Glycative Stress Research

Online edition : ISSN 2188-3610
Print edition : ISSN 2188-3602
Received : September 7, 2017
Accepted : October 13, 2017
Published online : December 31, 2017

Original article

Reducing effect of the long term intake of water chestnut (*Trapa bispinosa* Roxb.) pericap extract on glycative stress in the placebo-controlled double blinded clinical trial and in vitro inhibitory actions on low-density lipoprotein (LDL) glycation.

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Abstract

***Objectives*:** We focused on the glycation and glycated stress-reducing action of *Trapa bispinosa* Roxb. (Water chestnut) peel extract (TBE), and examined its action in humans after long-term ingestion.
***Methods*:** This was a placebo-controlled double-blind study, which included 25 healthy Japanese males and females, aged 30 years or older, who were divided into two groups: the trial group (13 subjects: 47.5 ± 7.0 years) and the control group (12 subjects: 48.5 ± 4.6 years). Subjects ingested the test product containing 100 mg TBE or the control product for 12 weeks. We set the observation dates as before the ingestion, and 4, 8, and 12 weeks after the ingestion, and tested blood biochemistry, skin fluorescence (advanced glycation end-products; AGEs), and skin viscoelasticity. In addition, we examined if TBE suppressed the glycation of LDL *in vitro*.
***Results*:** In the test group, serum pentosidine significantly decreased at week 4 ($p < 0.05$), HbA1c increase was significantly suppressed at week 8 ($p < 0.05$), and skin viscoelasticity index R2 improved significantly at week 8 ($p < 0.05$), compared to the control group. Both systolic and diastolic blood pressures were significantly lower at week 4 in the trial group ($p < 0.05$). The subgroup analysis of female subjects (seven in the trial group: 45.7 ± 8.0 years, six in the control group: 50.1 ± 5.1 years) showed that at week 8, the trial group had significantly lower HbA1c, total cholesterol, and triglyceride levels than the control group ($p < 0.05$). There were no adverse events during the trial period associated with the formulation used for the trial. In the LDL glycation suppression test, after the addition of TBE, a concentration-dependent decrease of CML production was confirmed.
***Conclusions*:** After long-term ingestion of TBE, glycation stress decreased, and lipid metabolism in females improved. Its potential to prevent lifestyle-related diseases was indicated.

***KEY WORDS*:** *Trapa bispinosa* Roxb. (Water chestnut), placebo-controlled double-blind study, pentosidine, LDL-cholesterol, skin elasticity

Introduction

In recent years, *in vivo* glycation stress has been suggested as a risk factor that promotes aging and disease progression [1)]. Glycation stress encompasses a series of reactions such as the glycation reaction, in which reducing sugars and aldehydes react with proteins and amino acids to produce glycation products such as carbonyl compound and advanced glycation end products (AGEs). The accumulation of these AGE products causes a load on cells and tissues, reduces the function of functional protein, and furthermore, increases inflammation and diseases [2)]. Diseases associated with glycation are diverse: diabetic nephropathy, cataracts [3)], arteriosclerosis [4)], osteoporosis [5)], Alzheimer's disease [6)], infertility [7)], sclerema [8)], etc. As such, prevention of the generation and accumulation of AGEs and promotion of the decomposition and metabolism of generated AGEs reduce glycative stress and lead to an extended healthy life span and improved quality of life (QOL).

The authors discovered a strong anti-glycative and glycation product decomposition effect of *Trapa bispinosa* Roxb. (Water chestnut) peel extract (hereinafter referred to as

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TBE) and have studied and reported on the possible glycative stress countermeasures [9]. Water chestnut is an annual water grass from the family Lythraceae and genus *Trapa*, and dried fruit peel is often used in tea or as herbal medicine. The peel extract of *Trapa Japonica* FLEROV., a species closely related to Water chestnut, has been shown to suppress glycometabolism enzymes such as amylase and glucosidase, and has been reported to suppress the postprandial increase in blood glucose levels in rats [10]. Similarly, the extract of *Trapa natans*, another closely related species, decreased blood glucose concentrations in streptozotocin-induced diabetic rats [11]. The authors have also reported that a single administration of TBE to humans suppressed the increase in postprandial blood glucose levels [12]. These results indicate that ingesting *Trapa* extracts may be connected to improved glycometabolism. However, there is no report on the clinical effects of long-term TBE administration in humans. Therefore, we implemented a long-term trial of TBE in humans, and examined its reduction effects on glycative stress and other actions.

Methods

Trial substance

In accordance with the previous report [9], TBE was obtained as follows: Water chestnut fruit peels were dried, sterilized, and ground, and the solution was extracted using hot water approximately six times the weight of dried Water chestnut peels; then, dextrin was added at a ratio of 67 : 33 to the dry weight, and spray dried.

1. Long-term ingestion test in humans

Trial product properties

Test foodstuff for the TBE ingestion group was formulated into an opaque hard capsule of dark caramel color with 100 mg of the TBE powder obtained in the above method. The control foodstuff for the control group appeared the same outside, but without TBE, so that it could not be differentiated from the test foodstuff. Both formulations were prepared at Sunsho Pharmaceutical Co., Ltd. (Fuji, Shizuoka, Japan). Compositions and nutritional components of each formulation are shown in ***Table 1***.

Subjects

The present test subjects were 30 Japanese male and female employees of Hayashikane Sangyo Co., Ltd. (Shimonoseki, Yamaguchi, Japan) with ages ranging between 30 and 60 years. We obtained written consent to participate in the trial from 90 candidates who wished to participate after they receiving all the details. For each of these 90 candidates, we performed a background survey and a screening test through measuring AGE deposition on skin using an AGE Reader (DiagnOptics, Groningen, Netherlands), and selected the top 30 candidates with the highest skin AGE depositions. However, those with clearly outlying values were excluded.

Trial design

The trial period was September to November 2014. The trial was performed under the guidance of the trial supervisory physician and attending physician. The trial design was a placebo-controlled, double-blind comparative test with two groups (the control and test groups). The test group (15 subjects) took a formulation containing 100 mg of TBE (the test foodstuff) and the control group (15 subjects) took a formulation without TBE (the control foodstuff) once a day before breakfast for 12 weeks.

We performed the following tests before the start of trial, and four, eight, and 12 weeks after the start of the trial: physical examination, blood biochemistry, skin fluorescence AGE measurement, skin viscoelasticity measurement, N^{ε}-(carboxymethyl) lysine (CML) in stratum corneum, and anti-aging QOL common questionnaire (AAQol). Trial participants recorded the presence and degree of adverse events, test foodstuff ingestion, lifestyle habits, and meal and exercise habits during the trial period.

Table 1-1. Composition of the test diets.

	Test diet (TBE)	Control diet
TBE (mg)	100	0
Corn starch (mg)	187	287
Calcium stearate (mg)	3	3
Total (mg)	290	290

TBE, water chestnut (*Trapa bispinosa* Roxb.) pericarp extract.

Table 1-2. Nutritional facts of the test diets.

	Test diet (TBE)	Control diet
Calories (kcal)	1.03	1.03
Protein (g)	0.0014	0.0009
Carbohydrate (g)	0.25	0.25
Lipid (g)	0.003	0.003

TBE, water chestnut (*Trapa bispinosa* Roxb.) pericarp extract.

Test methods

1) Blood biochemistry

Fasting blood glucose, HbA1c, total cholesterol (TC), low-density lipoprotein-cholesterol (LDL-C), high-density lipoprotein-cholesterol (HDL-C), triglyceride (TG), and pentosidine were measured. We contracted LSI Medience Corporation (Chiyoda-ku, Tokyo, Japan) to measure serum pentosidine via high performance liquid chromatography (HPLC), while the other items were measured at Shimonoseki Medical Association (Shimonoseki, Yamaguchi, Japan).

2) Skin function measurement

1. Skin AGEs-derived fluorescence measurement

In accordance with the previous report, we evaluated the skin AGE depositions with AGE reader to determine the index of glycation [13]. The measurement site was approximately 10 cm from the elbow on the inner side of right upper arm. Measurement sites were cleaned with an alcohol swab, and the mean of three measurements was used. Measurement values were expressed as an Auto Fluorescence (AF) value.

2. Corneum CML measurement

In accordance with the previous report [14], adhesive film (Corneum checker: Asahi Biomed, Chiyoda-ku, Tokyo, Japan) was attached to the skin on the inner side of right upper arm, and the stratum corneum was sampled (tape stripping method). This was repeated three times on the same site, and the CML in the corneum protein was measured.

3. Skin viscoelasticity test

In accordance with the previous report [15], skin viscoelasticity was evaluated using a Cutometer (MPA580: Courage & Khazaka, Cologne, Germany). The measurement site was approximately 10 cm from the elbow on the inner side of right upper arm. The skin surface was suctioned into the probe opening under negative pressure, and the length of the skin suctioned into the opening was measured using a prism. The skin height recovery following extension and retraction was used as the R2 index, and the ratio of the elastic portion during retraction (R7) was obtained. The test result was the mean of three measurements after removing the highest and lowest R2 from five measurements taken at the same site.

3) Subjective symptoms and physical examination

Evaluations of subjective symptoms were divided into "physical symptoms" and "mental symptoms", and the Anti-Aging QOL Common Questionnaire (AAQol) [16] was used for this evaluation (1 to 5 points). We also measured height (cm), weight (kg), systolic and diastolic blood pressure (mmHg), pulse (beats/minute), and body mass index (BMI).

2. LDL glycation suppression test

Trial product adjustment

TBE was dissolved in distilled water for the test. Aminoguanidine sulfate (AG; Wako Pure Chemical Industries, Osaka, Japan), a glycation reaction inhibitor, was used as a positive control.

