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Original article

Anti- glycative effect of Japanese sake (seishu): Prevention of advanced glycation end product (AGE) formation.

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Abstract

***Purpose*:** This study aimed as examining the use of anti-glycation to inhibit the formation of fluorescent advanced glycation end products (AGEs) and glycation intermediates and to clarify the functionality of Japanese sake (seishu), as an anti-glycative food material.

***Methods*:** The anti-glycative effect of inhibiting the AGE and intermediate formation was evaluated by a glycation model (Glucose/HSA) reaction system, which was made from the reaction of glucose and human serum albumin (HSA). The fluorescent AGE amount was measured with the condition of excitation wavelength; 370 nm and detection wavelength; 440 nm. Then, 3-deoxyglucosone (3DG), glyoxal (GO) and methylglyoxal (MGO) were then measured by HPLC in the reaction solution of Glucose / HSA using the HPLC.

***Results*:** Japanese sake (seishu) significantly inhibited the formation of fluorescent AGEs, GO and MGO in the reaction solution of Glucose / HSA. However, Japanese sake did not inhibit the formation of 3DG.

***Conclusion*:** It was observed that Japanese sake (seishu) had an anti-glycative effect in the Glucose/HSA reaction system to inhibit the formation of fluorescent AGEs and the formation of GO and MGO, which were glycation intermediates.

***KEY WORDS*:** Glycative stress, advanced glycation end products, glycation intermediates and Japanese sake (seishu).

Introduction

Glycation is also called the Maillard reaction, which is a chemical reaction between amino acids and reducing sugars. This reaction is non-enzymatic and irreversible. While the glycation *in vivo* reaction progresses, Amadori compounds are produced. Amadori compounds undergoe dehydration and hydrolysis. Through carbon bond cleavage, dicarbonyl compounds, one of the glycation intermediates, is produced. Glycation intermediates and amadori compounds go through many reaction pathways, oxidation, dehydration, and condensation. Finally, advanced glycation end products are formed. As for the advanced glycation end product (AGE) production pathways, many pathways of carbonyl compounds are known [1,2]: glyoxal (GO), methylglyoxal (MGO) and 3-deoxyglucosone (3DG). It is reported that the production and accumulation of AGEs *in vivo* alter the structures and functions of proteins. This could trigger lifestyle-related diseases and symptoms of aging [1,2]. Therefore, this stress on *in vivo* tissue is called glycative stress [3]; as AGEs are produced through glucose, stress is placed on organisms. Countermeasures could include the inhibition of hyperglycemia, the inhibition of the formation of AGEs and the acceleration of the decomposition and excretion of AGEs.

The function of food in regulating the human organism is regarded as the third function, being followed by the functions of energy source and food preference. The food that strongly exhibits the third function is called functional food. It has been reported [4] that Japanese sake (seishu) has multiple effects of the third function type including the prevention of high blood pressure and osteoporosis. Constituents of Japanese sake that contribute to this functionality have already been identified and the mechanism of the action has been clarified [5]. In recent years, diversified functional food materials (anti-glycative food materials) have been developed for glycative stress management [6-10]. It is inferred from this background that Japanese sake (seishu), as a fermented food, has the possibility of inhibiting the production of AGEs [11].

Japanese sake (seishu) is classified by the laws and regulations of the National Tax Agency of Japan: they are defined mainly by brewing process [12]. The three main sorting

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principals are: whether distilled alcohol is added, whether they are brewed in the way of ginjo-zukuri and the rice-polishing ratio. Ginjo-shu is a type of sake brewed at low temperature, over a long time, from milled white rice and has a fragrance specific to itself. Junmai-ginjo-shu is a type of sake in which distilled alcohol is not added. Honjozo-shu is a type of sake in which distilled alcohol is added. These two have differences in their respective brewing processes. Furthermore, how many times hiire, pasteurization or heat sterilization are performed in the brewing process is different depending on the type of sake.

This study examined the functions of Japanese sake, as an anti-glycation food material, for inhibiting the formation of fluorescent AGEs, and glycation intermediates, 3DG, GO and MGO formation.

