Glycative Stress Research

Online edition : ISSN 2188-3610
Print edition : ISSN 2188-3602
Received : April 13, 2020
Accepted : May 31, 2021
Published online : June 30, 2021
doi:10.24659/gsr.8.2_98

Original article

Evaluation of the effects of mixed herb extract on skin based on anti-glycation effect: A randomized, double-blind, placebo-controlled, parallel-group study

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Abstract

***Purpose*:** One of the measures against glycative stress is a method of promoting the decomposition and metabolism of accumulated advanced glycation end products (AGEs). We screened a large number of plants using indicators such as the action of directly cleaving the AGE crosslinks and the enhancing action for the oxidized protein hydrolase (OPH) activity that is involved in the decomposition of the glycated proteins. As a result, we identified that *Trigonella foenum-graecum* (fenugreek, seeds), *Foeniculum vulgare* (fennel, seeds) and *Hibiscus sabdariffa* (hibiscus, calyxes and bracts) have both actions. In addition, it has been reported that the mixed herb extract (MHE) consisting of these 3 herbs demonstrated various anti-glycation effects and physiological functions in our exploratory clinical trial with two groups, 100 mg/day (low-dose group) and 300 mg/day (high-dose group), in the previous study. Therefore, in this study, we conducted a randomized, placebo-controlled parallel-group study with the same amount of MHE as the low-dose group in the previous study to verify its anti-glycation effect and its effects on the skin.

***Method*:** The subjects were 40 Japanese women (20 in the MHE group, 20 in the placebo group) aged 40 to 64 years who had high amounts of AGEs deposits on the skin (as measured by AGE Reader mu). MHE was used as the test food, which was administered in soft capsules of 100 mg per day for 12 weeks. The subjects underwent blood tests, urine tests, skin-related tests, physical tests, body measurements and medical interviews before administration as well as after 6 and 12 weeks of administration. Note that this study has been conducted upon approval by an ethics review committee. (UMIN Clinical Trial ID: UMIN000037855).

***Results*:** During the study, 4 subjects withdrew and 1 was subject to the trial exclusion criteria, therefore, the number of subjects analyzed was 19 in the MHE group and 16 in the placebo group. A subgroup analysis was performed for pentosidine, the primary outcome, with 10 subjects in the MHE group and 6 subjects in the placebo group who were below the mean value of all the subjects for analysis, excluding 1 subject whose result was judged to be an outlier using the interquartile range. A significant decrease was observed only in the MHE group when comparing the results from pre-administration to after 12 weeks of administration. A significant decrease was also observed in the group compared to the placebo group. A subgroup analysis was conducted for N^{ε}-(carboxymethyl) lysine (CML) with 7 subjects in the MHE group and 5 subjects in the placebo group whose BMI were 22.0 or higher, excluding 1 subject whose result was judged to be an outlier using the interquartile range. A tendency to decrease was observed only in the MHE group when comparing the results from pre-administration to after 12 weeks of administration. In the results of imaging analysis by VISIA for all cases, only the MHE group showed a decrease in the wrinkle parameter after administration compared to the pre-administration levels, and the group also indicated a significant tendency to decrease against the placebo group. In addition, improvement effects were observed in the MHE group in the secondary outcomes of skin texture and brown spots.

***Conclusion*:** Administration of MHE improved glycation indices as well wrinkles, texture and brown spots of the skin. It is considered that the AGE crosslink cleaving activity and the OPH activity enhancing action of the MHE may have contributed to these effects.

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KEY WORDS: mixed herb extract, anti-glycation, advanced glycation end products (AGEs), crosslink cleaving, oxidized protein hydrolase (OPH)

Introduction

In 1912, LC Maillard reported that a brown substance was produced by a non-enzymatic chemical reaction between amino acids and sugars [1]. This browning reaction is called the Maillard reaction, which was named after its discoverer. This reaction had long been studied in the field of food as one of the causes of browning and flavor changes that occur during heating, but it was later found that this reaction also occurs in the living body. Hemoglobin A1c (HbA1c), which is a reaction product of hemoglobin, first received attention as an *in vivo* Maillard reaction and it has been clinically applied as an index of glycemic control. Currently, the Maillard reaction that occurs in the living body is called glycation, and the substances that are finally produced by the progress of the glycation reaction are called advanced glycation end products (AGEs). Thus far, dozens of structures have been reported [2].

Accumulation of AGEs and glycation reaction intermediates due to aging and metabolic disorders is thought to play a part in various diseases such as diabetic complications and arteriosclerosis [3, 4]. The stress on the living body caused by these AGEs and their reaction intermediates is called "glycative stress" and is positioned as one of the risk factors for aging [5]. The measures against glycative stress include inhibition of postprandial hyperglycemia, inhibition of glycation reaction and the decomposition and excretion of generated AGEs. Anti-glycation materials for these measures have been researched and developed in recent years. Most of the conventional anti-glycation materials are inhibitors of the production of AGEs. They have been identified in plant extracts (extract mixture of dokudami, hawthorn berries, Roman chamomile, and grape leaves) [6] and mangosteen [7], etc. On the other hand, actions such as decomposing AGEs and cleaving AGE crosslinks have been confirmed in pomegranates [8] and water chestnuts [9]. In addition, it has been reported that plant extracts [10] have the effect of enhancing the activity of OPH (oxidized protein hydrolase), which is an enzyme involved in the decomposition of proteins that were modified by glycation. There are few reports on anti-glycation materials that focus on these effects (cleavage and the promotion of metabolism of AGE crosslinks) and even fewer reports have been made on materials that have both effects.

Focusing on the decomposition of AGEs, we screened a large number of plants using such indicators as the action of cleaving AGE crosslinks and the enhancing action of OPH activity. As a result, we identified *Trigonella foenum-graecum* (fenugreek, seeds), *Foeniculum vulgare* (fennel, seeds) and *Hibiscus sabdariffa* (hibiscus, calyxes and bracts) as plants that demonstrated high effectiveness in both effects [11]. In addition, an exploratory clinical trial was conducted where these mixed herb extracts (MHE) consisting of fenugreek, fennel, and hibiscus were administered to 2 groups: a low-dose group with a daily intake of 100 mg and a high-dose group with a daily intake of 300 mg [12]. In the study, MHE was continuously administered for 12 weeks to women 40 to 64 years of age with high skin AGEs deposit levels. As a result, a significant decrease of pentosidine, a type of AGEs, was observed in both the low-dose and the high-dose groups compared to the pre-administration levels. Furthermore, the glucose metabolism index (fasting blood sugar) improved in both groups, and the liver function indices (AST, ALT, γ-GTP) were also improved only in the high-dose group.

Therefore, this study was designed as a randomized controlled trial and conducted to examine the details of the effects of MHE ingestion, which reduces glycative stress. The subjects were administered a daily intake of 100 mg of MHE for 12 weeks and underwent examinations on anti-glycation effects as well as various skin tests, and various clinical tests.