Preparing the glycation LDL suppression measurement sample

We referred to the previous report [17], and obtained glycated LDL by reacting human LDL (Biochem Thecnologies, Inc., King of Prussia, PA, USA) with glyoxal (GO). Briefly, 0.5 mg/mL human LDL and 1.0 mmol/L GO were mixed into 10 mmol/L phosphate buffer solution (PBS, pH 7.4), and the trial solution of arbitrary concentration was added. Then, the solution was incubated at 60°C for 16 hours. This solution was used as a glycated-LDL measurement solution. We used a system in which distilled water was added instead of test solution as the experimental control, and a system in which AG sulfate solution was added as the positive control group.

CML measurement

CircuLex CML/N^{ε}-(Carboxymethyl) lysine enzyme-linked immunosorbent assay (ELISA) Kit (CycLex Co., Ltd., Ina, Nagano, Japan) was used to measure CML, according to previously reported method [9]. Briefly, the measurement sample was diluted four times using the dilution buffer from the kit, and 60 µL of anti-CML monoclonal antibody solution was added to the CML-HSA (human serum albumin) standard solution from the kit, blank solution, or 60 µL ELISA measurement sample, and mixed well. We added 100 µL of mixed solution to the wells of CML-BSA (bovine serum albumin) immobilizing microplates from the kit, which was then incubated by shaking for one hour at room temperature. The solution from each well was discarded, and the remains were washed four times with 200 µL washing buffer containing 0.2% Tween 20. As a secondary antibody, 100 µL horseradish peroxidase (HRP) labeled anti-mouse IgG polyclonal antibody from the kit was added to each well, and was incubated for one hour at room temperature while shaking. After the reaction ended, each well was washed according to the same procedure. We added 100 µL of tetra-methylbenzidine (TMB) substrate solution from the kit to each well, and incubated by shaking for 10 minutes at room temperature. After the reaction, we added reaction stop solution containing 1.0 N H_2SO_4 and stirred. Using a microplate reader, we measured absorbance 450 nm (dominant wavelength) / 540 nm (reference wavelength). The CML concentration in each measurement sample was calculated from a calibration curve prepared from the CML-HSA reference solution in the kit.

Ethical considerations

This trial strictly adhered to ethical principles and the personal information protection act based on the Declaration of Helsinki, was reviewed by the "Ethics Review Committee regarding Research using Human Subjects" of Doshisha University, and was conducted in accordance with the policy upon approval (approval number: dou-dai-rin-otsu 2014-No.203).

Statistical analysis

Results are presented as mean ± standard deviation (SD). We used Dunnett's test to compare with the ingestion start time (0 w), and the unpaired t test or Tukey-Kramer test to compare between groups. A significance level of less than 5% in the two-tailed test was considered significant.

Results

1. Long-term ingestion test in humans

Full analysis set

One subject in the trial group dropped out since the subject had ingested less than 80% of the trial foodstuff. One subject in the trial group and three subjects in the control group had routine use of pharmaceutical products (two cases) and were outside of the healthy range in lipid related items (out of healthy range cases), and the trial supervisor physician excluded them as ineligible. This left 13 cases in the trial group (47.5 ± 7.0 years) and 12 cases in the control group (48.5 ± 4.6 years).

Test results

1) Blood biochemistry

Results of the blood biochemistry tests are shown in ***Table 2***.

Serum pentosidine levels significantly decreased on weeks 4 and 12 in the trial group ($p < 0.05$, ***Fig. 1-a***). Comparison of the changes between groups showed that the trial group had significantly lower values on week 4 compared to the control group ($p < 0.05$). Comparison of changes on week 12 between the trial and control groups showed that there was a decreasing trend in the trial group but this difference was not significant ($p = 0.085$).

Before-after comparison of changes in HbA1c showed that there was a significant increase in the control group on week 8 ($p < 0.05$, ***Fig. 1-b***). A comparison between the trial and control groups showed that the trial group had significantly lower values on week 8 ($p < 0.05$).

Fasting blood glucose, TC, HDL-C, LDL-C, and TG did not show significant changes during the course in both trial and control groups (***Fig. 1-c***, ***d***, ***e***).

2) Skin function measurement

Table 3 shows the results of the skin function measurements.

There were no significant changes in skin fluorescence AGEs in the trial and control groups during the trial course. There also were no significant differences in the mean and the changes between the groups.

There was a significant decrease in the corneum CML in both the trial and control groups on weeks 4, 8, and 12, compared to week 0 ($p < 0.01$). However, there was no significant difference in the mean and changes between the groups.

The mean skin viscoelasticity index R2 showed a significant increase in the trial group on week 8 and in the control group on week 12, compared to the mean measured on week 0 (***Fig. 2-a***). A comparison of the groups showed that the mean in the trial group on week 8 was significantly higher than the control group ($p = 0.004$). Furthermore, mean skin viscoelasticity index R7 of the trial group showed significant increases on week 8 and week 12 compared to that on week 0 (***Fig. 2-a***). A comparison of the changes showed that in both trial and control groups, measured changes in R7 significantly increased on week 8 and week 12.

3) Subjective symptoms and physical information

Table 4-1 and ***4-2*** show the evaluation of physical and mental symptoms, determined using the AAQol.

Among the 34 physical symptoms, the trial group showed significant improvement over the control group in "stiff shoulders" on week 4, and "Lethargy", "No feeling of good health", "Thirst", and "Edematous" on week 8.

Among the 22 mental symptoms, the trial group showed significant improvement over the control group in "Lapse of memory" on week 8, and "Inability to concentrate" on week 4.

The results of the physical measurements, changes in systolic and diastolic blood pressure, and changes in pulse are shown in ***Table 5***. Weight, basal metabolic rate, percentage skeletal muscle, percentage body fat, BMI, and heart rate did not change significantly with ingestion in either the trial or control groups.

There was no significant difference between groups in the mean weight, basal metabolic rate, percentage skeletal muscle, percentage body fat or BMI.

A comparison of changes in systolic and diastolic blood pressures showed that in week 4, the trial group had significantly lower values than the control group ($p < 0.05$, ***Fig. 3***).

Subgroup analysis

We performed a subgroup analysis of all female subjects in the full analysis set (seven in the trial group: mean age of 45.7 ± 8.0 years, six in the control group: mean age of 50.1 ± 5.1 years).

1) Skin function measurement

Table 6 and ***Fig. 4*** show the results of the skin function measurement. In the trial group, changes in both the R2 and R7 measures of the skin viscoelasticity significantly increased in weeks 8 and 12 ($p < 0.05$). The control group did not show a significant increase compared to the start of the experiment.

2) Blood biochemistry

Table 6 and ***Fig. 5*** show the results of the blood biochemistry tests. A comparison of changes in serum pentosidine showed a significant decrease in the trial group compared to the control group in week 4 ($p < 0.05$). In the trial group, HbA1c, TC, and TG levels were significantly lower in week 8 than those in the control group. LDL-C levels were also lower in the trial group than in the control group, but the difference was not significant (8w: $p = 0.072$).

3) Subjective symptoms and physical information

There was no significant improvement in the self-evaluation of physical symptoms using the AAQol in the trial and the control group.

There were no significant changes for either group in weight, basal metabolic rate, percentage skeletal muscle, percentage body fat, BMI, blood pressure, or heart rate. In addition, there were no significant differences between the two groups.

Trial product safety

During the trial period and after the trial, there were no adverse events related to the ingestion of the formulation used for the trial, and there were no events that were identified as "correlated" in terms of a causal relationship with the trial foodstuff.

Table 2. erum parameters.

Item	Group	average value ± SD				average variation (vs 0w) ± SD		
		0 w	4 w	8 w	12 w	4 w	8 w	12 w
Glucose (mg/dL)	Control	97.1 ± 8.4	97.5 ± 10.5	95.5 ± 8.0	95.3 ± 12.5	0.4 ± 5.1	-1.6 ± 5.2	-1.8 ± 9.0
	Test	93.9 ± 12.7	95.8 ± 9.6	96.4 ± 10.4	96.6 ± 10.3	1.9 ± 5.6	2.5 ± 7.2	2.7 ± 5.2
HbA1c (%)	Control	5.5 ± 0.4	5.6 ± 0.4	5.6 ± 0.4	5.6 ± 0.4	0.09 ± 0.16	**0.16 ± 0.13 #**	0.12 ± 0.19
	Test	5.5 ± 0.2	5.5 ± 0.2	5.5 ± 0.2	5.6 ± 0.3	0.00 ± 0.10	**0.04 ± 0.11 ***	0.08 ± 0.17
TC (mg/dL)	Control	208.1 ± 44.8	208.9 ± 45.0	212.7 ± 42.7	221.6 ± 45.3	0.7 ± 14.0	4.5 ± 14.4	13.4 ± 24.9
	Test	219.9 ± 33.3	215.9 ± 25.7	216.0 ± 40.1	221.9 ± 36.3	-4.0 ± 18.6	-3.9 ± 13.6	2.0 ± 21.0
TG (triglyceride) (mg/dL)	Control	81.8 ± 34.2	74.6 ± 36.4	80.0 ± 32.4	82.8 ± 38.9	-7.3 ± 13.8	-1.8 ± 10.6	0.9 ± 32.7
	Test	102.9 ± 71.9	74.0 ± 40.3	100.0 ± 105.7	89.3 ± 51.1	-28.9 ± 36.3	-2.9 ± 49.9	-13.6 ± 24.8
HDL-C (mg/dL)	Control	65.1 ± 16.9	66.1 ± 19.7	67.3 ± 21.0	67.6 ± 20.5	1.0 ± 6.5	2.2 ± 7.2	2.5 ± 7.9
	Test	68.9 ± 17.5	69.6 ± 15.8	67.5 ± 19.0	68.0 ± 16.7	0.8 ± 4.9	-1.3 ± 4.7	-0.9 ± 4.7
LDL-C (mg/dL)	Control	128.1 ± 43.7	126.7 ± 41.4	126.3 ± 38.7	131.4 ± 37.0	-1.4 ± 11.2	-1.8 ± 10.7	3.3 ± 15.5
	Test	126.9 ± 30.7	125.6 ± 23.5	121.2 ± 32.6	127.2 ± 31.8	-1.3 ± 20.0	-5.7 ± 8.8	0.2 ± 20.6
LDL-C/HDL-C	Control	2.2 ± 1.1	2.1 ± 1.0	2.1 ± 1.0	2.1 ± 0.9	-0.07 ± 0.26	-0.07 ± 0.25	-0.04 ± 0.25
	Test	2.0 ± 0.7	1.9 ± 0.7	1.9 ± 0.7	2.0 ± 0.8	-0.03 ± 0.19	-0.03 ± 0.16	0.05 ± 0.33
Pentosidine (μg/mL)	Control	102.0 ± 19.9	97.9 ± 13.0	104.2 ± 9.6	96.4 ± 16.3	-4.0 ± 14.8	2.3 ± 16.8	-5.6 ± 17.7
	Test	120.9 ± 20.2	**104.9 ± 18.0#**	112.9 ± 19.2	**102.9 ± 19.1#**	**-16.0 ± 9.8*#**	-7.9 ± 17.7	**-18.0 ± 16.9#**

Subjects: n = 12 (Control group), n = 13 (Test group). Measured value: average value ± SD. *: $p < 0.05$ indicated comparisons between control and test by unpaired Student's t-test. #: $p < 0.05$ indicated comparisons vs 0w by Dunnett's test. TC, total cholesterol; TG, triglyceride; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; SD, standard deviation.