Methods

Materials

Subjects were thirty kinds of seishu, Japanese sake (twenty Junmai-ginjo-shu and ten Honjozo-shu, ***Table1***). In the Junmai-ginjo-shu, the employed materials were only rice, koji rice (malted rice) and water, and the rice-polishing ratio was below sixty percent. In Honjozo-shu, the employed materials were rice, koji rice (malted rice), water and distilled alcohol, and the rice-polishing ratio was below seventy percent. Two conditions had been decided beforehand: to purchase Japanese sake of the subject materials for a thorough evaluation of the species differences in ginjo-zukuri and the addition of distilled alcohol, excluding other factors:

1) Hiire pasteurization, was performed twice during the manufacturing process.

2) Nihonshu-do, the Sake Meter Value, was between +0 ~ 7 to eliminate considerable differences in sweetness. Nihonshu-do is also an indicator of sake sweetness, using specific gravity: a Nihonshu-do of zero is specific gravity zero and lighter than zero is designated + (plus). The thirty kinds of sake, subject materials of this study, were assigned identity number ID, S1-S30.

Reaction model

As previously reported [13], this study used a Glucose/HSA reaction model system and evaluated its effects; combining 0.2 mol/L glucose, 8 mg/mL human serum albumin (HSA) and 25 mmol/L phosphate buffer solution (PBS) with pH 7.4 and then incubating at 60 °C for forty hours. Sake subject material was then added; the volume concentration of sake was 1/10.

The effect of ethanol was examined in the Glucose/HSA reaction model system. The final concentration of ethanol was adjusted; 0%, 1%, 3%, 5%, 8%, 9.95%. For the reaction

Table 1. Japanese sake (seishu) sample profile.

ID	Class	Product name	Brewery	Location	Cont (%)	SMV	RPR (%)
S1	J	Kyo no Tokuri	Kizakura	Kyoto • Fushimi	14	+1	60
S2	J	Kyo no Shizuku	Kizakura	Kyoto • Fushimi	16	+2	60
S3	H	Hontsukuri Kappa Cup	Kizakura	Kyoto • Fushimi	16	0	65
S4	H	Rikugen	Higashiyama Shuzo	Kyoto • Fushimi	16	+2	65
S5	J	Ninteki	Higashiyama Shuzo	Kyoto • Fushimi	16	+2	50
S6	J	Miyakotsuru	Miyakotsuru Shuzo	Kyoto • Fushimi	15	+3	60
S7	J	Hana no Izanai	Shoutoku Shuzo	Kyoto • Fushimi	12	+3	60
S8	J	Tamanohikari Yamahai Shikomi	Tamanohikari Shuzo	Kyoto • Fushimi	16	+1	60
S9	J	Tamanohikari Iwai	Tamanohikari Shuzo	Kyoto • Fushimi	16.4	+2	60
S10	H	Tomio	Kitagawa Honke	Kyoto • Fushimi	15	+7	68
S11	J	Tomio Tanshu Yamadanishiki	Kitagawa Honke	Kyoto • Fushimi	15	+1	55
S12	J	Tomio Gion Komachi	Kitagawa Honke	Kyoto • Fushimi	15	+3	58
S13	H	Nouvelle Gekkeikan	Gekkeikan	Kyoto • Fushimi	15	+3	60
S14	J	Momo no Shizuku	Matsumoto Shuzo	Kyoto • Fushimi	15	+5	58
S15	J	Eikun Koto Sennen	Saito Shuzo	Kyoto • Fushimi	15	+3	55
S16	J	Kinshi Masamune	Kinshi Masamune	Kyoto • Fushimi	15	+3	55
S17	H	Marutake Ebisu	Sasaki Shuzo	Kyoto • Rakuchu	15	+4	65
S18	J	Jurakudai	Sasaki Shuzo	Kyoto • Rakuchu	15	+4	60
S19	H	Tokusen Gekkeikan	Gekkeikan	Kyoto • Fushimi	16	+2 ~ 4	65
S20	J	Sumiya Yahei	Tsuji Honten	Okayama • Bichu	15	+4	55
S21	J	Kyo no Hana	Tatsu Izumi Shuzo	Fukushima • Aizu Wakamastu	15	+2	60
S22	J	Rinzen	Tamanohikari Shuzo	Kyoto • Fushimi	16	+3	60
S23	J	Nechi Otokoyama	Watanabe Shuzo	Niigata • Nechi	15	+1	55
S24	J	Karaku Kyo no Koto	Shoutou Shuzo	Kyoto • Fushimi	15	+3	60
S25	J	Yuki no Bosha	Saiya Shuzouten	Akita • Yuri Honjo	16	0	55
S26	H	Kikuyoi	Aoshima Shuzo	Shizuoka • Fujieda	15	+4	57
S27	H	Hakkaisan	Hakkaisan	Niigata • Uonuma	15	+4	55
S28	H	Tamagawa	Kinoshita Shuzo	Kyoto • Tango	15	+1.5	65
S29	H	Shimeharitsuru Tsuki	Miyao Shuzo	Niigata • Murakami	15	+4	55
S30	J	Tenmei	Akebono Shuzo	Fukushima • Aizu Wakamatsu	16	+4	55