Method

Subjects

Eighty Japanese women 40 to 64 years of age, who were publicly recruited by TTC Co., Ltd. to be paid volunteers, were screened to select the subjects of this study, where a randomized controlled trial was conducted. Screening tests were performed on the applicants. Among the applicants who did not meet the exclusion criteria and satisfied the inclusion criteria, the top 40 people with high levels of skin AGEs deposits (measured by AGE Reader mu) were selected. Thirty-two exclusion criteria were established, including: 1) those who have a smoking habit, 2) those who have a habit of drinking alcohol 4 times or more a week, 3) those who sleep less than 5 hours on average and 4) who regularly use anti-glycation foods and anti-glycation cosmetics. In addition, during the trial period, 11 restrictions that the subjects had to comply were established, including: restrictions on excessive exercise, dieting or overeating that greatly deviates from their daily life, keeping lifestyle habits such as sleeping and bathing consistent as much as possible, and maintaining their regular daily life.

Test Food

There were two types of test foods; the MHE food that contained a mixed herb extract (product name: Satonaceil, Karada Lab, Inc., Kamigyo-ku, Kyoto, Japan) and placebo foods. These were formed as color-coated soft capsules so that they could not be distinguished by flavor, color and other features. In addition, the soft capsule was designed to contain 100 mg of MHE in one capsule, and the daily intake was specified as one soft capsule. The test food composition and nutritional constituents are listed in ***Table 1*** and ***Table 2***, respectively.

Table 1. Test food composition (Compounded amount: mg/capsule)

	MHE food	Placebo food
Saflower oil	169	159
MHE (Satonaceil)	100	–
Dextrin	–	100
Gelatin	113	113
Glycerine	45	45
Beeswax	14	14
Glycerine fatty acid ester	14	15
Caramel color	7	10
Soybean lecithin	3	3
Edible fat and oil	–	6
Total	465	465

MHE (mixed herb extract).

Table 2. Test food nutritional constituents (Administration amount per day: per capsule)

		MHE food	Placebo food
Energy	(kcal)	2.78	2.78
Protein	(g)	0.11	0.10
Lipid	(g)	0.20	0.20
Carbohydrate	(g)	0.12	0.15
Sodium	(mg)	0.36	0.03

MHE (mixed herb extract).

Trial Design

The clinical trial was designed as a randomized, double-blind, placebo-controlled, parallel-group study. The group allocator created an allocation table using random numbers, and randomly allocated the selected 40 people into two groups of 20 people each. The allocation table was sealed by the group allocator and kept sealed and stored until it was time to open the allocation table. The subjects, as a general rule, ingested an MHE capsule or a placebo capsule once a day over 12 weeks on an empty stomach with water or lukewarm water without chewing. Blood tests/urine tests, skin-related tests, physical tests, body measurements and medical interviews were conducted prior to administration (week 0) and after 6 and 12 weeks of administration for a total of 3 times.

The subjects took a bath the day before the tests and went to bed by 12:00 am. Also, taking a shower or bath was prohibited on the day of testing. In addition, the subjects fasted, except for water, on the day prior to testing, from 10:00 pm until the end of testing on the following day. The time of their visit was set to the same time for every visit so that the time of the test would be the same every time (within ± 2 hours).

The trial period was from July 2019 to January 2020. Skin-related tests except glycation indices, as well as blood pressure measurements, blood drawings, etc. were conducted at DRC Co., Ltd. and Ikiiki Clinic, and skin-related glycation indices were tested by AGE Laboratories Co., Ltd. (Shimogyo-ku, Kyoto).

Endpoints

As the primary outcomes, we measured the glycation indices including skin AGEs deposit levels, internal body AGEs levels and blood testing (pentosidine, N^{ε}-(carboxymethyl) lysine (CML), 3-deoxyglucosone (3DG), glyoxal (GO), and methylglyoxal (MGO)). In addition, skin imaging analysis (wrinkles) by VISIA, insulin resistance index (HOMA-IR), and blood tests for liver functions (AST (GOT) and ALT (GPT)) were performed.

The secondary outcomes were: skin viscoelasticity, skin color difference, skin imaging analysis using VISIA, blood tests on glucose metabolism and liver functions (fasting blood sugar, HbA1c, insulin and γ-GTP) and medical interviews (Anti-Aging QOL Common Questionnaire).

In addition, various clinical tests, body measurements, and physical tests were performed to evaluate the safety of the product. Clinical tests were performed for the following various parameters: total bilirubin, direct bilirubin, indirect bilirubin, ALP, LDH, high-sensitivity CRP, total protein, albumin, A/G ratio, creatinine, urea nitrogen, uric acid, triglyceride, free fatty acids, total cholesterol, LDL cholesterol, HDL cholesterol, non-HDL cholesterol, atherogenic index, Na, K, Cl, Ca, Fe and blood cell count (leukocyte count, erythrocyte count, hemoglobin amount, hematocrit value, platelet count, erythrocyte index, hemogram) as well as general urine tests (specific gravity, pH, urobilinogen, bilirubin, ketones, proteins, sugars, occult blood, leukocyte esterase, nitrite). For the body measurements and physical tests, height (first time only), weight, body mass index (BMI), body fat percentage, abdominal circumference, hip circumference, waist-hip ratio, blood pressure, and pulse rate were measured.

The subjects recorded the ingestion status of the test food, any adverse events, and any changes to lifestyle (diet, exercise, alcohol intake, etc.) in their journals.

Testing Method

Subjective Symptoms (Questionnaire Items)

The Anti-Aging QOL Common Questionnaire [13] was used for an evaluation of subjective symptoms. They were divided into 33 items for "physical symptoms" and 21 items for "mental symptoms." This evaluation was made on a five-point scale with the best condition being rated as 1 point and the worst condition being rated as 5 points.

Body Measurements and Physical Testing

In addition to height, body weight, blood pressure, and pulse rate, body fat percentage was measured via the bioelectrical impedance analysis, using Inbody 720 (Inbody Japan Inc., Koto-ku, Tokyo, Japan), a body composition analyzer.

Blood Testing

Among blood test parameters, glycation indices such as pentosidine, CML, 3DG, GO, and MGO were measured at AGE Laboratories Co., Ltd. under the supervision of the Glycative Stress Research Center, Graduate School of Life and Medical Sciences, Doshisha University while other parameters were measured at LSI Medience Corporation (Chiyoda-ku, Tokyo).

Skin-related Testing

The skin-related tests were conducted after washing the measurement areas of the skin. The skin AGEs deposit levels and internal body AGEs levels were measured in a constant temperature and humidity chamber (20 to 22°C, 50 ± 5%). Then, after acclimation for 20 minutes, images were captured by VISIA, and skin viscoelasticity and color difference were measured in the same constant temperature and humidity chamber.

1) Skin AGEs deposit levels

The skin AGEs deposit levels were measured using AGE Reader mu (Diagnoptics Technologies B.V., Groningen, Netherlands). The area for measurement was the inner side of the forearm (about 10 cm from the olecranon toward the forearm).

2) Internal body AGEs levels

An AGEs sensor (Air Water Biodesign Inc., Kobe, Hyogo, Japan) was used to measure AGEs in the body. The area for measurement was the middle finger of the left hand.

3) Skin viscoelasticity

Skin viscoelasticity was measured using Cutometer MPA580 (Courage + Khazaka electronic GmbH, Cologne, Germany), a skin viscoelasticity measurement device. The measurement area was the inner side of the upper arm (about 10 cm from the olecranon toward the upper arm).

