursing care, and dementia.

The height, body weight, and blood pressure were measured. The body mass index (BMI) was calculated using the height and body weight. Information concerning the lifestyle was collected by a self-administered questionnaire. Dietary assessment was measured by using a brief self-administered diet-history questionnaire (BDHQ). A food variety score (ranging from 0 to 8) was calculated to represent dietary diversity, counting on intake frequency of eights food groups; potatoes, fish, meat, eggs, soybeans and soybean products, fruits, seaweeds and mushrooms. SAF is a non-invasive measurement of AGEs. The accumulation of AGEs in the skin was evaluated with an AGE Reader™ (DiagnOpitics, Netherlands). Statistical analysis of output data included Pearson and correlation and the Student's t-test were carried out using SPSS software.

Results: The mean ages of the study population in males and females were 76.9 ± 4.9 and 76.8 ± 4.8 years, respectively, showing no significant differences. Mean SAF scores in males and females were 2.17 ± 0.32 and 1.97 ± 0.32 respectively and differences were detected ($p = 0.06$). Mean food variety scores in males and females were 4.7 ± 1.2 and 5.6 ± 1.2 respectively and significant differences were observed. This survey showed that total energy intake was negatively correlated with SAF in males. On the contrary it was positively correlated with SAF in females. Fat intake was also positively correlated with SAF in females. Food variety was negatively correlated with SAF in males but not in females. Fish intake was negatively correlated with SAF in males. Mushrooms intake was negatively correlated with SAF in both males and females. In addition, Snack intake was positively correlated with SAF in females.

Conclusion: Although this study was limited by a small sample size, our findings suggest that food consumption patterns and dietary diversity are associated with SAF in older adults. Further research is required to verify the findings.

Acknowledgements: Supported by SIP Project ID 14533567. Approved by the Doshisha University Ethics Committee (#14089).

13. The potentiality of reducing the skin glycation stress by uptake of a mixed herb tea extract

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Objectives: High glycation stress conditions cause formation and accumulation of advanced glycation endproducts (AGEs) in the body, and thus play a role as a risk factor in the pathogenesis of diseases, and age-related conditions like elasticity reduction and loss of clearness in the skin. AGE formation causes a yellow-brownish change of tissue and physical deterioration, especially in the collagen tissue of the skin, bone and cartilage, thus it is a topic in the field of aesthetics, cosmetics and health promotion. This research looked for the herb tea which has anti-glycation activity for I type collagen (Col) or human serum albumin (HSA). And this research, when a mixed herb tea extract powder was taken in, a potentiality of reducing a skin glycation stress was studied.

Methods: The sample used the hot water extract thing of 81 kinds of commercial herb tea. AGE-derived fluorescence was measured using a glucose/HSA and glucose/Col model. Briefly, various concentrations of the test product extract or aminoguanidine (AG) in aqueous solution were added to phosphate buffer solution (pH 7.4), glucose and HSA or Col. And the material was incubated at 60°C for 40 hours or 10 days. AGE-derived fluorescence was measured microplate reader at an excitation 370 nm / fluorescence 440 nm. Pentosidine and N^{ε}-carboxymethyl-lysine (CML) measured by the enzyme-linked immunosorbent assay. The strong herb tea of the anti-glycation activity was selected, and the mixed herb tea extract powder was prepared. And we conducted a double-blind, placebo-controlled, parallel group study of the mixed herb tea extract powder were carried out for the 21 healthy womens of a premenopausal.

Results: 11 of 81 kinds of herb tea exract had an anti-glycation activity. In a glucose collagen model, was an anti-glycation activity of 20 times or more of AG. There was inhibition activity in four kinds, chinese blackberry (*Rubus suavissimus*), persimmon (leaf) (*Diospyros kaki*), kuma bamboo grass (*Sasa veitchii*), and banabá (*Lagerstroemia speciosa*), about a production of CML and pentosidine. In the 12-week uptake study of these four kinds mixed herb tea extract powder of 300 mg/day, CML in a corneum of the left cheek ($p = 0.085$) and the blood pentosidine ($p = 0.039$) decreased compared with the placebo foods uptake group. In the subclass analyses, the viscoelasticity index (R2) ($p = 0.011$) of the left cheek increased eight weeks afterward. And, blood CML decreased 12 weeks afterward ($p = 0.017$).

Conclusion: The uptake clinical study of a mixed herb tea extract powder had the same effect as an *in vitro* anti-glycation. The mixed herb tea extract uptake of herb tea had a potentiality of a skin glycation stress.

Acknowledgements: Pre-registered with UMIN (#000012820).