Cont, alcohol content (%); SMV, Sake Meter Value (Nihonshu-do); RPR, rice polishing ratio (%); J, Junmai-ginjo; H, Honjozo.

model without glucose, an equivalent quantity of distilled water was added instead.

Fluorescent AGE measurement

As previously reported, fluorescent AGEs was measured [14], using a Glucose/HSA reaction system; combining 0.1 mol/L PBS 500 μL (pH 7.4), distilled water 100 μL, 40 mg/mL HSA (Sigma Chemical Co. Ltd, St. Louis, MO, USA) 200 μL, 2.0 mol/L glucose water solution 100 μL. Sake subject material 100 μL, to which distilled water was added, at 1/10 volume concentration, and was diluted to 1.0 mL as the total amount. The solution was incubated at 60 °C for forty hours. Reference or Ref was produced and reacted; the same amount of distilled water as the material was added and then wit as incubated at 60 °C for forty hours. The fluorescent AGEs of each reaction solution were measured by Microplate Reader Varioscan® Flash (Thermo Scientific, Waltham, MA); measuring a fluorescence intensity with Ex, Excitation wavelength of 370 nm and an Em, Emission wavelength of 440 nm. The fluorescence intensity of the sulfuric acid aqueous solution, in the case of 5 μg/mL quinine sulfate 0.1N sulfuric acid aqueous solution, was designated 1,000. The formation rate percentage of fluorescent AGEs was calculated as a relative rate where the fluorescence intensity value of Ref was one hundred percent.

Quantitative analysis of 3DG, GO and MGO

As previously reported, 3DG, GO and MGO were measured by high performance liquid chromatography (HPLC) [13]. The sample was prepared from materials for a measurement of 200 μL, distilled water 300 μL and 20 mg/mL 2, 3-pentanedione 25 μL as the internal standard substance. They were then combined, stirred and mixed. After adding 500 μL of 6.0% perchloric acid and mixing them, the sample was centrifuged at 12,000 rpm (13,200 g) for ten minutes. After centrifugation, 800 μL of supernatant liquid was dispensed and 1,000 μL of saturated sodium hydrogen carbonate solution was added and mixed. As a labeling reagent, 1.0 mg/mL 2, 3-diaminonaphthalene (Dojindo Laboratories, Kumamoto, Japan) 100 μL of 50% methanol solution was added, mixed and was then incubated at room temperature for twenty four hours and left to rest. 3DG, GO and MGO in the supernatant were measured by HPLC under the following analysis condition: the amount of injection: 20 μL, column to use: Imtakt Unison UK-Phenyl (3 mm × 75 mm I.D), eluent 50mmol/L, phosphoric acid : acetonitrile = 89 : 11, current flow rate: 1.0 mL/min, column temperature: 40 °C, and Emission wavelength: 268 nm.

The quantity of 3DG, GO and MGO (μg/mg HSA) per 1 mg of reaction solution HSA were calculated from the concentration which was calculated based on quantitative analysis. Each generation rate was indicated as a relative value when the Ref value was designated at one hundred percent.