4) Skin color difference

The skin color difference was measured using a CM-2600d, a spectrophotometer, and a CM-SA (Konica Minolta, Inc., Minato-ku, Tokyo, Japan), a skin analysis software package. The areas for measurement were the cheek and the inner side of the upper arm.

5) Imaging analysis of the facial skin

Imaging analysis of the facial skin was conducted by using the VISIA Evolution (Canfield Imaging Systems, Inc., NJ, USA).

Statistical Analysis

The analysis was performed using the IBM SPSS Statistics 23 (IBM Japan, Ltd., Chuo-ku, Tokyo). The results are expressed as a mean value ± standard deviation for the subject background factors and safety endpoints and as a mean ± standard error of the mean for others. The test methods for the measurement items are as follows: Dunnett's multiple comparison test was used if the measurements were conducted 3 times pre-administration as well as 6 and 12 weeks post-administration, and if the measurements were conducted twice, a paired t-test was used to compare the results from each post-administration point to pre-administration test value. For intergroup comparisons, a two-sample t-test was used. The test methods of the questionnaire items are as follows: if the measurements were conducted 3 times pre-administration as well as 6 and 12 weeks post-administration, the result of Wilcoxon signed-rank sum test was adjusted with the Holm method and the score at each post-administration point was compared with those from pre-examination. The Mann-Whitney U test was used for intergroup comparisons. The significance level was considered to be significant at a risk rate of less than 5% and marginally significant at less than 10% for all two-tailed tests.

Ethical Standards

The study complied with the ethical principles set forth in the Helsinki Declaration and Japan's Personal Information Protection Act, and was conducted in accordance with the Ethical Guidelines for Medical Research Involving Human Subjects (public notice of Ministry of Education, Culture, Sports, Science and Technology/Ministry of Health, Labor and Welfare) and the guidance for the Ethical Guidelines for Medical Research Involving Human Subjects (Ministry of Education, Culture, Sports, Science and Technology, Ministry of Health, Labor and Welfare). The documents to be examined were submitted to the Aisei Hospital Ueno Clinic Research Ethics Committee (Taito-ku, Tokyo) in advance, and were examined and approved on July 25, 2019. Informed consent was obtained from trial subjects prior to screening tests, and the subjects sufficiently understood the content of the testing plan and voluntarily expressed their willingness to participate in the test by submitting the consent form. The outline of this study was registered in the UMIN Clinical Trials Registry system (UMIN-CTR) (UMIN Clinical Trial ID: UMIN000037855).

Results

Target Group for Analysis

Figure 1 shows the tracking flow diagram for trial subjects. Subject recruitment began in July 2019. Forty subjects, who had been selected from the screening tests held on September 4th and 5th, were randomly allocated to the MHE group of 20 subjects and to the placebo group of 20 subjects and were now in this study. Later, one participant voluntarily withdrew from the study to undergo surgery and long-term physical rehabilitation due to an injury caused by a fall, and another participant withdraw due to pregnancy. In addition to the above, another participant withdrew from the study to start treatment for suspected glaucoma during the study period, and another participant withdrew due to a work-related reason. Excluding the above four withdrawals, one more subject was excluded from the analysis because her intake rate of the test food was less than 80%, in addition to her noncompliance with the restrictions. Therefore, a total of 35 subjects, 19 in the MHE group and 16 in the placebo group, were included in the analysis. The backgrounds of these subjects are shown in ***Table 3***.

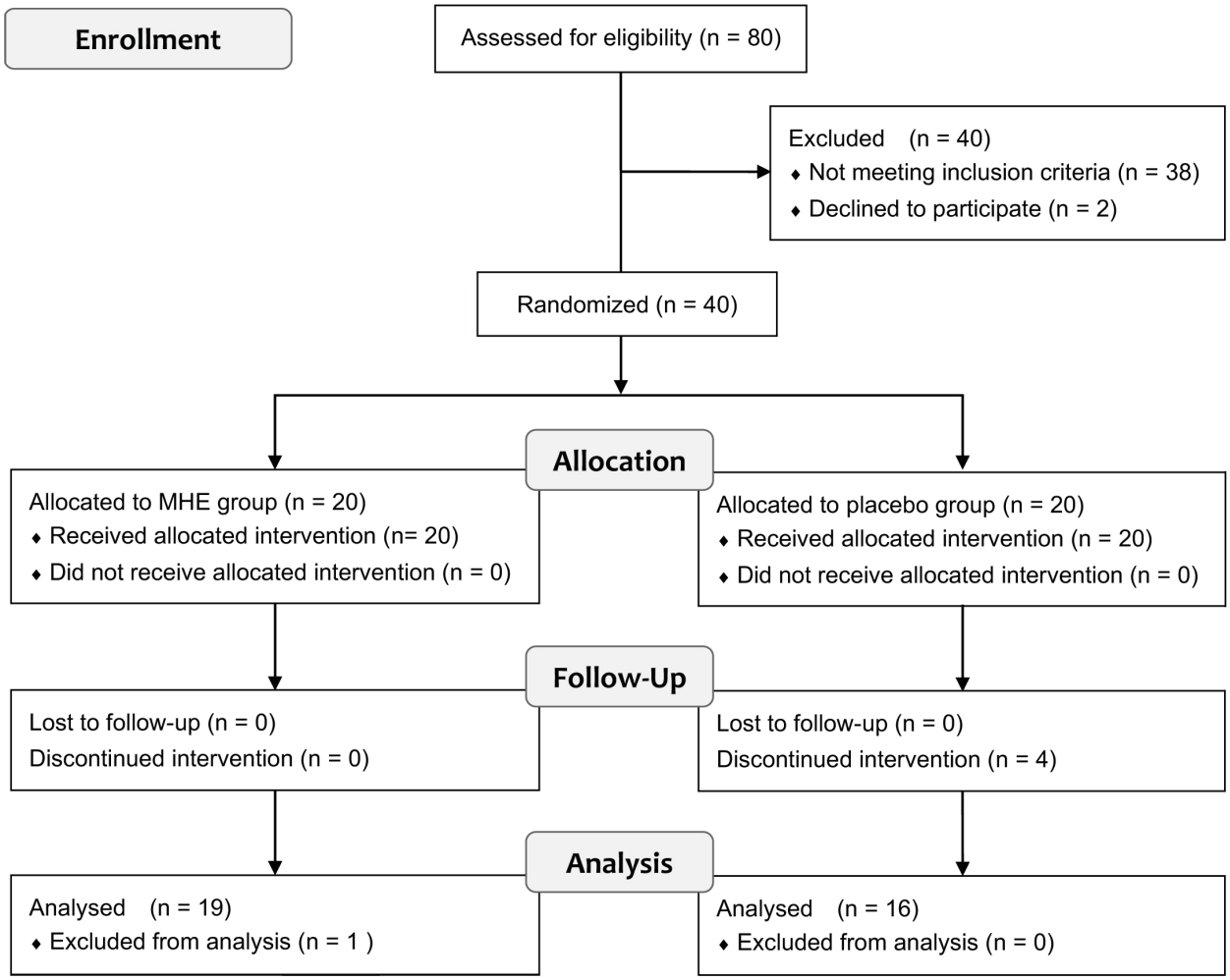

Fig. 1. **Tracking flow diagram for trial subjects.**
MHE (mixed herb extract).