Table 3. Skin function test.

Item	Group	average value ± SD				average variation (vs 0w) ± SD		
		0 w	4 w	8 w	12 w	4 w	8 w	12 w
Skin fluorescent AGEs	Control	2.11 ± 0.28	2.13 ± 0.24	2.15 ± 0.26	2.03 ± 0.16	0.01 ± 0.24	0.04 ± 0.29	-0.09 ± 0.25
	Test	2.01 ± 0.23	2.05 ± 0.26	2.04 ± 0.26	1.99 ± 0.23	0.04 ± 0.28	0.03 ± 0.15	-0.02 ± 0.20
CML content in stratum corneum (μg/mg Protein)	Control	16.72 ± 4.02	**9.85 ± 2.92#**	**8.76 ± 4.66#**	**7.66 ± 3.41#**	**-6.87 ± 3.61#**	**-7.96 ± 5.11#**	**-9.06 ± 4.79#**
	Test	17.31 ± 5.61	**10.90 ± 5.16#**	**9.37 ± 5.46 #**	**8.28 ± 3.61#**	**-6.41 ± 6.92#**	**-7.94 ± 9.41#**	-9.03 ± 7.25
Skin elasticity test (R2)	Control	0.86 ± 0.05	0.86 ± 0.03	0.87 ± 0.02	**0.89 ± 0.03 #**	0.01 ± 0.04	0.01 ± 0.06	0.04 ± 0.06
	Test	0.87 ± 0.03	0.87 ± 0.03	**0.90 ± 0.03*#**	0.89 ± 0.03	0.00 ± 0.03	0.03 ± 0.02	0.02 ± 0.03
Skin elasticity test (R7)	Control	0.64 ± 0.06	0.65 ± 0.05	0.68 ± 0.04	0.69 ± 0.03	0.01 ± 0.03	**0.04 ± 0.04 #**	**0.05 ± 0.05#**
	Test	0.67 ± 0.05	0.68 ± 0.03	**0.71 ± 0.03#**	**0.70 ± 0.04#**	0.01 ± 0.03	**0.05 ± 0.03 #**	**0.04 ± 0.03 #**

Subjects: n = 12 (Control group), n = 13 (Test group). Measured value: average value ± SD. *: $p < 0.05$ indicated comparisons between control and test by unpaired Student's t-test . #: $p < 0.05$ indicated comparisons vs 0w by Dunnett's test. AGEs, advanced glycation end products; CML, N^{ε}-(carboxymethyl)lysine; SD, standard deviation.

Table 4-1. Physical subjective symptom score in AAQol.

Item	Group	average value ± SD				average variation (vs 0w) ± SD		
		0 w	4 w	8 w	12 w	4 w	8 w	12 w
Tired eye	Control	3.42 ± 0.79	3.50 ± 1.00	3.33 ± 0.78	3.00 ± 0.85	0.08 ± 0.51	-0.08 ± 0.51	-0.42 ± 0.51
	Test	2.85 ± 0.80	2.77 ± 0.83	**2.46 ± 0.66** *	2.77 ± 0.93	-0.08 ± 0.64	-0.38 ± 0.77	-0.08 ± 0.76
Blurry eyes	Control	3.00 ± 1.18	3.00 ± 0.85	2.92 ± 0.90	2.67 ± 1.07	0.25 ± 1.29	0.17 ± 1.34	-0.08 ± 1.38
	Test	**2.08 ± 0.86** *	**2.23 ± 0.93** *	**2.15 ± 0.90** *	2.46 ± 1.05	0.15 ± 0.55	0.08 ± 0.64	0.38 ± 0.65
Eye pain	Control	2.00 ± 1.35	2.08 ± 1.16	2.00 ± 1.35	1.92 ± 1.08	0.08 ± 0.67	0.00 ± 0.43	-0.08 ± 0.79
	Test	1.69 ± 0.75	1.92 ± 0.86	1.85 ± 0.55	1.92 ± 0.76	0.23 ± 0.73	0.15 ± 0.69	0.23 ± 0.73
Stiff shoulders	Control	2.83 ± 1.27	2.92 ± 1.24	2.75 ± 1.06	2.42 ± 1.00	0.08 ± 0.29	-0.08 ± 0.51	-0.42 ± 0.51
	Test	3.23 ± 1.17	3.00 ± 1.22	2.77 ± 1.36	3.00 ± 1.22	**-0.23 ± 0.44** *	-0.46 ± 0.78	-0.23 ± 0.83
Muscular pain/stiffness	Control	1.92 ± 0.90	2.42 ± 0.90	2.17 ± 0.72	**1.92 ± 0.67** *	0.50 ± 0.80	0.25 ± 0.97	0.00 ± 0.85
	Test	2.77 ± 1.17	2.85 ± 1.21	2.85 ± 1.34	2.85 ± 0.99	0.08 ± 1.12	0.08 ± 1.26	0.08 ± 0.64
Palpitations	Control	1.50 ± 0.52	1.67 ± 0.49	1.67 ± 0.65	1.58 ± 0.51	0.17 ± 0.39	0.17 ± 0.39	0.08 ± 0.29
	Test	1.69 ± 1.03	1.69 ± 0.95	1.77 ± 0.73	2.00 ± 0.82	0.00 ± 0.41	0.08 ± 0.64	0.31 ± 0.63
Shortness of breath	Control	1.67 ± 0.65	1.67 ± 0.49	1.73 ± 0.65	1.58 ± 0.67	0.00 ± 0.43	-0.08 ± 0.67	-0.08 ± 0.51
	Test	1.77 ± 0.93	1.85 ± 0.80	1.92 ± 0.76	2.00 ± 0.91	0.08 ± 0.76	0.15 ± 0.80	0.23 ± 0.60
Tendency to gain weight	Control	2.58 ± 1.08	2.67 ± 0.89	2.25 ± 0.97	2.42 ± 1.00	0.08 ± 0.51	-0.33 ± 0.65	-0.17 ± 0.58
	Test	2.54 ± 1.27	2.85 ± 1.21	2.69 ± 1.03	2.69 ± 0.95	0.31 ± 0.75	0.15 ± 0.90	0.15 ± 0.69
Weight loss ;thin	Control	1.75 ± 0.87	1.75 ± 0.97	2.00 ± 0.85	2.00 ± 0.95	0.00 ± 0.85	0.25 ± 0.75	0.25 ± 0.87
	Test	1.69 ± 0.63	1.54 ± 0.66	1.62 ± 0.65	1.54 ± 0.66	-0.15 ± 0.8	-0.08 ± 0.28	-0.15 ± 0.55
Lethargy	Control	2.58 ± 1.00	2.75 ± 1.06	2.75 ± 0.87	2.50 ± 0.80	0.17 ± 0.83	0.17 ± 0.58	-0.08 ± 0.79
	Test	2.69 ± 0.85	2.69 ± 0.85	2.31 ± 1.03	2.54 ± 0.78	0.00 ± 0.82	**-0.38 ± 0.65** *	-0.15 ± 0.69
No feeling of good health	Control	2.08 ± 1.00	2.17 ± 0.94	2.42 ± 1.00	2.17 ± 0.83	0.08 ± 0.79	0.33 ± 0.49	0.08 ± 0.79
	Test	2.15 ± 0.99	2.08 ± 0.76	1.85 ± 0.69	1.92 ± 0.86	-0.08 ± 0.76	**-0.31 ± 0.75** *	-0.23 ± 0.73
Thirst	Control	1.92 ± 1.08	2.08 ± 1.00	2.08 ± 1.00	2.08 ± 1.00	0.17 ± 0.58	0.17 ± 0.58	0.17 ± 0.58
	Test	2.15 ± 1.07	2.23 ± 0.83	1.85 ± 0.69	2.23 ± 0.83	0.08 ± 0.49	-0.31 ± 0.75	0.08 ± 0.64