Statistical analysis

Statistical analysis was performed three times. The results were indicated as the mean value ± the standard deviation. The comparison between the mean value of the thirty kinds of seishu, Japanese sake, and Ref, the comparison between Junmai-ginjo-shu and Honjozo-shu and the comparison between Japanese sakes were all evaluated by a student's t-test. The comparison between each seishu and Ref were evaluated using Dunnett's test, a multiple comparison test.

Results

The formation of fluorescent AGEs and the influence of ethanol in glucose/HSA

In adding ethanol to the Glucose/HSA reaction system, the formation of Fluorescent AGEs increased in a store-dependent manner (***Fig. 1***). In the reaction of ethanol and HSA without glucose, the formation of AGEs was not recognized.

The inhibitory effect of Japanese sake on Fluorescent AGE formation

Fig. 2 shows the production rate of fluorescent AGEs in the case of adding seishu, Japanese sake, to the Glucose / HSA reaction system. The production rate of fluorescent AGEs for each of the thirty kinds of seishu, Japanese sake, was significantly lower than the production rate of Ref ($p < 0.01$, ***Fig. 2-A***). The average production rate of fluorescent AGEs for the thirty kinds of seishu, Japanese sake was significantly lower than the production rate of Ref ($p < 0.01$, ***Fig. 2-B***). Between Junmai-ginjo-shu and Honjozo-shu, no significant difference in the production rate was recognized (***Fig. 2-C***). Between the twenty kinds of Junmai-ginjo-shu and Ref, the lowest production rate of fluorescent AGEs was shown by S7. S7 exhibited a significantly lower production rate of fluorescent AGEs than S15 ($p < 0.01$, ***Fig. 2-D***).

The inhibitory effect of Japanese sake on the formation of glycation intermediates (3DG, GO and MGO)

Fig. 3 shows the 3DG production rate in the case of adding seishu, Japanese sake, to the Glucose / HSA reaction system. There were no significant differences recognized in the AGE production rate between all the subject materials of the thirty kinds of Japanese sake, and Ref (***Fig. 3-A***). There was no significant difference recognized between Junmai-ginjo and Honjozo in AGE production rate (***Fig. 3-B***). There were no significant differences recognized between S7 and S15 in AGE production rate (***Fig. 3-C***).

Fig. 4 shows the GO production rate in the case of adding seishu, Japanese sake, to the Glucose / HSA reaction system. The average GO production rate for the thirty kinds of seishu, Japanese sake, was significantly lower than that of Ref ($p < 0.01$, ***Fig. 4-A***). There was no significant difference recognized between Junmai-ginjo and Honjozo in GO production rate (***Fig. 4-B***). S7 showed a significantly lower rate than S15 ($p < 0.01$, ***Fig. 4-C***).

Fig. 5 shows the MGO production rate in the case of adding seishu, Japanese sake, to the Glucose / HSA reaction system. The average MGO production rate for the thirty kinds of seishu, Japanese sake, was significantly lower than that of Ref ($p < 0.01$, ***Fig. 5-A***). There was no significant difference recognized between Junmai-ginjo and Honjozo in MGO production rate (***Fig. 5-B***). S7 showed a significantly lower rate than S15 ($p < 0.01$, ***Fig. 5-C***).