14. Survey of fluorescence wavelength range reflecting human tissue aging

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Objectives: To determine the fluorescence wavelength range reflecting the degree of tissue aging in the human body, the skin fluorescent spectrum was measured at 375 ± 5 nm excitation and 420-750 nm emission.
Methods: Skin fluorescence data and measurements reflecting aging as well as data on lifestyle were collected from 65 healthy people (mean chronological age: 40.9 ± 17.2 years). The correlation of fluorescence intensity, chronological age, skin auto fluorescence (AF), tissue age (vascular age and stiffness), and lifestyle behavior risks were assessed using Pearson's correlation coefficient (r) which was then age-adjusted (rage). This study was conducted after approval by the Doshisha University Ethics Committee for Scientific Research Involving Human Subjects (#832).
Results: Chronological age (420-627.7 nm; rmax = 0.372, p = 0.002 at 507.5 nm), skin AF (473.0-502.1 nm; rmax = 0.275, p = 0.028 at 485.8 nm), vascular age (447.3-532.6 nm; rmax = 0.317, p = 0.031 at 485.3 nm), and number of risky lifestyle behaviors (436.2-525.5 nm; rmax = 0.325, p = 0.024 at 484.0 nm) were all positively correlated with relative fluorescence. Further, only number of risky lifestyle behaviors (421.3-525.5 nm; rage max = 0.336, p = 0.024 at 451.0 nm) remained positively correlated with relative fluorescence after age-adjusted analysis.
Conclusion: Our present findings suggest that several ranges of fluorescence intensity may be positively correlated with vascular age. Further, the degree of human tissue aging might be reflected by spectrofluorimetry values.
Acknowledgements: Supported by SIP Project ID 14533567.

15. Anti-glycation effect of *Geranium dielsianum* (miskamiska) extracts

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Objectives: *Geranium dielsianum* (miskamiska) is a plant of the family of *Geraniaceae*. The plant has traditionally been used as folk medicine for diabetes treatment at the Andes area in Peru. Our study aims to examine the anti-glycation effects of the hydroalcoholic extract of miskamiska (MISKAMISKA®, TOWA CORPORATION, Tokyo, Japan) *in vitro*.

Methods: Aminoguanidine, a known inhibitor of glycation, as a positive control and distilled water as a negative control for miskamiska were used. The experiments were practiced on the following 3 conditions.

1. Inhibition of fluorescent advanced glycation end-products (AGEs) formation by glucose and human serum albumin (HSA).

AGEs were prepared by incubation of glucose and HSA with miskamiska extract in phosphate buffer (pH 7.4) at 60°C. The inhibition of the formation of fluorescent AGEs by miskamiska extract was analyzed with fluorescence spectroscopy.

2. Inhibition of fluorescent AGEs formation by glucose and Collagen Type I.

AGEs were prepared by incubation of glucose and Collagen Type I from bovine skin with miskamiska extract in phosphate buffer (pH 7.4) at 60°C. The inhibition of the formation of fluorescent AGEs by miskamiska extract was analyzed with fluorescence spectroscopy.

3. General inhibition of N^{ε}-(carboxymethyl)lysine (CML), non-fluorescent AGEs, formation by glucose and Collagen Type I.

AGEs were prepared by incubation of glucose and Collagen Type I from bovine skin with miskamiska extract in phosphate buffer (pH 7.4) at 60°C. The general inhibition of CML by miskamiska extract was analyzed with enzyme linked immunoassay.

Results: Miskamiska extract exhibited the dose-dependent inhibition of AGE formation and anti-glycation activity was higher than that of aminoguanidine in each assay.

Conclusion: It suggests that the miskamiska extract has actions contributing to the beauty and anti-aging.

16. “Mangostin aqua” inhibits the formation of AGEs and improves skin conditions

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Objectives: The inhibition of advanced glycation end-products (AGEs) by daily meals is believed to become an effective prevention for lifestyle-related diseases. In the present study, the inhibitory effect of hot water extracts of mangosteen (*Garcinia mangostana* L.) pericarp (Mangostin aqua; Nippon Shinyaku Co., LTD.) on the formation of AGEs, *in vitro* and *in vivo*, and the remedial effect on skin conditions were measured.
Methods: Hot water was added to dried mangosteen pericarp, filtered, concentrated, added 33% dextrin for solid content of extract, and spray-dried to obtain the “Mangostin aqua”. To examine the inhibition of AGE formation, collagen and fructose was incubated in the presence of “Mangostin aqua”. Then, the inhibition of pentosidine formation was examined. Furthermore, the tablet contained 100 mg “Mqangostin aqua”, once a day, was administrated to 11 healthy women (32-48 years) for 12 weeks. Routine blood and skin function testing were evaluated every 4 weeks. AGE accumulation in the skin was estimated by measuring fingertip autofluorescence (SHARP), skin elasticity was measured with Cutometer (MPA580), and moisture content was measured with Robo Skin Analyzer (RSA50).
Results: The formation of melanoidin decreased depending on the addition of “Mangostin aqua” during collagen incubation with fructose. “Mangostin aqua” and Maclurin glycoside (rhodanthenone B) isolated from “Mangosntin aqua” inhibited the formation of pentosidine. Oral administration of “Mangostin aqua” at 100 mg/day to volunteer subjects for 12 weeks reduced the serum pentosidine contents, although fasting the blood glucose (mg/dl) and HbA1c (%) levels did not change. The oral administration of “Mangostin aqua” significantly reduced the skin autofluorescence intensity, demonstrating that “Mqangostin aqua” also reduced AGE accumulation in the skin. Furthermore, the elasticity of the skin was also improved comparing with before “Mangostin aqua” administration. The average moisture content of the skin in the subject before “Mangostin aqua” administration increased in a time-development manner.
Conclusion: These results demonstrate that intakes of “Mangostin aqua” reduced the glycation stress results in the improvement of skin condition.