Table 3. Baseline demographic and clinical characteristics

	Unit	All subjects	MHE group	Placebo group	p-value
Target size		35	19	16	(t-test)
Age	years of age	50.6 ± 6.5	50.1 ± 6.2	51.3 ± 7.0	0.577
Height	cm	157.6 ± 5.1	157.5 ± 5.4	157.7 ± 4.8	0.893
Weight	kg	53.7 ± 9.6	54.7 ± 9.7	52.5 ± 9.5	0.491
Body fat	%	29.4 ± 6.7	29.7 ± 6.9	29.1 ± 6.5	0.815
BMI	kg/m^2	21.6 ± 3.3	22.0 ± 3.5	21.0 ± 3.0	0.371
Skin AGEs deposit level		2.22 ± 0.29	2.19 ± 0.22	2.26 ± 0.35	0.460

Measured value: mean ± standard deviation. p-value: intergroup comparison of the MHE group and the Placebo group by two-sample t-test. MHE (mixed herb extract), BMI (body mass index), AGEs (advanced glycation end products).

Primary Outcomes

The results of the primary outcomes are shown in ***Table 4*** and ***Table 5***.

In all cases, among the glycation indices, a significant decrease was observed in pentosidine and CML in the MHE group when comparing the results from pre-administration to after 12 weeks of administration. However, the average of each measured value of the 2 parameters decreased in the placebo group as well, although it was not significant, therefore, no significant difference was observed between the groups. As a result of skin imaging analysis by VISIA, wrinkles on the right side of the face significantly decreased in the MHE group when comparing the results from pre-administration to after 12 weeks of administration, while no significant change was observed in the placebo group (***Fig. 2***). At this time, a tendency to decrease was observed in the MHE group compared to the placebo group ($p = 0.075$). HOMA-IR was significantly higher in both groups, however, no significant difference was observed between the groups (MHE group: $p = 0.039$, placebo group: $p < 0.001$, intergroup: $p = 0.162$). There were no other significant changes in the primary outcomes before and after administration and between groups.

Table 4. Primary outcomes

Parameter		Unit	Reference range	Group	0W	6W	12W
Skin AGEs deposit level	AF		–	MHE	2.19 ± 0.05	2.09 ± 0.06	2.17 ± 0.06 ††
				Placebo	2.26 ± 0.09	2.21 ± 0.10 †	2.27 ± 0.09
Internal body AGEs level	AF		–	MHE	0.506 ± 0.014	0.517 ± 0.010	0.557 ± 0.022 ††
				Placebo	0.505 ± 0.014	0.523 ± 0.017 †	0.489 ± 0.020
Imaging analysis by VISIA	Skin wrinkles (right cheek)		–	MHE	0.390 ± 0.051	0.307 ± 0.045 #	0.309 ± 0.054 #
				Placebo	0.412 ± 0.069	0.424 ± 0.063	0.428 ± 0.048
Glucose metabolism	HOMA-IR		1.6 or below	MHE	0.973 ± 0.116	1.059 ± 0.104	1.161 ± 0.111 #
				Placebo	0.719 ± 0.073	0.834 ± 0.070	1.034 ± 0.067 ##
Liver function	AST (GOT)	U/L	10 - 40	MHE	18.7 ± 1.1	20.3 ± 1.3	20.2 ± 1.0
				Placebo	18.8 ± 0.9	19.5 ± 1.1	19.9 ± 1.0
	ALT (GPT)	U/L	5 - 45	MHE	15.3 ± 2.0	17.8 ± 2.1	17.1 ± 2.0
				Placebo	13.1 ± 0.7	14.3 ± 0.8	14.8 ± 1.0

Subjects: n = 19 (MHE group), n = 16 (Placebo group). † Placebo group of AGEs-related value in 8W is 15 subjects because one subject was missing their value(s). †† MHE group of AGEs-related value in 12W is 18 subjects because one subject was missing their value(s). Measurement value: mean ± standard error of the mean. * $p < 0.05$, ** $p < 0.01$: Comparison with the Placebo group (two-sample t-test). # $p < 0.05$, ## $p < 0.01$: Comparison with before administration (Dunnett's test). MHE (mixed herb extract), AGEs (advanced glycation end products), AF (autofluorescence), HOMA-IR (homeostasis model assessment for insulin resistance), AST (GOT): aspartate aminotransferase (glutamic oxaloacetic transaminase), ALT (GPT): alanine aminotransferase (glutamic-pyruvic transaminase).

Table 5. Blood AGEs-related testing

Parameter		Unit	Group	0W	12W
AGEs in blood	Pentosidine	ng/mL	MHE	8.15 ± 0.46	7.30 ± 0.49 ##
			Placebo	8.27 ± 0.60	7.38 ± 0.32
	CML	μg/mL	MHE	0.79 ± 0.04	0.70 ± 0.04 ##
			Placebo	1.05 ± 0.22	0.86 ± 0.13
	3-Deoxyglucosone (3DG)	μg/mL	MHE	0.183 ± 0.020	0.169 ± 0.012
			Placebo	0.192 ± 0.031	0.184 ± 0.011
	Glyoxal (GO)	ng/mL	MHE	46.5 ± 2.6	47.1 ± 2.2
			Placebo	42.4 ± 3.5	47.7 ± 2.6
	Methylglyoxal (MGO)	ng/mL	MHE	28.6 ± 2.3	28.5 ± 2.7
			Placebo	28.3 ± 2.7	28.1 ± 3.4

Subjects: n = 19 (MHE group), n = 16 (Placebo group). Measurement value: mean ± standard error of the mean. * $p < 0.05$, ** $p < 0.01$: Comparison with the Placebo group (two-sample t-test). # $p < 0.05$, ## $p < 0.01$: Comparison with before administration (paired t-test). MHE (mixed herb extract). AGEs (advanced glycation end products), CML (N^{ε}-(carboxymethyl) lysine).

For the glycation indices, subgroups were defined with 7 subjects from the MHE group and 5 from the placebo group, whose levels of skin AGEs deposits were equal to or higher than the mean of the analyzed subjects. The changes in the levels of skin AGEs deposits in this subgroup are shown in ***Fig. 3***. A tendency to decrease was observed in the MHE group (pre-administration: 2.44 ± 0.02 → after 12 weeks: 2.29 ± 0.07, p = 0.051), while no significant change was observed in the placebo group (pre-administration: 2.70 ± 0.11 → after 12 weeks: 2.58 ± 0.12, p = 0.305).

Excluding 1 subject in the placebo group who was judged to be an outlier using the interquartile range, other subgroups were defined for pentosidine with 10 subjects from the MHE group and 6 from the placebo group, whose levels were below the mean of the analyzed subjects. As a result, only the MHE group demonstrated a significant decrease when comparing the results from pre-administration to after 12 weeks of administration. In terms of an intergroup comparison, a significant decrease in the amount of change was observed in the MHE group compared to that of the placebo group (***Fig. 4***).