Item	Group	average value ± SD				average variation (vs 0w) ± SD		
		0w	4w	8w	12w	4w	8w	12w
Skin problems	Control	2.17 ± 0.72	**1.92 ± 0.79 ***	2.33 ± 0.89	2.08 ± 0.9	-0.25 ± 0.62	0.17 ± 0.39	-0.08 ± 0.67
	Test	2.58 ± 0.67	2.62 ± 0.87	2.54 ± 0.78	2.69 ± 0.75	0.23 ± 1.01	0.15 ± 0.8	0.31 ± 1.11
Anorexia	Control	1.58 ± 0.67	**1.25 ± 0.45 ***	1.25 ± 0.45	1.50 ± 0.52	**-0.33 ± 0.49 ***	-0.33 ± 0.65	-0.08 ± 0.67
	Test	1.69 ± 0.85	1.77 ± 0.73	1.69 ± 0.63	1.62 ± 0.65	0.08 ± 0.49	0.00 ± 0.41	-0.08 ± 0.28
Early satiety	Control	1.67 ± 0.78	1.42 ± 0.67	1.50 ± 0.52	1.67 ± 0.49	-0.25 ± 0.45	-0.17 ± 0.58	0.00 ± 0.6
	Test	1.83 ± 0.83	1.69 ± 0.48	1.85 ± 0.69	1.77 ± 0.73	0 ± 0.71	0.15 ± 0.55	0.08 ± 0.64
Epigastralgia	Control	1.83 ± 0.83	1.75 ± 0.97	1.75 ± 1.14	1.83 ± 0.83	-0.08 ± 0.51	-0.08 ± 0.79	0 ± 0.60
	Test	1.75 ± 0.62	1.92 ± 0.49	2.00 ± 0.58	1.69 ± 0.63	0.31 ± 0.75	0.38 ± 0.65	0.08 ± 0.28
Liable to catch cold	Control	1.92 ± 0.67	2.00 ± 0.74	2.08 ± 0.90	2.18 ± 0.60	0.08 ± 0.51	0.17 ± 0.58	0.08 ± 0.79
	Test	2.38 ± 1.04	2.15 ± 0.99	2.31 ± 0.95	2.38 ± 0.96	-0.23 ± 0.73	-0.08 ± 0.49	0.00 ± 0.58
Coughing and sputum	Control	1.92 ± 0.90	1.92 ± 0.90	2.00 ± 1.21	2.33 ± 0.89	0.00 ± 0.43	0.08 ± 0.67	0.42 ± 0.67
	Test	2.15 ± 1.14	2.46 ± 1.13	2.23 ± 0.93	2.38 ± 0.96	0.31 ± 0.63	0.08 ± 0.86	0.23 ± 0.93
Diarrhea	Control	1.75 ± 0.97	1.75 ± 0.87	1.67 ± 0.89	1.75 ± 0.75	0.00 ± 0.43	-0.08 ± 0.29	0.00 ± 0.95
	Test	2.31 ± 0.95	2.23 ± 0.83	2.23 ± 0.83	2.23 ± 0.60	-0.08 ± 0.49	-0.08 ± 0.64	-0.08 ± 0.64
Constipation	Control	1.75 ± 1.06	1.58 ± 0.90	1.67 ± 0.98	1.83 ± 1.03	**-0.17 ± 0.58 ***	-0.08 ± 0.29	0.08 ± 0.51
	Test	2.15 ± 0.99	2.46 ± 1.20	2.23 ± 0.93	2.38 ± 0.96	0.31 ± 0.48	0.08 ± 0.28	0.23 ± 0.73
Hair loss	Control	2.00 ± 0.95	2.08 ± 1.00	1.83 ± 1.03	1.83 ± 0.94	0.08 ± 0.79	-0.17 ± 0.72	-0.17 ± 0.58
	Test	2.23 ± 0.73	2.69 ± 0.85	2.31 ± 0.75	2.46 ± 0.66	0.46 ± 0.52	0.08 ± 0.28	0.23 ± 0.73
Gray hair	Control	3.25 ± 1.14	3.25 ± 1.22	3.42 ± 1.31	3.17 ± 1.34	0.00 ± 0.6	0.17 ± 0.72	-0.08 ± 0.90
	Test	2.92 ± 1.38	3.00 ± 1.35	2.77 ± 1.24	3.08 ± 1.32	0.08 ± 0.49	-0.15 ± 0.38	0.15 ± 0.55
Headache	Control	2.17 ± 1.27	1.92 ± 0.79	2.00 ± 0.85	1.83 ± 0.72	-0.25 ± 0.87	-0.17 ± 1.19	-0.33 ± 1.15
	Test	2.23 ± 0.83	2.54 ± 1.13	2.31 ± 0.95	2.31 ± 0.95	0.31 ± 0.75	0.08 ± 0.28	0.08 ± 0.64
Dizziness	Control	1.83 ± 0.94	1.92 ± 1.08	1.92 ± 0.90	1.67 ± 0.65	0.08 ± 0.51	0.08 ± 0.29	-0.17 ± 0.83
	Test	2.00 ± 0.91	2.00 ± 1.00	2.00 ± 0.82	2.08 ± 0.76	0.00 ± 0.41	0.00 ± 0.58	0.08 ± 0.49

Item	Group	average value ± SD				average variation (vs 0w) ± SD		
		0 w	4 w	8 w	12 w	4 w	8 w	12 w
Tinnitus	Control	1.75 ± 1.22	1.83 ± 1.19	1.75 ± 1.14	1.92 ± 1.08	0.08 ± 0.67	0.00 ± 0.43	0.17 ± 0.58
	Test	1.85 ± 0.69	1.92 ± 0.86	1.69 ± 0.63	1.77 ± 0.60	0.08 ± 0.76	-0.15 ± 0.38	-0.08 ± 0.49
Hearing difficulty	Control	2.08 ± 0.90	2.08 ± 0.90	1.92 ± 0.90	1.92 ± 0.90	0.00 ± 0.00	-0.17 ± 0.58	-0.17 ± 0.58
	Test	1.69 ± 0.63	1.62 ± 0.51	1.46 ± 0.52	1.77 ± 0.44	-0.08 ± 0.28	-0.23 ± 0.44	0.08 ± 0.49
Lumbago	Control	2.17 ± 0.94	**2.25 ± 0.87** *	**2.00 ± 0.85** *	**1.75 ± 0.62** *	0.08 ± 0.79	-0.17 ± 0.58	**-0.42 ± 0.67** *
	Test	2.85 ± 1.34	3.23 ± 1.24	2.92 ± 1.12	3.23 ± 1.24	0.38 ± 0.96	0.08 ± 0.95	0.38 ± 0.96
Arthralgia	Control	**1.42 ± 0.51** *	**1.50 ± 0.52** *	**1.50 ± 0.67** *	**1.42 ± 0.51** *	0.08 ± 0.29	0.08 ± 0.67	0.00 ± 0.43
	Test	2.08 ± 0.76	2.08 ± 0.76	2.23 ± 0.93	2.38 ± 1.12	0.00 ± 0.82	0.15 ± 0.38	0.31 ± 0.85
Edematous	Control	**1.83 ± 0.83** *	2.25 ± 1.29	2.08 ± 1.08	**1.67 ± 0.78** *	0.42 ± 0.79	0.25 ± 0.62	-0.17 ± 0.39
	Test	2.69 ± 1.03	2.54 ± 1.05	2.38 ± 0.96	2.62 ± 1.04	-0.15 ± 0.90	**-0.31 ± 0.63** *	-0.08 ± 1.04
Easily breaking into a sweat	Control	2.75 ± 1.06	2.33 ± 0.98	2.17 ± 1.03	**1.92 ± 0.90** *	-0.42 ± 1.00	-0.58 ± 0.79	**-0.83 ± 0.94** *
	Test	2.85 ± 1.34	3.15 ± 1.34	2.54 ± 1.13	2.92 ± 1.19	0.31 ± 1.03	-0.31 ± 0.75	0.08 ± 0.86
Frequent urination	Control	2.25 ± 1.06	2.33 ± 1.23	1.83 ± 1.34	1.83 ± 0.83	0.08 ± 0.67	-0.42 ± 0.67	-0.42 ± 0.79
	Test	2.23 ± 1.09	2.23 ± 1.09	2.23 ± 1.01	2.23 ± 0.83	0.00 ± 0.41	0.00 ± 0.58	0.00 ± 0.71
Hot flash	Control	1.42 ± 0.51	1.58 ± 0.67	**1.25 ± 0.45** *	1.50 ± 0.52	0.17 ± 0.58	**-0.17 ± 0.39** *	0.08 ± 0.29
	Test	1.62 ± 0.65	1.62 ± 0.65	1.77 ± 0.73	1.92 ± 0.86	0.00 ± 0.00	0.15 ± 0.38	0.31 ± 0.48
Cold skin	Control	2.08 ± 0.90	2.00 ± 0.95	2.00 ± 1.04	2.08 ± 1.00	-0.08 ± 0.51	-0.08 ± 0.67	0.00 ± 0.60
	Test	2.38 ± 1.12	2.46 ± 1.13	2.54 ± 1.05	2.62 ± 0.87	0.08 ± 0.95	0.15 ± 0.69	0.23 ± 0.83

Subjects: n = 12 (Control group), n = 13 (Test group). Measured value: average value ± SD. *: $p < 0.05$ significant difference in improvement in comparison between groups. AAQol, Anti-Aging QOL Common Questionnaire; SD, standard deviation.

Table 4-2. Mental subjective symptom score in AAQOL.