17. Development of anti-glycation assay methods for assessment of functional phytochemicals and zoochemicals

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Objectives: Advanced Glycation End-products (AGEs) play important roles in the development of chronic diseases and aging processes. A number of natural and synthetic compounds have been proposed as inhibitors against the generation of AGEs. Recently, much attention has been focused on the benefit of phytochemicals with anti-glycation properties. The aim of this study is to evaluate inhibitory effects of phytochemicals and zoochemicals on the formation of AGEs, we developed the following 7 types of anti-glycation assay methods.

Protein	Reducing sugars	Detection
1. Skin Collagen-gel	Glucose or Fructose	Fluorescence
2. Skin Collagen-gel	Glyceraldehyde	Fluorescence
3. Bone Collagen-gel	Glyceraldehyde	Fluorescence
4. Type II Collagen-gel	Glyceraldehyde	Fluorescence
5. Elastin Solution	Glyceraldehyde	Fluorescence
6. BSA Solution	Glyceraldehyde	Fluorescence
7. Coated-BSA	Glyceraldehyde or Glyoxal	ELISA using re-sRAGE

(BSA, bovine serum albumin; RAGE, Receptor for AGEs; re-sRAGE, recombinant soluble RAGE)

Methods: Phytochemicals or zoochemicals were dissolved in phosphate-buffered saline (PBS), and evaluated for their anti-glycation activity in the following three systems. (1) Glycation of collagen-gel with glucose or fructose; 50 μL of 0.5% collagen-gel were formed in a 96-well plate (with clear black bottom), 40 μL of 10-100 μg/mL of samples were added to the plate wells, followed by the addition of 10 μL of 200 mM glucose or 200 mM fructose in PBS. The plate was then incubated at 37°C for 4 weeks. After incubation, the fluorescence (emission and excitation wavelength at 440 and 370 nm, respectively) was measured in a fluorescence plate reader. (2) Glycation of collagen-gel with glyceraldehyde; Instead of glucose or fructose, 500 mM glyceraldehydes was used and the plate was incubated at 37°C for 2 days. (3) Glycation of bone-collagen-gel with glyceraldehyde; 500 mM glyceraldehyde was used and the plate was incubated at 37°C for 1 day. (4) Glycation of Type II-collagen-gel with glyceraldehyde; 500 mM glyceraldehyde was used and the plate was incubated at 37°C for 1 day. (5) Glycation of elastin solution with glyceraldehyde; 500 mM glyceraldehyde was used and the plate was incubated at 37°C for 1 day. (6) Glycation of BSA with glyceraldehyde; 500 mM glyceraldehyde was used and the plate was incubated at 37°C for 1 day. (7) A 96-well plate was coated with 10 μg/mL of BSA solution and samples were added to the wells of the plate, then the plate was treated with glyceraldehyde or glyoxal for glycation. Detection of AGEs was performed by ELISA using re-sRAGE.

Results: (1): Crude extracts from *Houttuynia cordata*, and asparagus inhibited these glycation in a dose-dependent manner. Gallic acid and fucoxanthin also inhibited the glycation of collagen in a concentration-dependent manner. (2): Glyceraldehyde significantly accelerated the reaction rate. Results could be obtained in only 1 day. (3) and (4): Bone type I and cartilage type II collagen gels were also glycated by the addition of glyceraldehyde, but the fluorescence intensity were difference. Aminoguanidine inhibited these reactions in a concentration-dependent manner. (5) and (6): Elastin in the vessel wall and skin tissue is also glycated by the addition of glyceraldehyde. Polyphenols derived from Aronia, Haskap, Bilberry inhibited these reactions in a concentration-dependent manner. (7): AGEs were detected by ELISA using re-sRAGE in BSA, which had been glycated with glyceraldehyde or glyoxal. Lysine and arginine inhibited these reactions in a concentration-dependent manner.

Conclusion: Our study demonstrated that 7 newly developed assay methods are useful for wide-ranging evaluation of anti-glycation activity of phytochemicals and zoochemicals. Especially, it is notable that the inhibition of the formation of RAGE-reactive-AGEs by some phytochemicals could be evaluated, because the binding of AGEs to RAGE is known to deteriorate various cell functions and has been implicated in the pathogenesis of diabetic vascular complications by reducing sugars.

Acknowledgements: Supported by SIP Project ID 14533567.