Excluding 1 subject in the placebo group who was judged to be an outlier using the interquartile range, other subgroups consisting of the analyzed subjects were defined for CML with 7 subjects from the MHE group and 5 from placebo group, whose BMI was 22.0 or higher. As a result, only the MHE group demonstrated a tendency to decrease when comparing the results from pre-administration (0.81 ± 0.06 μg/mL) to after 12 weeks of administration (0.70 ± 0.07 μg/mL, p = 0.068), while no significant change was observed in the placebo group (pre-administration: 0.78 ± 0.07 μg/mL → after 12 weeks: 0.78 ± 0.08 μg/mL, p = 0.983, ***Fig. 5***).

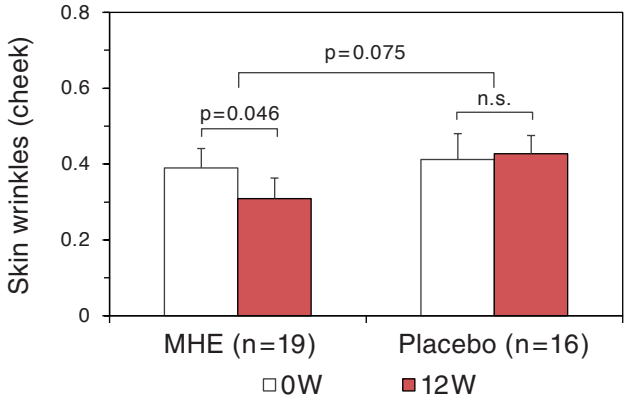

Fig. 2. **Changes in the skin (cheek) wrinkles measured by VISIA.**

Measured value: mean ± standard error of the mean. Analysis: Dunnett's test (intragroup) and two-sample t-test (intergroup). MHE (mixed herb extract).

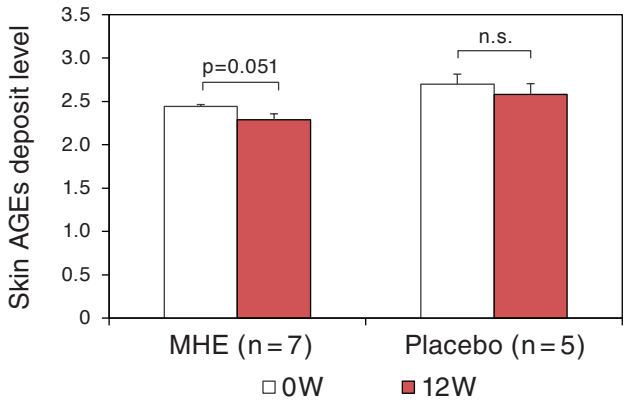

Fig. 3. **Changes in the skin AGEs deposit measured by AGE Reader mu: subgroup analysis.**

Measured value: mean ± standard error of the mean. Analysis: Dunnett's test (intragroup) and two-sample t-test (intergroup). MHE (mixed herb extract), AGEs (advanced glycation end products).

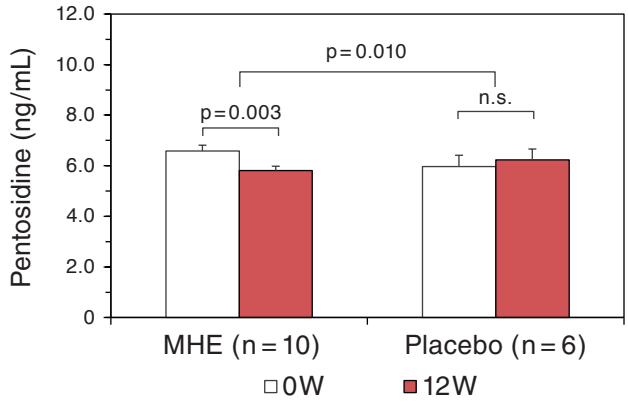

Fig. 4. **Changes in blood pentosidine: subgroup analysis.**

Measured value: mean ± standard error of the mean. Analysis: paired t-test (intragroup) and two-sample t-test (intergroup). MHE (mixed herb extract).

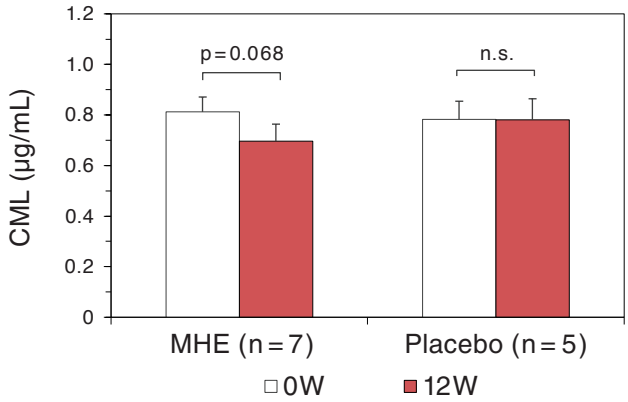

Fig. 5. **Changes in blood CML: subgroup analysis.**

Measured value: mean ± standard error of the mean. Analysis: paired t-test (intragroup) and two-sample t-test (intergroup). MHE (mixed herb extract), CML (N^{ε}-(carboxymethyl) lysine).

Excluding 1 subject in the placebo group who was judged to be an outlier using the interquartile range, subgroups were defined for GO with 19 subjects from the MHE group and 15 from the placebo group. As a result, the placebo group demonstrated an increasing tendency (pre-administration: 40.1 ± 2.8 ng/mL → after 12 weeks: 47.1 ± 2.7 ng/mL, p = 0.050), while the MHE group showed almost no change (pre-administration: 46.5 ± 2.6 ng/mL → after 12 weeks: 47.1 ± 2.2 ng/mL, p = 0.841, ***Fig. 6***).

Secondary Outcomes

For the secondary outcomes of skin viscoelasticity, skin color differences, imaging analysis of the skin using VISIA, blood testing (fasting blood sugar, HbA1c, insulin and γ-GTP) and medical interviews (Anti-Aging QOL Common Questionnaire), improvements were observed in the following 2 parameters (***Table 6***).

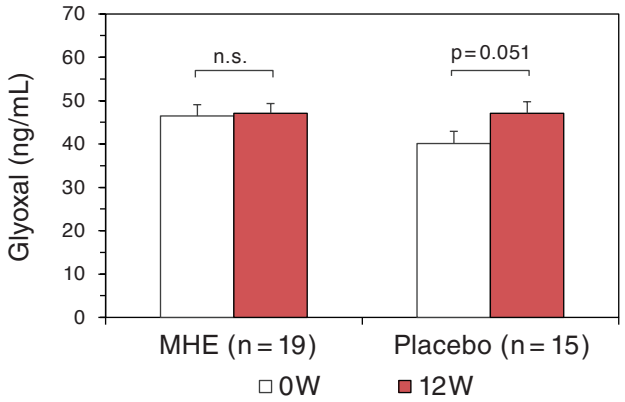

Fig. 6. **Changes in blood glyoxal: subgroup analysis.**

Measured value: mean ± standard error of the mean. Analysis: paired t-test (intragroup) and two-sample t-test (intergroup). MHE (mixed herb extract).