Item	Group	average value ± SD				average variation (vs 0w) ± SD		
		0 w	4 w	8 w	12 w	4 w	8 w	12 w
Irritability	Control	2.58 ± 1.16	2.58 ± 1.00	2.67 ± 1.15	2.67 ± 0.98	0.00 ± 0.43	0.08 ± 0.79	0.08 ± 0.67
	Test	2.31 ± 0.85	2.23 ± 0.60	2.23 ± 0.93	2.38 ± 0.77	-0.08 ± 0.49	-0.08 ± 0.64	0.08 ± 0.49
Easily angered	Control	2.42 ± 1.16	2.50 ± 1.09	2.42 ± 1.08	2.25 ± 0.87	0.08 ± 0.67	0.00 ± 1.04	-0.17 ± 0.72
	Test	2.31 ± 0.63	2.08 ± 0.64	2.23 ± 0.73	2.31 ± 0.75	-0.23 ± 0.44	-0.08 ± 0.76	0.00 ± 0.58
Loss of motivation	Control	2.33 ± 0.98	2.17 ± 0.94	1.83 ± 0.72	2.08 ± 0.79	-0.17 ± 0.94	-0.50 ± 0.90	-0.25 ± 0.62
	Test	2.00 ± 0.58	1.92 ± 0.64	1.92 ± 0.64	2.00 ± 0.71	-0.08 ± 0.28	-0.08 ± 0.49	0.00 ± 0.58
No feeling of happiness	Control	1.92 ± 0.67	2.17 ± 0.72	2.00 ± 0.74	1.75 ± 0.62	0.25 ± 0.45	0.08 ± 0.29	-0.17 ± 0.72
	Test	1.69 ± 0.63	1.85 ± 0.55	1.85 ± 0.69	2.00 ± 0.71	0.15 ± 0.38	0.15 ± 0.55	0.31 ± 0.48
Nothing to look forward to in life	Control	1.75 ± 0.62	1.83 ± 0.58	1.67 ± 0.49	1.83 ± 0.58	0.08 ± 0.51	-0.08 ± 0.51	0.08 ± 0.51
	Test	1.85 ± 0.69	1.85 ± 0.69	1.77 ± 0.60	2.08 ± 0.76	0.00 ± 0.00	-0.08 ± 0.64	0.23 ± 0.44
Daily life is not enjoyable	Control	1.83 ± 0.72	2.08 ± 0.79	1.75 ± 0.75	1.83 ± 0.72	0.25 ± 0.45	-0.08 ± 0.29	0.00 ± 0.74
	Test	1.77 ± 0.60	1.85 ± 0.55	1.85 ± 0.69	2.00 ± 0.71	0.08 ± 0.28	0.08 ± 0.28	0.23 ± 0.44
Lose confidence	Control	1.75 ± 0.62	1.92 ± 0.79	1.75 ± 0.75	1.67 ± 0.65	0.17 ± 0.58	0.00 ± 0.43	-0.08 ± 0.67
	Test	2.00 ± 0.71	2.08 ± 0.76	1.92 ± 0.76	2.00 ± 0.82	0.08 ± 0.49	-0.08 ± 0.28	0.00 ± 0.58
Reluctance to talk with others	Control	1.75 ± 0.62	2.08 ± 0.79	1.83 ± 0.72	1.67 ± 0.65	0.33 ± 0.49	0.08 ± 0.51	-0.08 ± 0.79
	Test	1.85 ± 0.55	2.00 ± 0.58	1.92 ± 0.64	1.92 ± 0.76	0.15 ± 0.38	0.08 ± 0.49	0.08 ± 0.49
Depressed	Control	2.00 ± 0.85	2.08 ± 0.79	1.67 ± 0.78	1.75 ± 0.75	0.08 ± 0.51	-0.33 ± 0.49	-0.25 ± 0.62
	Test	1.85 ± 0.55	1.92 ± 0.64	1.85 ± 0.69	2.00 ± 0.82	0.08 ± 0.49	0.00 ± 0.41	0.15 ± 0.55
Feeling of uselessness	Control	2.17 ± 0.58	2.08 ± 0.67	1.75 ± 0.75	1.73 ± 0.65	-0.08 ± 0.51	-0.42 ± 0.67	**-0.58 ± 1.00** *
	Test	1.92 ± 0.64	2.08 ± 0.64	1.92 ± 0.49	2.08 ± 0.76	0.15 ± 0.38	0.00 ± 0.58	0.15 ± 0.55
Shallow sleep	Control	2.42 ± 1.24	2.42 ± 1.00	2.25 ± 1.29	2.08 ± 1.16	0.00 ± 0.74	-0.17 ± 0.83	-0.33 ± 0.78
	Test	2.46 ± 1.27	2.46 ± 1.27	2.46 ± 1.27	2.46 ± 1.20	0.00 ± 0.58	0.00 ± 0.41	0.00 ± 0.58
Difficulty in falling asleep	Control	2.33 ± 1.44	2.08 ± 1.00	2.08 ± 1.24	1.83 ± 1.03	-0.25 ± 0.87	-0.25 ± 0.75	-0.50 ± 0.80
	Test	2.23 ± 1.09	2.23 ± 0.93	2.15 ± 1.07	2.15 ± 0.90	0.00 ± 0.71	-0.08 ± 0.28	-0.08 ± 0.64

Item	Group	average value ± SD				average variation (vs 0w) ± SD		
		0w	4w	8w	12w	4w	8w	12w
Pessimism	Control	2.33 ± 0.78	2.08 ± 0.67	2.00 ± 0.74	2.00 ± 0.60	-0.25 ± 0.87	**-0.33 ± 0.65** *	-0.33 ± 0.65
	Test	2.08 ± 0.64	2.00 ± 0.91	2.23 ± 0.83	2.08 ± 0.86	-0.08 ± 0.49	0.15 ± 0.38	0.00 ± 0.58
Lapse of memory	Control	2.58 ± 0.79	2.58 ± 0.79	2.58 ± 1.00	2.25 ± 0.75	0.00 ± 0.60	0.00 ± 0.43	-0.33 ± 0.49
	Test	2.92 ± 0.49	2.62 ± 0.51	2.31 ± 0.85	2.54 ± 0.66	-0.31 ± 0.48	**-0.62 ± 0.77** *	-0.38 ± 0.65
Inability to concentrate	Control	2.08 ± 0.67	2.25 ± 0.75	2.00 ± 0.60	1.75 ± 0.62	0.17 ± 0.39	-0.08 ± 0.29	-0.33 ± 0.49
	Test	2.23 ± 0.60	2.08 ± 0.64	2.08 ± 0.64	2.23 ± 0.73	**-0.15 ± 0.38** *	-0.15 ± 0.38	0.00 ± 0.58
Inability to solve problems	Control	2.00 ± 0.74	2.00 ± 0.6	1.83 ± 0.72	1.75 ± 0.62	0.00 ± 0.74	-0.17 ± 0.58	-0.25 ± 0.45
	Test	2.08 ± 0.49	2.08 ± 0.49	2.00 ± 0.71	2.00 ± 0.71	0.00 ± 0.41	-0.08 ± 0.49	-0.08 ± 0.64
Inability to make judgments readily	Control	2.08 ± 0.79	1.83 ± 0.58	1.83 ± 0.72	1.83 ± 0.72	-0.25 ± 0.62	-0.25 ± 0.45	-0.25 ± 0.45
	Test	2.23 ± 0.60	2.00 ± 0.58	1.92 ± 0.64	2.08 ± 0.76	-0.23 ± 0.44	-0.31 ± 0.48	-0.15 ± 0.80
Inability to sleep because of worries	Control	2.08 ± 1.16	3.58 ± 6.16	2.17 ± 1.19	1.91 ± 0.83	1.50 ± 5.55	0.08 ± 0.67	-0.33 ± 1.15
	Test	2.31 ± 1.11	2.31 ± 0.75	2.23 ± 1.01	2.08 ± 0.86	0.00 ± 0.82	-0.08 ± 0.49	-0.23 ± 0.60
A sense of tension	Control	2.25 ± 1.14	2.25 ± 1.22	2.17 ± 1.11	2.08 ± 1.08	0.00 ± 0.43	-0.08 ± 0.79	-0.17 ± 0.72
	Test	2.23 ± 0.83	2.46 ± 0.78	2.15 ± 0.90	2.31 ± 0.75	0.23 ± 0.73	-0.08 ± 0.95	0.08 ± 0.49
Feeling of anxiety for no special reason	Control	1.92 ± 0.79	1.75 ± 0.87	2.00 ± 0.74	1.92 ± 0.67	-0.17 ± 0.58	0.08 ± 0.51	0.00 ± 0.43
	Test	1.85 ± 0.69	1.85 ± 0.55	1.92 ± 0.76	2.00 ± 0.82	0.00 ± 0.41	0.08 ± 0.28	0.15 ± 0.38
Vague feeling on fear	Control	1.83 ± 0.72	1.50 ± 0.52	1.67 ± 0.49	1.75 ± 0.62	-0.33 ± 0.49	-0.17 ± 0.58	-0.08 ± 0.51
	Test	1.69 ± 0.63	1.69 ± 0.63	1.77 ± 0.60	1.85 ± 0.80	0.00 ± 0.41	0.08 ± 0.28	0.15 ± 0.38
Stress	Control	2.75 ± 1.06	2.67 ± 1.07	2.50 ± 1.17	2.64 ± 1.03	-0.08 ± 0.67	-0.25 ± 0.87	-0.33 ± 0.78
	Test	2.62 ± 0.96	2.69 ± 0.85	2.46 ± 1.05	2.62 ± 0.65	0.08 ± 0.49	-0.15 ± 0.55	0.00 ± 0.82

Subjects: n = 12 (Control group), n = 13 (Test group). Measured value: average value ± SD. *: $p<0.05$ significant difference in improvement in comparison between groups. AAQol, Anti-Aging QOL Common Questionnaire; SD, standar deviation.

Table 5. Anthropometry and physical examination.