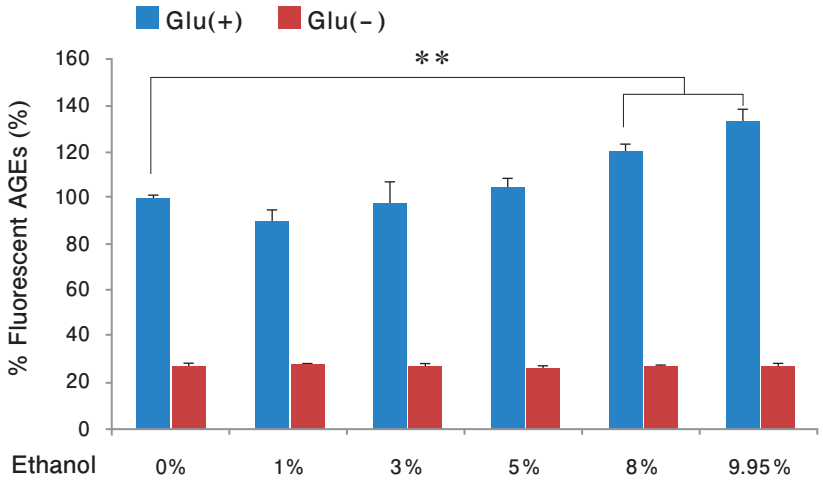

Fig. 1. **Effect of ethanol on HSA-derived AGE fluorescence.**

Glu(+): Glucose 0.2 mol/L and HSA 8 mg/mL was incubated at 60°C, for 40 hours. Glu(−): HSA was incubated without glucose. Results are expressed as mean ± SD (n = 3), ** $p < 0.01$, Dunnett test. HSA, human serum albumin; AGE, advanced glycation end product; SD, standard deviation.