1) Skin texture (face)

Figure 7 shows the changes in skin texture (face).

There were significant decreases in the MHE group (pre-administration: 0.112 ± 0.013 → after 12 weeks: 0.096 ± 0.015, p = 0.001), whereas the placebo group did not show significant change (pre-administration: 0.113 ± 0.011 → after 12 weeks: 0.113 ± 0.010, p = 0.995). In an intergroup comparison, the changes in the MHE group showed a tendency to decrease when compared to the changes in the placebo group (p = 0.055).

2) Brown spots on the skin (face)

Figure 8 shows the changes in brown spots on the skin (face).

In all cases, the MHE group demonstrated a significant decrease in comparison with the placebo group (p = 0.032). The following data shows that neither group showed a significant change: MHE group (pre-administration: 0.606 ± 0.014 → after 12 weeks: 0.595 ± 0.013, p = 0.120) and the placebo group (pre-administration: 0.618 ± 0.015 → after 12 weeks: 0.625 ± 0.012, p = 0.274).

Safety Endpoints

Although some of the safety endpoints (clinical test values, body measurement values and physical test values) showed significant changes between pre- and post-administration during the period of the study, all of them were slight changes and were not considered that they did not constitute a clinical problem (***Table 7***). There were 31 adverse events observed in the MHE group (*e.g.* headache, stiff shoulders, menstrual pain), and 17 adverse events in the placebo group (*e.g.* cold, menstrual pain, constipation). All adverse events were mild, except for "hematoma (left thumb cuticle)" in the MHE group and "left distal radius fracture" and "gastroenteritis" in the placebo group. The physicians determined that all adverse events were not associated with the test food.

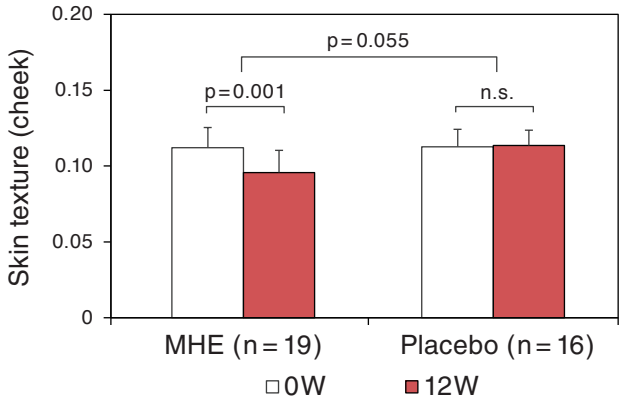

Fig. 7. **Changes in the skin (cheek) texture measured by VISIA.**

Measured value: mean ± standard error of the mean. Analysis: Dunnett's test (intragroup) and two-sample t-test (intergroup). MHE (mixed herb extract).

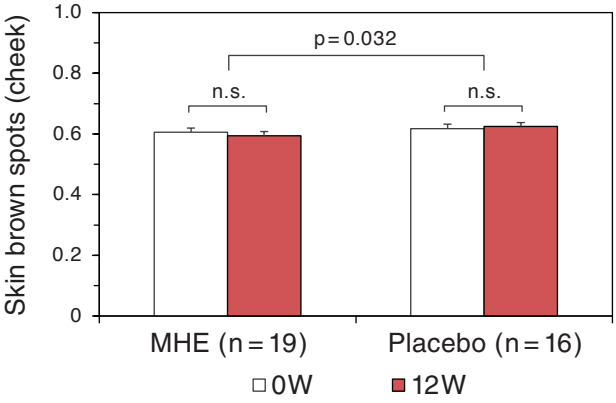

Fig. 8. **Changes in the skin (cheek) brown spots measured by VISIA.**

Measured value: mean ± standard error of the mean. Analysis: Dunnett's test (intragroup) and two-sample t-test (intergroup). MHE (mixed herb extract).

Table 6. Secondary outcomes

Parameter		Unit	Reference range	Group	0W	6W	12W
Skin viscoelasticity (Cheek)	R2			MHE	0.851 ± 0.014	0.841 ± 0.012	0.849 ± 0.012
				Placebo	0.838 ± 0.014	0.835 ± 0.010	0.833 ± 0.009
	R7			MHE	0.553 ± 0.018	0.540 ± 0.016	0.555 ± 0.017
				Placebo	0.538 ± 0.016	0.535 ± 0.015	0.541 ± 0.014
Color difference (Cheek)	L*			MHE	65.3 ± 0.4	65.7 ± 0.4 ##	66.2 ± 0.4 ##
				Placebo	65.6 ± 0.8	66.0 ± 0.8	66.4 ± 0.7 ##
	a*			MHE	10.53 ± 0.32	10.44 ± 0.26	10.56 ± 0.35
				Placebo	9.58 ± 0.52	9.69 ± 0.48	9.96 ± 0.42
	b*			MHE	17.9 ± 0.3	17.2 ± 0.4	16.8 ± 0.4 ##
				Placebo	19.0 ± 0.6	18.1 ± 0.5 #	18.1 ± 0.6 #
	Melanin Index			MHE	1.11 ± 0.02	1.05 ± 0.03 ##	1.01 ± 0.03 ##
				Placebo	1.18 ± 0.05	1.13 ± 0.05 ##	1.11 ± 0.05 ##
	Hb SO_2 Index	%		MHE	57.2 ± 1.2	59.6 ± 1.2 ##	61.9 ± 1.3 ##
				Placebo †	59.2 ± 2.1	61.2 ± 1.8 #	63.6 ± 1.9 ##
Color difference (Upper arm)	L*			MHE	68.9 ± 0.4	68.9 ± 0.4	69.3 ± 0.4 ##
				Placebo	67.8 ± 0.7	67.7 ± 0.7	68.3 ± 0.7 ##
	a*			MHE	5.82 ± 0.29	5.42 ± 0.25 #**	5.47 ± 0.23 #
				Placebo	6.14 ± 0.36	6.29 ± 0.39	6.22 ± 0.34
	b*			MHE	16.4 ± 0.5	16.4 ± 0.4	17.1 ± 0.4 ##
				Placebo	16.9 ± 0.6	17.3 ± 0.6	17.7 ± 0.6 ##
	Melanin index			MHE	0.82 ± 0.03	0.81 ± 0.03	0.83 ± 0.03
				Placebo	0.90 ± 0.06	0.92 ± 0.06	0.92 ± 0.06
	Hb SO_2 index	%		MHE	58.9 ± 1.6	60.6 ± 1.7	62.8 ± 1.5 #
				Placebo †	57.2 ± 2.2	58.7 ± 2.2	63.2 ± 2.5 #
Imaging analysis by VISIA	Brown Spots			MHE	0.606 ± 0.014	0.606 ± 0.013	0.595 ± 0.013 *
				Placebo	0.618 ± 0.015	0.622 ± 0.014	0.625 ± 0.012
	Pores			MHE	0.196 ± 0.035	0.214 ± 0.038	0.228 ± 0.042 #
				Placebo	0.164 ± 0.024	0.166 ± 0.024	0.172 ± 0.023
	Porphyrin			MHE	0.028 ± 0.009	0.027 ± 0.009	0.027 ± 0.008
				Placebo	0.037 ± 0.009	0.034 ± 0.008	0.031 ± 0.006
	Red			MHE	127.1 ± 13.1	109.5 ± 10.2 ##	111.8 ± 12.0 #
				Placebo	127.1 ± 9.9	122.3 ± 11.6	113.6 ± 8.8 #
	Spots			MHE	0.307 ± 0.019	0.301 ± 0.021	0.309 ± 0.025
				Placebo	0.361 ± 0.029	0.360 ± 0.030	0.355 ± 0.032
	Texture			MHE	0.112 ± 0.013	0.095 ± 0.011 ##*	0.096 ± 0.015 ##
				Placebo	0.113 ± 0.011	0.111 ± 0.012	0.113 ± 0.010
	UV Spots			MHE	0.259 ± 0.013	0.260 ± 0.013	0.274 ± 0.012 ##
				Placebo	0.278 ± 0.019	0.279 ± 0.021	0.291 ± 0.019 #
Glucose metabolism	Glucose	mg/dL	70 - 109	MHE	81.6 ± 1.3	83.2 ± 1.6	81.0 ± 1.3
				Placebo	81.5 ± 1.6	81.2 ± 1.5	81.8 ± 1.6
	HbA1c [NGSP]	%	4.6 - 6.2	MHE	5.65 ± 0.07	5.62 ± 0.07	5.63 ± 0.07
				Placebo	5.59 ± 0.07	5.56 ± 0.07	5.61 ± 0.07
	Insulin	μU/mL	1.7 - 10.4	MHE	4.79 ± 0.54	5.13 ± 0.48	5.75 ± 0.51 #
				Placebo	3.53 ± 0.33	4.14 ± 0.31	5.11 ± 0.32 ##
Liver function	γ-GTP	U/L	30 or below	MHE	19.5 ± 3.9	19.9 ± 4.5	18.6 ± 4.1
				Placebo	14.5 ± 1.6	13.9 ± 1.4	14.1 ± 1.7