Item	Group	average value ± SD				average variation (vs 0w) ± SD		
		0 w	4 w	8 w	12 w	4 w	8 w	12 w
weight (kg)	Control	60.8 ± 9.7	61.1 ± 9.7	60.9 ± 9.4	60.5 ± 9.5	0.3 ± 0.7	0.1 ± 1.4	-0.3 ± 1.8
	Test	60.2 ± 10.9	60.3 ± 10.6	60 ± 10.5	59.9 ± 10.3	0.1 ± 0.8	-0.2 ± 0.7	-0.3 ± 1.0
Basal metabolic rate (kcal)	Control	1382.4 ± 218.2	1383.3 ± 219.2	1377.5 ± 213.9	1369.8 ± 218.5	0.9 ± 20.9	-4.9 ± 30.7	-12.6 ± 35.4
	Test	1352.9 ± 230.4	1352.5 ± 225.4	1346.9 ± 223.3	1342.9 ± 219.8	-0.3 ± 15.0	-6 ± 14.6	-9.9 ± 19.4
percentage skeletal muscle (%)	Control	29.9 ± 2.5	29.5 ± 2.3	29.1 ± 2.2	29.0 ± 2.4	-0.4 ± 1.6	-0.8 ± 1.6	-0.9 ± 1.7
	Test	28.3 ± 2.9	28 ± 3.0	27.9 ± 2.8	27.8 ± 2.8	-0.2 ± 0.5	-0.4 ± 0.6	-0.5 ± 0.6
BMI	Control	21.7 ± 2.5	21.8 ± 2.5	21.8 ± 2.3	21.6 ± 2.3	0.1 ± 0.3	0.0 ± 0.5	-0.1 ± 0.6
	Test	22.4 ± 3.3	22.4 ± 3.1	22.3 ± 3.2	22.2 ± 3.1	0.1 ± 0.3	-0.1 ± 0.3	-0.1 ± 0.3
percentage body fat (%)	Control	23.5 ± 3.3	24.2 ± 2.8	24.9 ± 2.7	24.9 ± 2.9	0.6 ± 2.1	1.3 ± 1.9	1.4 ± 2.4
	Test	26.1 ± 4.6	26.4 ± 4.9	26.7 ± 4.6	27.0 ± 4.2	0.3 ± 0.9	0.6 ± 1.3	0.9 ± 1.6
Systolic blood pressure (mmHg)	Control	116.3 ± 14.2	124.0 ± 20.2	119.4 ± 17.0	122.3 ± 16.4	**7.8 ± 10.8 #**	3.2 ± 5.8	6.1 ± 5.3
	Test	122.0 ± 15.7	120.4 ± 17.8	124.6 ± 18.6	126.9 ± 20.7	**-1.6 ± 10.6 ***	2.7 ± 11.2	4.9 ± 13.8
Diastolic blood pressure (mmHg)	Control	76.2 ± 11.3	79.9 ± 14.5	78.7 ± 14.1	80.8 ± 11.7	3.7 ± 6.9	2.5 ± 4.9	4.7 ± 5.4
	Test	79.8 ± 14.7	76.6 ± 12.7	77.9 ± 10.7	79.1 ± 11.8	**-3.2 ± 7.3 ***	-1.9 ± 8.2	-0.7 ± 7.7
Heart Rate (beats/min)	Control	68.4 ± 9.5	71 ± 9.0	71.0 ± 8.8	70.2 ± 8.4	2.6 ± 3.6	2.6 ± 3.6	1.8 ± 5.2
	Test	66.1 ± 8.3	65.1 ± 8.6	65.8 ± 7.0	64.0 ± 7.0	-1 ± 5.1	-0.3 ± 4.0	-2.2 ± 5.2

Subjects: n = 12 (Control group), n = 13 (Test group). Measured value: average value ± SD. *: $p < 0.05$ indicated comparisons between control and test by unpaired Student's t-test. #: $p < 0.05$ indicated comparisons vs 0w by Dunnett's test. BMI, body mass index; SD, standard deviation.

Table 6. Stratified analysis in female subjects.

Item	Group	average value ± SD				average variation (vs 0w) ± SD		
		0 w	4 w	8 w	12 w	4 w	8 w	12 w
Skin elasticity test (R2)	Control	0.86 ± 0.05	0.88 ± 0.01	0.88 ± 0.01	0.89 ± 0.02	0.01 ± 0.05	0.01 ± 0.05	0.03 ± 0.04
(R2)	Test	0.87 ± 0.03	0.88 ± 0.03	**0.91 ± 0.03#**	**0.90 ± 0.02#**	0.01 ± 0.03	**0.04 ± 0.03#**	**0.03 ± 0.02#**
Skin elasticity test (R7)	Control	0.65 ± 0.07	0.66 ± 0.06	0.69 ± 0.05	0.69 ± 0.04	0.01 ± 0.03	0.03 ± 0.04	0.03 ± 0.05
(R7)	Test	0.67 ± 0.06	0.69 ± 0.03	**0.72 ± 0.04#**	**0.72 ± 0.04#**	0.02 ± 0.04	**0.05 ± 0.03#**	**0.05 ± 0.04#**
HbA1c (%)	Control	5.28 ± 0.44	5.38 ± 0.40	5.43 ± 0.38	5.35 ± 0.27	0.10 ± 0.18	0.15 ± 0.18	0.07 ± 0.21
	Test	5.49 ± 0.17	5.44 ± 0.21	5.46 ± 0.21	5.49 ± 0.28	-0.04 ± 0.11	**-0.03 ± 0.10***	0.00 ± 0.18
TC (mg/dL)	Control	201.3 ± 39.6	205.0 ± 42.9	214.0 ± 43.0	224.8 ± 51.4	3.7 ± 19.0	12.7 ± 13.6	23.5 ± 28.3
	Test	211.7 ± 24.2	208.6 ± 24.9	202.9 ± 32.8	215.7 ± 27.1	-3.1 ± 13.4	**-8.9 ± 15.6***	4.0 ± 13.9
TG (mg/dL)	Control	54.5 ± 17.9	42.8 ± 14.2	53.9 ± 16.8	58.2 ± 12.8	**-11.7 ± 6.3#**	-0.7 ± 7.5	3.7 ± 10.7
	Test	63.1 ± 36.1	52.3 ± 23.7	**51.4 ± 35.3#**	57.0 ± 23.7	-10.9 ± 17.2	**-11.7 ± 9.9***	-6.1 ± 16.2
LDL-C (mg/dL)	Control	110.7 ± 26.7	109.5 ± 28.5	112.0 ± 30.3	118.8 ± 31.6	-1.2 ± 12.1	1.3 ± 7.8	8.2 ± 16.0
	Test	114.9 ± 19.3	114.0 ± 21.8	105.7 ± 18.5	115.1 ± 21.0	-0.9 ± 15.3	-9.1 ± 10.7	0.3 ± 12.9

Subjects: n = 12 (Control group), n = 13 (Test group). Measured value: average value ± SD. *: $p < 0.05$ indicated comparisons between control and test by unpaired Student's t-test. #: $p < 0.05$ indicated comparisons vs 0w by Dunnett's test. TC, total cholesterol; TG, triglyceride; LDL-C, low-density lipoprotein cholesterol; SD, standard deviation.

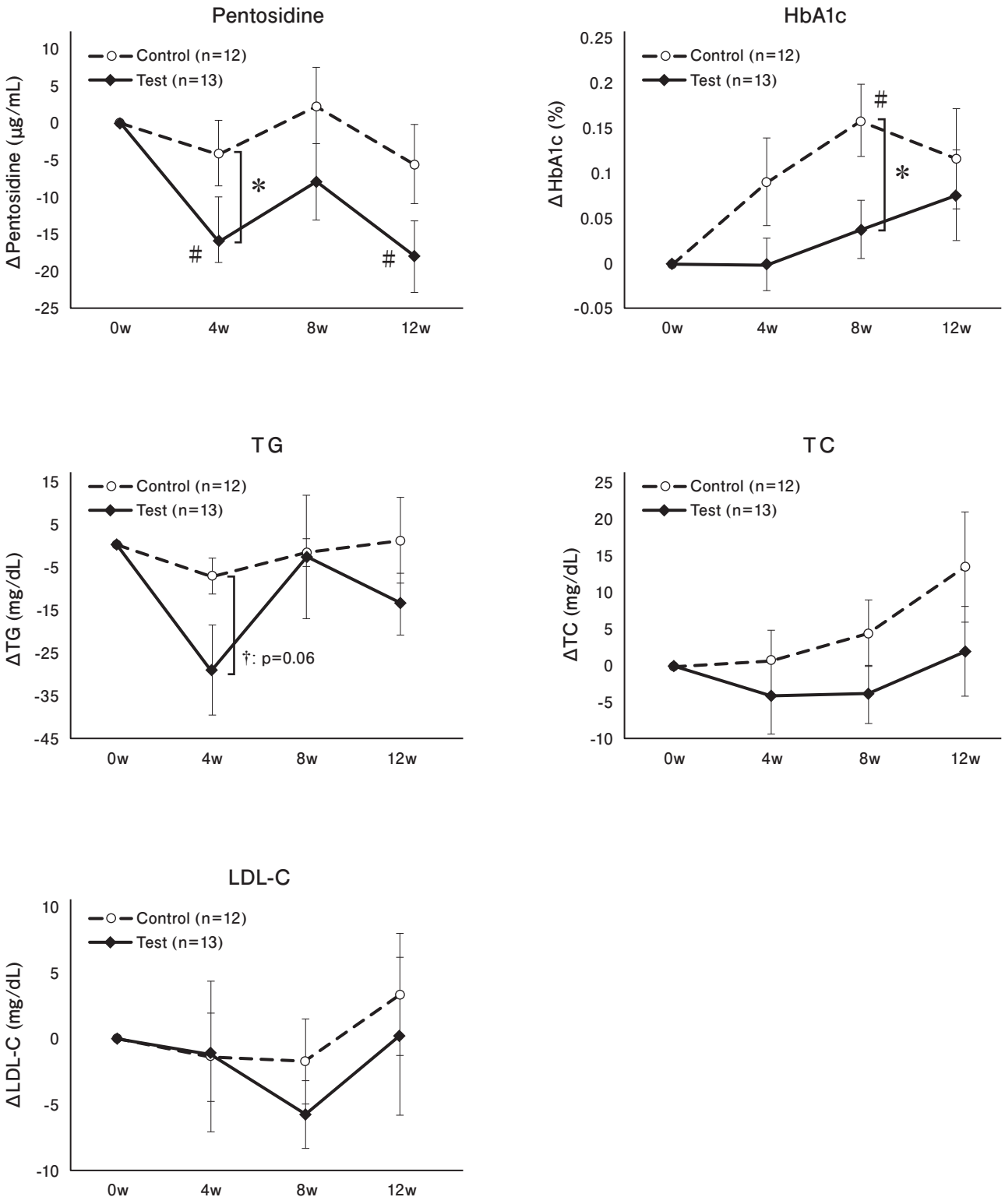

Fig. 1. **Changes of serum markers.**

a) Serum pentosidine, **b)** HbA1c, **c)** TG, **d)** TC, **e)** LDL-C. Bar indicates standard error. * $p < 0.05$ by t-test, † $p = 0.06$, #: $p < 0.05$ vs.0w by Dunnett's test. TG, triglyceride; TC, total cholesterol; LDL-C, low-density lipoprotein-cholesterol. †