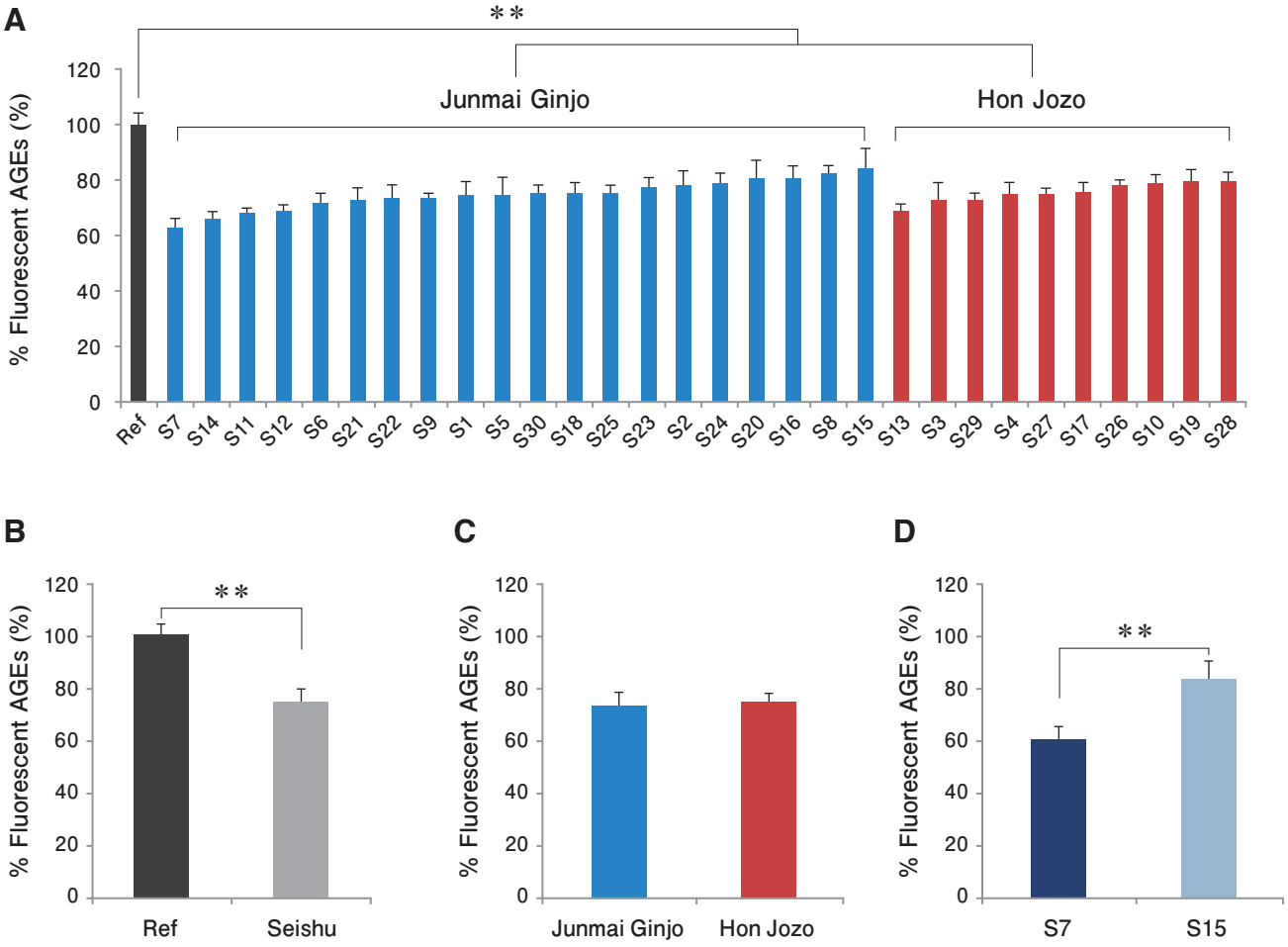

Fig. 2. **Influence of seishu (Japanese sake) on fluorescent AGEs in Glucose/ HSA.**

Glucose 0.2 mol/L and HSA 8 mg/mL was incubated at 60°C, for 40 hours in Glucose/ HSA reference (Ref). A: All the sake samples are significantly different from Ref. ** $p < 0.01$, Dunnett test. B: Average values of 30 kinds of seishu was compared with Ref. ** $p < 0.01$, Students t test. C: Comparison between Junmai-ginjo (20 kinds) and Honjozo (10 kinds). D: Comarison between S7 and S15. ** $p < 0.01$, Dunnett test, Junmai-ginjo, Japanese sake which used higher polished rice only; Honjozo, sake brewed without addition of saccharides. Results are expressed as mean ± SD (n = 3). HSA, human serum albumin; AGEs, advanced glycation end products; SD, standard deviation.

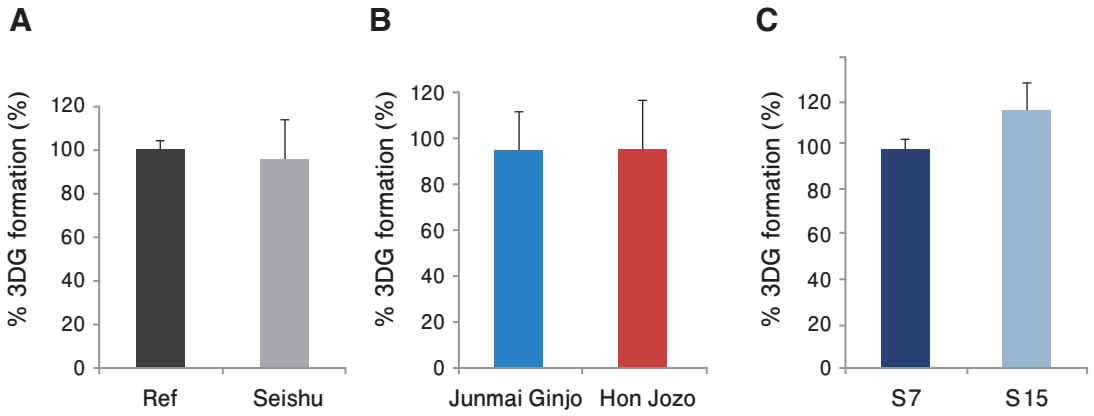

Fig. 3. **Influence of seishu (Japanese sake) on 3DG formation in Glucose/HSA.**

A: Comparion between Ref and average of seishu (30 kinds). B: Comparison between Junmai-ginjo (20 kinds) and Honjozo (10 kinds). C: Comarison between S7 and S15. Results are expressed as mean ± SD (n = 3). Ref, glucose 0.2 mol/L and HSA 8 mg/mL was incubated at 60°C, for 40 hours (Glucose/ HSA); HSA, human serum albumin; 3DG, 3-deoxyglucosone; SD, standard deviation.