Subjects: n = 19 (MHE group), n = 16 (Placebo group). † Placebo group of Hb SO2 Index is 15 subjects because one subject's value was subject to an error value elimination. Measurement value: mean ± standard error of the mean. * $p < 0.05$, ** $p < 0.01$: Comparison with the Placebo group (two-sample t-test). # $p < 0.05$, ## $p < 0.01$: Comparison with before administration (Dunnett's test). MHE (mixed herb extract), Hb (hemoglobin), UV (ultraviolet), NGSP (National Glycohemoglobin Standardization Program), γ-GTP (γ-glutamyltransferase).

Table 7. Physical testing and clinical chemistry testing

Parameter		Unit	Reference range	Group	0W	6W	12W
Body composition	Weight	kg		MHE	54.42 ± 9.58	54.81 ± 9.21	54.68 ± 9.01
				Placebo	52.46 ± 9.52	52.87 ± 9.41	52.92 ± 9.50 #
	BMI	kg/m^2		MHE	21.95 ± 3.44	22.07 ± 3.37	22.07 ± 3.31
				Placebo	21.02 ± 3.03	21.28 ± 2.95	21.20 ± 2.96 #
	Body fat	%		MHE	29.64 ± 6.76	30.74 ± 6.83 ##	29.97 ± 6.51
				Placebo	29.11 ± 6.50	30.36 ± 6.20 ##	29.77 ± 5.83
Blood pressure	Systolic	mmHg		MHE	112.2 ± 16.0	114.1 ± 18.4	114.8 ± 18.1
				Placebo	112.6 ± 15.1	109.2 ± 14.7	110.2 ± 12.2
	Diastolic	mmHg		MHE	77.6 ± 10.4	78.6 ± 13.9	77.1 ± 12.0
				Placebo	77.0 ± 8.1	74.6 ± 7.1	75.6 ± 6.4
Pulse rate		bpm		MHE	68.2 ± 10.1	72.8 ± 9.1 ##	71.0 ± 7.7 #
				Placebo	69.4 ± 11.1	70.2 ± 10.2	71.6 ± 7.3
Liver function	Total bilirubin	mg/dL	0.2 - 1.2	MHE	0.80 ± 0.43	0.67 ± 0.39 ##	0.74 ± 0.31
				Placebo	0.82 ± 0.24	0.70 ± 0.30 #	0.83 ± 0.25
	Direct bilirubin	mg/dL	0.0 - 0.2	MHE	0.11 ± 0.06	0.11 ± 0.05	0.11 ± 0.05
				Placebo	0.11 ± 0.03	0.11 ± 0.03	0.10 ± 0.04
	Indirect bilirubin	mg/dL	0.2 - 1.0	MHE	0.70 ± 0.40	0.57 ± 0.38 ##	0.64 ± 0.28
				Placebo	0.71 ± 0.23	0.59 ± 0.30	0.73 ± 0.23
	ALP	U/L	100 - 325	MHE	189.5 ± 60.6	200.5 ± 61.8 #	195.1 ± 57.5
				Placebo	199.8 ± 72.7	206.2 ± 74.0	203.4 ± 69.8
	LDH	U/L	120 - 240	MHE	176.5 ± 30.6	175.5 ± 30.6	174.1 ± 32.1
				Placebo	190.8 ± 28.7	185.1 ± 25.0 #	183.8 ± 24.2 #
Inflammation	High-sensitivity CRP	mg/dL		MHE	0.039 ± 0.036	0.070 ± 0.078	0.049 ± 0.100
				Placebo	0.057 ± 0.068	0.057 ± 0.076	0.044 ± 0.057
Protein	Total protein	g/dL	6.7 - 8.3	MHE	7.34 ± 0.34	7.32 ± 0.40	7.22 ± 0.31
				Placebo	7.24 ± 0.34	7.19 ± 0.34	7.22 ± 0.30
	Albumin	g/dL	3.8 - 5.2	MHE	4.51 ± 0.21	4.34 ± 0.22 ##	4.32 ± 0.16 ##
				Placebo	4.51 ± 0.21	4.41 ± 0.27	4.36 ± 0.16 ##
	A/G ratio		1.1 - 2.0	MHE	1.62 ± 0.18	1.49 ± 0.19 ##	1.51 ± 0.18 ##
				Placebo	1.67 ± 0.22	1.61 ± 0.22	1.55 ± 0.16 ##
Nitrogenous component	Creatinine	mg/dL	0.47 - 0.79	MHE	0.659 ± 0.066	0.632 ± 0.069 #	0.644 ± 0.075
				Placebo	0.642 ± 0.088	0.592 ± 0.084 ##	0.613 ± 0.094 #
	Urea nitrogen	mg/dL	8.0 - 20.0	MHE	11.98 ± 2.85	11.46 ± 2.64	12.08 ± 2.80
				Placebo	12.10 ± 3.03	12.31 ± 3.10	12.37 ± 2.68
	Uric acid	mg/dL	2.5 - 7.0	MHE	4.44 ± 1.04	4.48 ± 0.96	4.40 ± 0.99
				Placebo	3.96 ± 1.14	3.93 ± 1.20	3.94 ± 1.17
Lipid metabolism	Triglyceride	mg/dL	30 - 149	MHE	78.8 ± 51.1	85.3 ± 41.2	68.4 ± 30.0
				Placebo	71.5 ± 25.7	80.9 ± 38.0	67.4 ± 24.5
	Free fatty acid	mEq/L	0.10 - 0.90	MHE	1.008 ± 0.251	1.008 ± 0.279	1.031 ± 0.235
				Placebo	1.057 ± 0.197	1.004 ± 0.232	0.956 ± 0.201
	Total cholesterol	mg/dL	120 - 219	MHE	224.8 ± 25.8	227.4 ± 35.9	227.1 ± 28.3
				Placebo	223.0 ± 30.0	227.0 ± 36.2	227.0 ± 37.6
	LDL cholesterol	mg/dL	65 - 139	MHE	136.2 ± 20.3	138.1 ± 31.5	137.7 ± 25.4
				Placebo	137.9 ± 25.8	138.1 ± 29.8	137.9 ± 27.4
	HDL cholesterol	mg/dL	40 - 95	MHE	72.8 ± 10.2	72.2 ± 10.4	75.7 ± 10.8 #
				Placebo	70.8 ± 10.5	72.8 ± 12.1	75.6 ± 14.3 #
	Non-HDL cholesterol	mg/dL	90 - 149	MHE	152.0 ± 27.7	155.2 ± 35.9	151.4 ± 29.4
				Placebo	152.2 ± 29.0	154.3 ± 33.5	151.4 ± 29.6
	Atherogenic index		4.0 or below	MHE	2.153 ± 0.613	2.217 ± 0.744	2.060 ± 0.567
				Placebo	2.201 ± 0.583	2.176 ± 0.606	2.044 ± 0.482 ##