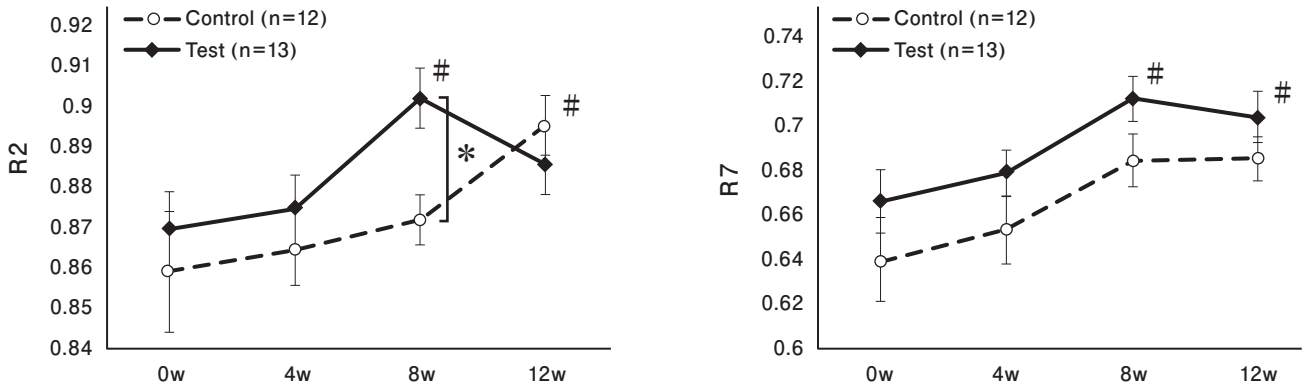

Fig. 2. **Changes of skin elasticity.**

a) R2, **b)** R7. Bar indicates standard error. * $p < 0.05$ by t-test, # $p < 0.05$ vs.0w by Dunnett's test.

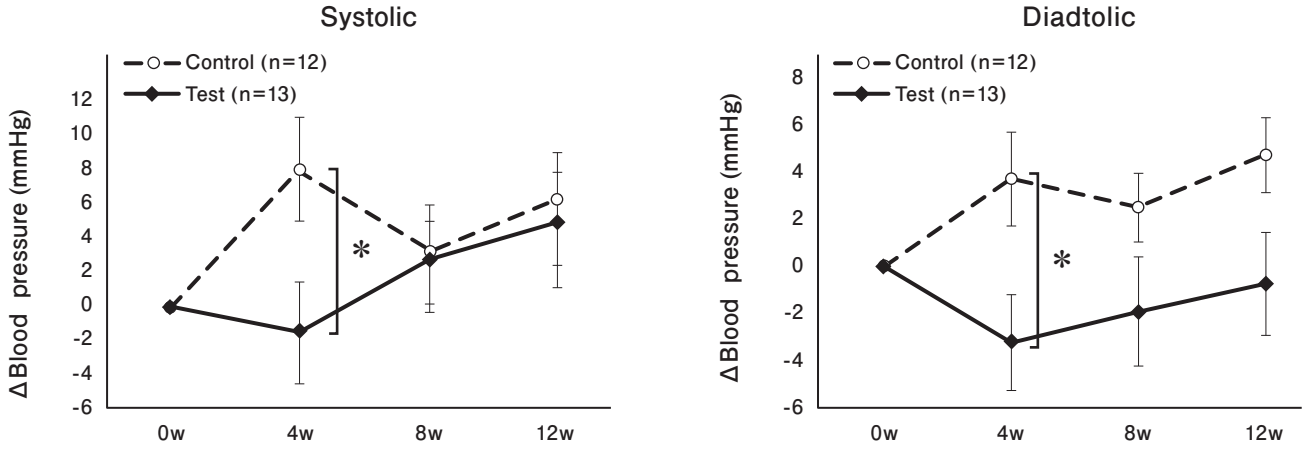

Fig. 3. **Changes of blood pressure.**

a) Systolic, **b)** Diastolic. Bar indicates standard error. *: $p<0.05$ by t-test.

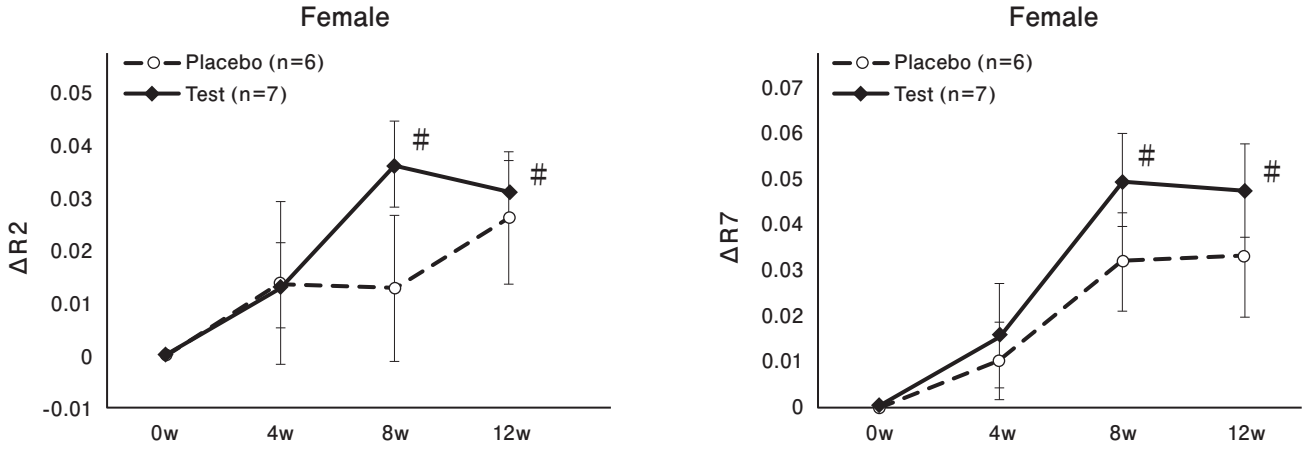

Fig. 4. **Changes of skin elasticity: Subclass analysis in female.**

a) R2, **b)** R7. Bar indicates standard error. # $p < 0.05$ vs.0w by Dunnett's test.

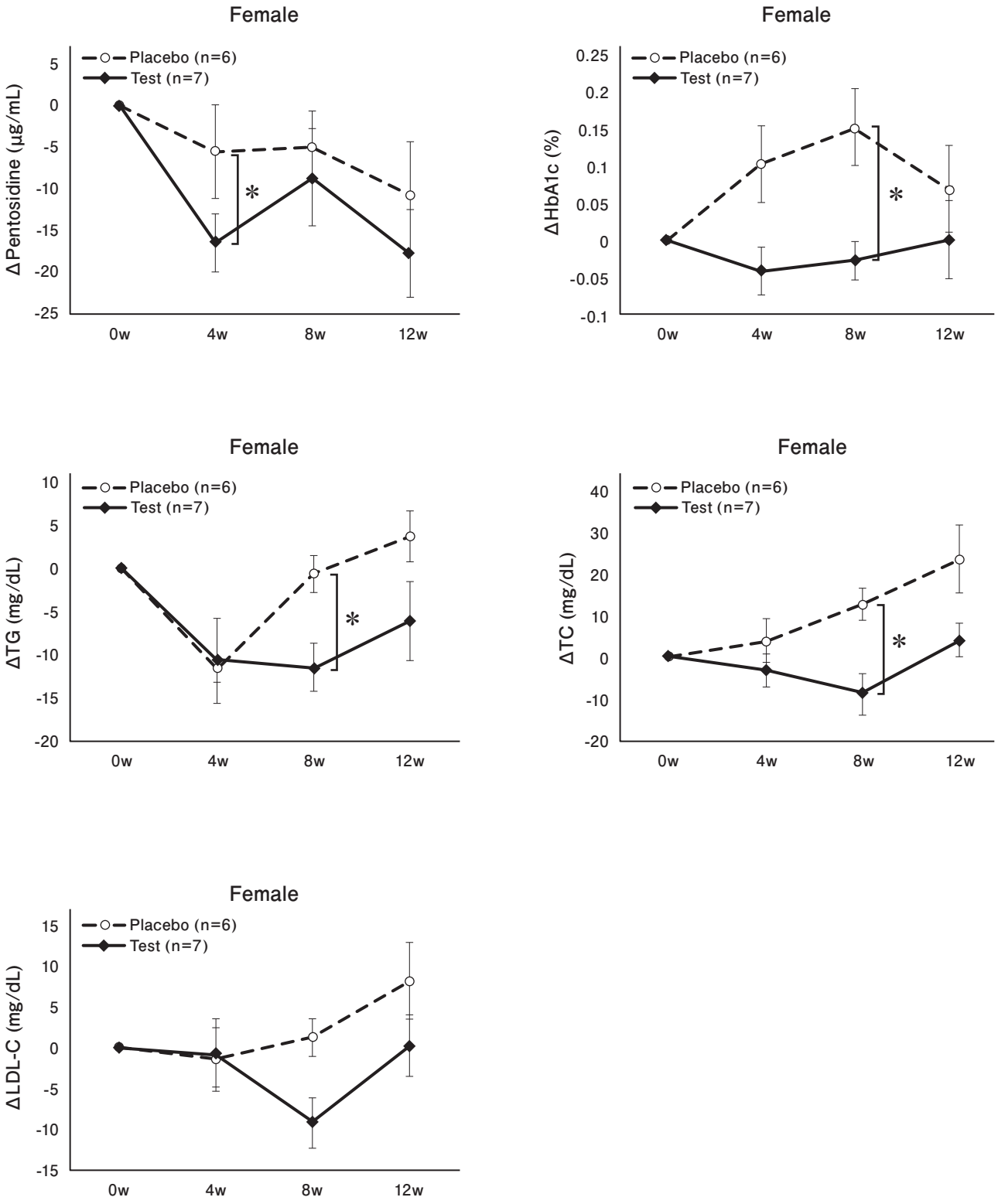

Fig. 5. **Changes of serum markers: Subclass analysis in female.**

a) Serum pentosidine, **b)** HbA1c, **c)** TG, **d)** TC, **e)** LDL-C. Bar indicates standard error. * $p < 0.05$ by t-test.