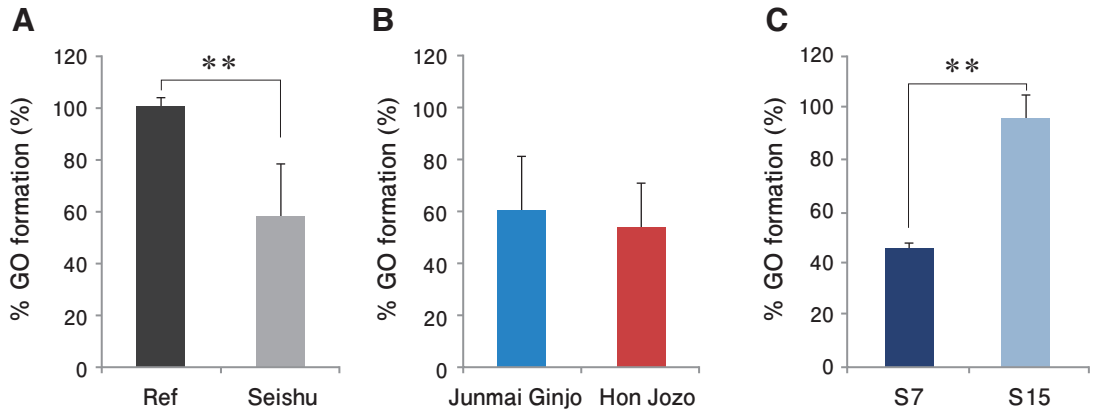

Fig. 4. **Influence of seishu (Japanese sake) on GO formation in Glucose/HSA.**

A: Comparion between Ref and average of seishu (30 kinds). ** $p < 0.01$ by Students t test. B: Comparison between Junmai-ginjo (20 kinds) and Honjozo (10 kinds). C: Comparison between S7 and S15. ** $p < 0.01$ by Dunnett test. Results are expressed as mean ± SD (n = 3). Ref, glucose 0.2 mol/L and HSA 8 mg/mL was incubated at 60°C, for 40 hours (Glucose/ HSA); HSA, human serum albumin; GO, glyoxal; SD, standard deviation.

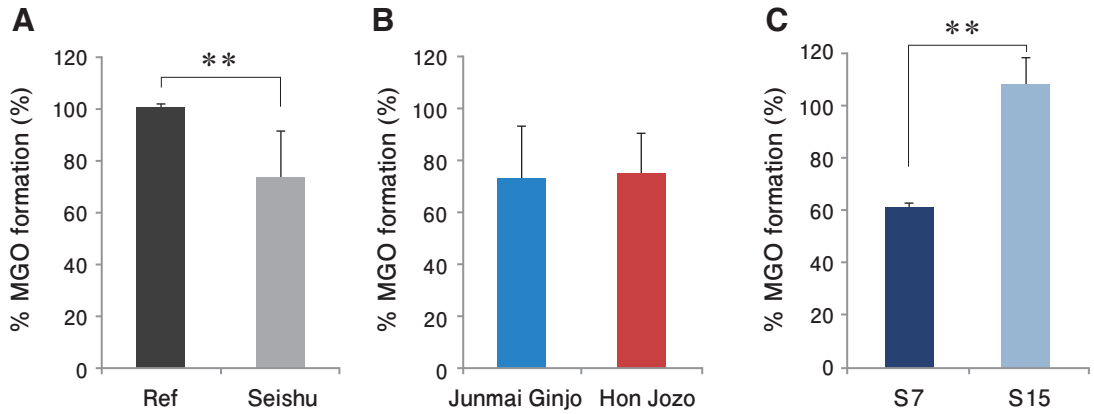

Fig. 5. **Influence of seishu (Japanese sake) on MGO formation in Glucose/HSA.**

A: Comparion between Ref and average of seishu (30 kinds). ** $p < 0.01$ by Students t test. B: Comparison between Junmai-ginjo (20 kinds) and Honjozo (10 kinds). C: Comarison between S7 and S15. ** $p < 0.01$ by Dunnett test. Results are expressed as mean ± SD (n = 3). Ref, glucose 0.2 mol/L and HSA 8 mg/mL was incubated at 60°C, for 40 hours (Glucose/ HSA); HSA, human serum albumin; MGO, methylglyoxal; SD, standard deviation.

Discussion

Evaluation of the influence of ethanol on the Glucose / HSA reaction system

Fig.1 shows the results of adding ethanol to the Glucose / HSA reaction system. The formation rate of fluorescent AGEs showed a significant rise when the ethanol concentration was above eight percent. When the ethanol concentration was high (above eight percent), it was recognized that the formation of fluorescent AGEs was accelerated. On the contrary, when the ethanol concentration was below five percent, there was no rise of the formation rate of fluorescent AGEs. Ordinary Japanese sake contains ethanol at 16 % [15]. The ethanol concentration (reaction concentration) in the Glucose / HSA solution was approximately 1.6 %. It was assumed that ethanol did not influence the formation of AGEs.