Subjects: n = 20 (MHE group), n = 16 (Placebo group). Measurement value: mean ± standard deviation. * $p < 0.05$, ** $p < 0.01$: Comparison with the Placebo group (two-sample t-test). # $p < 0.05$, ## $p < 0.01$: Comparison with before administration (Dunnett's test). MHE (mixed herb extract), BMI (body mass index), ALP (alkaline phosphatase), LDH (lactate dehydrogenase), CRP (C-reactive protein), A/G (albumin/globulin) LDL (low-density lipoprotein), HDL (high-density lipoprotein).

Discussion

The daily ingestion of 100 mg of MHE for 12 weeks in women 40 to 64 years of age with a high level of skin AGEs deposits significantly decreased the amount of pentosidine and CML in the MHE group. In addition, as a result of the analysis of subgroups, where one subject in the placebo group who was judged to be an outlier using the interquartile range was excluded, a tendency to increase in GO, a glycation reaction intermediate, was observed in the placebo group, while no change was observed in the MHE group. Although the cause of the increase in GO observed in the placebo group is unknown, it was suggested that the intake of the MHE led to an improvement in the suppression of GO increase. Pentosidine and CML, which are types of AGEs, are reportedly produced via glycation reaction intermediates. However, it has been revealed that GO constitutes an intermediate for some parts of them[14,15]. Therefore, it is possible that the MHE has an anti-glycation effect either by directly acting on the pentosidine and CML in the body or by indirectly blocking the formation of pentosidine and CML through the inhibition of GO production via the mechanism[11] that cleaves the α-diketone structure.

Our previous study found that a significant decrease in pentosidine was observed in both the low-dose group with a daily intake of 100 mg and the high-dose group with a daily intake of 300 mg over the period of 12 weeks when comparing pre- and post-administration ($p = 0.022$ and $p = 0.011$, respectively)[12]. This study also demonstrated a decrease in pentosidine in the MHE group, which is consistent with the results of previous study. The lowering action of these AGEs in the blood as well as glycation reaction intermediates may be due to the AGEs crosslink cleaving action and OPH activity enhancing action, which have been independently reported for each of the three types of herbs contained in MHE[11]. In particular, considering that OPH has demonstrated effectiveness in reducing CML and fluorescent AGEs[16], MHE may contribute to the promotion of decomposition and metabolism of AGEs in the body by enhancing OPH activity.

The significant decrease in facial wrinkles, a skin index, following the intake of MHE is also consistent with the previous study[12]. Furthermore, in this study, improvement effects were observed in skin (face) texture and brown spots. Thus far, the various effects of glycation on skin have been reported, including; decrease in skin elasticity due to glycated collagen being a possible cause of wrinkles, the involvement of stratum corneum AGEs in decreased skin texture quality, and the yellowing of the skin due to glycation[17,18]. Therefore, it is presumed that the effect of improving skin indices by ingesting the MHE is attributed to its effects of lowering AGEs and glycation reaction intermediates in the body.

Our previous study found that among the glucose metabolism indices, fasting blood sugar, significantly declined in both the low-dose group and the high-dose group. Moreover, HOMA-IR indicated a significant decrease in the high-dose group and a tendency to decrease in the low-dose group[12]. On the other hand, in this study, HOMA-IR became significantly higher in both groups, and fasting blood glucose did not show a significant change between pre- and post-administration. Although many of the studies on HbA1c, which is one of the glucose metabolism indices, have been conducted with diabetic subjects, it has been reported that the HbA1c level has seasonal fluctuations both in Japan and abroad[19,20], indicating a tendency of being higher from January through April (winter and spring) and lower from August to November (summer and fall)[21]. In the previous study, the HOMA-IR value at week 0 (pre-administration) may have been relatively high as week 0 was in February and week 12 (12 weeks after administration) was in May. On the other hand, during this study period, not only the MHE group but also the placebo group showed significantly higher HOMA-IR values. Since week 0 (pre-administration) was in September and week 12 (12 weeks after administration) was in December, the seasonal fluctuation of HOMA-IR from low to high value may have affected the test results. The cause of the difference in the results of fasting blood sugar is unknown.

In addition, regarding liver function indices, the previous study showed that improvement effects were observed only in the high-dose group, whereas no effect was observed in the low-dose group. It is suggested that the amount of intake was too small to bring about an improvement effect in this study.

Conclusion

Ingestion of MHE was found to improve skin wrinkles, texture and brown spots. The decrease in the AGEs level, particularly, CML and GO levels in the body due to the anti-glycation effect of MHE is thought to contribute to these beautiful skin effects. Therefore, this study demonstrated that ingestion of MHE decreased the AGEs level in people who had already accumulated AGEs in their bodies due to glycative stress, which led to potential beautiful skin effects. An excellent level of safety was confirmed, even with continuous ingestion of MHE.

Conflict of Interest Declaration

The clinical trial of this study was funded by Karada Lab, Inc. and properly conducted under the control of TTC Co., Ltd. In addition, the research costs for the quantification of blood glycation indices (pentosidine, CML, 3DG, GO and MGO) were funded by Karada Lab, Inc. and AGE Laboratories Co., Ltd. was contracted for the implementation. Dr. Sumio Kondo, who is affiliated with Medical Corporation Kenshokai Fukushima Healthcare Center, is the principal investigator for this study and performed the contracted work, and has no conflicts of interest to disclose.

Acknowledgments

The publication of this study was supported by the Research Foundation of Crude Drug gained from the same source of Medicine & Food. (Chuo-ku, Tokyo).

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