2. LDL glycation suppression test

Fig. 6 shows the results of the LDL glycation suppression test after TBE ingestion. The suppression action was expressed relative to the experimental control (100%) for each condition. By adding TBE, CML generation was suppressed in a concentration-dependent manner ($p < 0.05$). In a test with the positive control AG, CML generation was significantly suppressed at an AG concentration of 50 μg/mL ($p < 0.01$).

Discussion

Our research subject, TBE, is an extract obtained from the outer peel of the Water chestnut, a plant from the family Lythraceae and genus *Trapa* [18]. Fruit peel extract of Trapa plants has been used as a tea and herbal medicine for a long time [18]. Such food experience leads to the expectation that the Water chestnut peel is a safe plant material. However, there is currently no report on the efficacy of water chestnut peel extract when ingested over the long-term. Thus, we prepared TBE, consisting of Trapa peel hot water extract, and conducted a placebo-controlled, double-blind study in humans to examine the effects of ingesting 100 mg of TBE per day for 12 weeks.

Blood biochemistry in all subjects — 13 subjects in the trial group (47.5 ± 7.0 years) and 12 subjects in the control group (48.5 ± 4.6 years) — showed that compounds related to glycometabolism, specifically serum pentosidine, significantly decreased in the trial group, and that the change on week 4 in the trial group was significantly different from that in the control group. Similarly, HbA1c levels in the control group significantly increased, and were significantly higher than those observed in the trial group. Pentosidine is a type of AGEs generated through the glycation reaction, and HbA1c is a type of glycation product that is generated when a reducing sugar reacts with hemoglobin in the blood. According to a previous report [9], TBE has a strong ability to suppress glycation reactions and to decompose glycation products. As such, in the trial group, the generation and decomposition of pentosidine and HbA1c were suppressed, leading to a reduction in their concentrations. Furthermore, sustained hyperglycemic conditions are reported to promote AGE production [19]. TBE has an inhibitory effect on glucose decomposition via α-amylase and α-glucosidase inhibitory actions, and it slows the rise of postprandial blood glucose levels [12]. Therefore, continued ingestion of TBE may control increases in blood glucose levels. In the trial group, the glycation stress suppression effect is considered to be one of the mechanisms in which the glycation reaction did not actively occur due to the alleviation of postprandial hyperglycemia. As a comprehensive action, continued ingestion of TBE may suppress the generation and accumulation of AGEs in the blood.

Similarly, markers related to lipid metabolism in blood biochemistry did not show significant change in either groups in any subjects. Therefore, considering the sex-based differences in lipid metabolism, we conducted a subgroup analysis of female subjects (seven subjects in the trial group: 45.7 ± 8.0 years, and six subjects in the control group: 50.1 ± 5.1 years). Results showed that TG and TC levels in the trial group showed significant decreases. Furthermore, LDL-C levels in the trial group showed a decreasing trend compared to the control group, though the difference was not significant ($p = 0.07$). According to existing reports [20, 21], LDL is a target of a glycation reaction, where the lysine residue from the

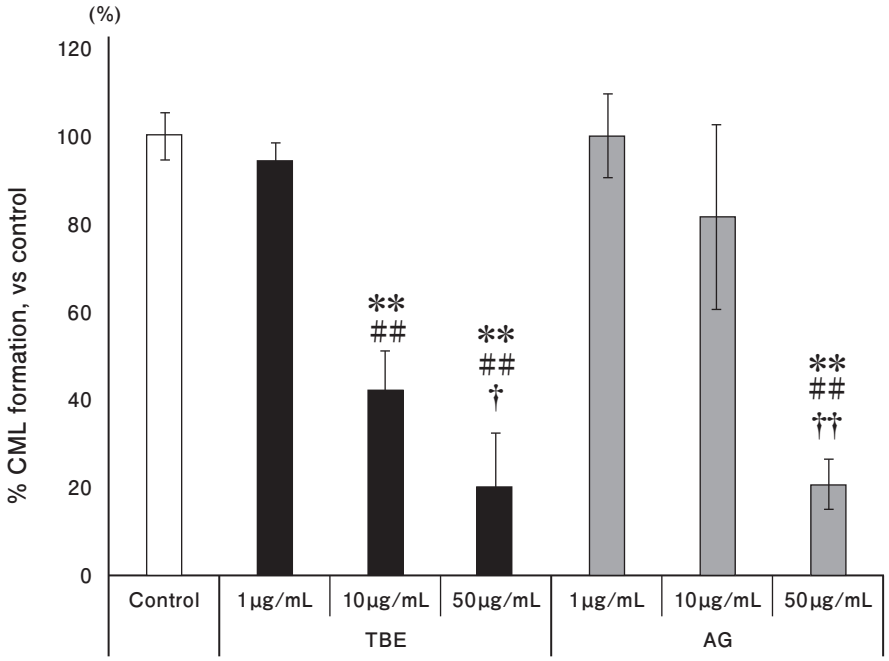

Fig. 6. **CML formation from LDL incubated with glyoxal.**

Bar indicates standard deviation. ** $p < 0.01$ vs Control, ## $p < 0.01$ vs 1 μg/mL, † $p < 0.05$, †† $p < 0.01$ vs 10 μg/mL by Tukey-Kramer test (n = 4 each).

APO structure protein (APO protein B-100) is glycated, and the binding energy to LDL receptor decreases notably. A decrease in the binding energy to the LDL receptor delays the clearance of LDL from the body, and becomes part of the increase in LDL levels in blood. Therefore, we performed an *in vitro* test to determine the glycation of LDL. The results showed that TBE significantly suppressed formation of the CML structure in LDL, showing high activity equal to or more than the positive control AG (***Fig. 6***). Based on the above report and the results of the *in vitro* test, it can be concluded that TBE suppresses the glycation of LDL, contributing to a decrease in blood LDL.

In terms of sex-based differences in lipid metabolism, generally, menopausal women around 50 years of age experience a sudden increase in blood LDL-C, and the rate of cardiovascular incidences increase rapidly compared to women pre-menopause [22]. This occurs because of the uptake of LDL-C by the liver, and due to the decrease in estrogen associated with metabolism that is found in menopausal women [23]. It is difficult to examine how TBE worked on female subjects based on the present trial only, but it likely affected female lipid metabolism; thus, we hope to study this further.

A skin function analysis of all subjects showed that the skin elasticity indicators R2 and R7, both improved during the trial period in both groups, while in the trial group, improvement in skin elasticity was noted earlier. Next, we assumed that there is a sex-based difference in skin conditions due to the differences in skin care and estrus cycle, and performed a subgroup analysis that only targeted female subjects. Results showed that measured values and changes in R2 and R7 in the trial group significantly improved in weeks 8 and 12 compared to week 0.

TBE possesses strong anti-glycative action and encourages the decomposition action of glycation products. Indeed, in the present trial, the reduction of serum pentosidine was confirmed. Cross-linked AGE pentosidine forms unnecessary crosslinks between collagens, reducing skin elasticity [24]. By reducing the accumulation of glycation products, skin elasticity may be improved [25]. Based on these reports, against the background of improved skin elasticity, the generation of glycation products is suppressed and decomposition is promoted by TBE, reducing the accumulation of glycation products.

A comparison of the diastolic and systolic blood pressures in the groups showed a significant decrease in the trial group at week 4. At week 12, the diastolic blood pressure in the trial group did not change significantly, but compared to the control group, it tended to be low ($p = 0.059$). Trapa japonica, which is closely related to the raw plant material for the trial foodstuff, Water chestnut (*T. bispinosa*), is said to have an angiotensin-converting enzyme (ACE) inhibiting effect [26]. Therefore, an improvement in systolic and diastolic blood pressures may be due to an antihypertensive effect that originates in an ACE inhibiting effect.

Since there was no health damage from ingestion during the course of the trial, TBE is considered a functional foodstuff that can be ingested over a long period of time.

When TBE is ingested for a long time, a decrease in pentosidine occurs. Moreover, the efficacy of TBE against lipid metabolism was also indicated; thus, a potential of TBE to improve glycometabolism and lipid metabolism was indicated in this study. This is because of a strong anti-glycative effect and glycation product decomposition effect of TBE; thus, TBE may be applied to prevent lifestyle diseases including glycative stress. In this study, we selected subjects through fluorescence measurements of skin accumulation of AGEs, but since our results indicated that there are changes in glucose and lipid related items, more accurate test results would be obtained by using glucose and lipid related items in subject selection. We intend to continue studying the effects and mechanism of TBE with respect to glycometabolism and lipid metabolism.

Conclusion

We conducted a clinical trial on healthy Japanese males and females to determine the efficacy of ingesting 100 mg TBE/day for 12 weeks. Results showed that serum pentosidine decreased while LDL-C was suppressed. Furthermore, a subgroup analysis of female subjects showed significant improvement in skin elasticity. As such, the ingestion of TBE reduces glycation stress and improves lipid metabolism, indicating that it is effective in preventing lifestyle-related diseases.

Conflict of interest statement

In performing this study, we received support from Hayashikane Sangyo Co., Ltd.

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