Evaluation of the inhibitory effects of Japanese sake on fluorescent AGE formation

It was recognized that seishu, Japanese sake, has inhibitory effects which reduce the formation of fluorescent AGEs. As for fluorescent AGEs which emit light, Pentoshin (Ex 335 nm / Em 385 nm) [16], Crosslin (Ex 379 nm / Em 463 nm) [17], and Pyrropyridine (Ex 370 nm / Em 455 nm) [18] are known. Seishu, Japanese sake, could have inhibitory effects on the formation of Pentoshin, Crosslin, and Pyrropyridine.

The aging, or fermenting period of Junmai-ginjo-shu is from four to five weeks, while Honjozo-shu was added to jozo-alcohol, brewed alcohol, during the three week process of the fermenting period [19-21]. However, in the Japanese sake used for this experiment, there was no significant change in AGE formation nor the length of fermenting period (***Fig. 2-C***) with the addition of jozo-alcohol.

Evaluation of Japanese sake, in terms of its inhibitory effects on the formation of glycation intermediates

It has been suggested that AGEs are formed via five intermediate compounds [2]. This study examined three of the five formation paths: 3DG, GO and MGO [13]. Furthermore, this study inferred concretely the glycation paths that Japanese sake inhibits.

3DG is formed by the dehydration and hydrolysis of amadori compounds. GO is formed by the autoxidation of glucose [22]. MGO is reported to have two formation paths [1]: autoxidation of amadori compounds and the cracking of triose phosphate in glycolysis. The average formation rate of the glycation intermediates, in the cases of the thirty kinds of Japanese sake, showed no significant differences from the formation rates of Ref (shown in ***Fig. 3-A***). The formation rates of GO and MGO were significantly lower than that of Ref ($p < 0.01$, ***Fig. 4-A, 5-A***). It was concluded from these results that seishu, Japanese sake, has no inhibitory effects on 3DG formation. However, seishu, Japanese sake, could have inhibitory effects on GO and MGO formation by autoxidation. This experiment was an *in vitro* experiment, so that the inhibition of MGO formation through the glycolysis system could be excluded. Japanese sake contains ferulic acid, which is a phenolic compound and has antioxidative effects. It was assumed that the ferulic acid [23] and phenolic compounds [24] contained in Japanese sake would be involved in the inhibitory action on GO and MGO formation.

Biological responses to glycation intermediates have been reported widely in the literature. When the concentration of 3DG rises, the risks of diabetic retinopathy and nephropathy also rise [25]. It has been suggested that GO could induce inflammatory damage in cells [26]. It has been reported that MGO-derived AGEs can trigger the apoptosis of cells [27]. This study clarified that seishu, Japanese sake has inhibitory effects on GO and MGO formation. Thus, seishu, or Japanese sake could reduce inflammatory damage to cells caused by GO and apoptosis of cells caused by MGO.

GO reacts with lysine and this reaction forms N^{ε}-carboxymethyllysine (CML), which is one of AGEs [1]. CML is a non-fluorescent AGEs and the formation of CML is accelerated by glycation stress or oxidative stress accentuation [28]. It has been reported that the addition of CML-collagen to human skin fibroblasts would induce apoptosis [29]. Furthermore, it was also reported that glycation causes CML to accumulate in certain layers of the skin and is involved in damage to transparent skin [30]. Seishu, Japanese sake, showed inhibitory effects on GO formation and has the possibility of indirectly reducing CML formation and of inhibiting both apoptosis and skin aging caused by CML.

Conclusion

Japanese sake (seishu) showed, in the Glucose/HSA reaction system, its inhibitory effects on the formation of fluorescent AGEs and the formation of GO and MGO, which are glycation intermediates. Japanese sake (seishu) did not inhibit the formation of 3DG.

Statement of conflict of interest

Non contributory.

Acknowledgments

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