Glycative Stress Research

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Original Artcle

Anti-glycation effect of pomegranate (*Punica granatum* L.) extract: An open clinical study

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Abstract

***Objective*:** The purpose of the present study is to elucidate the effect of pomegranate (*Punica granatum* L.) extracts on glycation stress reduction in an open clinical study without control subjects.
***Methods*:** Subjects were 10 post-menopausal females (aged 58.5 ± 3.9) who received oral administration of 100mg/day pomegranate extract (PE) for 12 weeks. At 0, 8 and 12 weeks the following glycative stress markers were analyzed: blood glucose, HbA1c, glycoalbumin, 3-deoxyglucosone (3DG), N^{ε}-(carboxymethyl) lysine (CML), pentosidine, and skin fluorescence from advanced glycation end products (AGEs).
***Results*:** HbA1c significantly was decreased at 8 and 12 weeks after PE administration. Glycoalbumin, 3DG and pentosidine showed significant reduction at 8 weeks, but not at 12 weeks. Serum CML increased at 12 weeks. No changes were noted in skin fluorescence AGEs intensity and skin elasticity. No severe adverse effect was observed during the test period.
***Conclusions*:** The 12-week oral administration of PE to healthy menopausal females showed a decrease in the glycative stress markers, indicating the potential effect of PE in reducing glycative stress.

***KEY WORDS*:** glycative stress, pomegranate (*Punica granatum* L.), advanced glycation end products (AGEs), 3-deoxyglucosone (3DG), N^{ε}-(carboxymethyl) lysine (CML), pentosidine.

Introduction

Glycation reaction, also called the Maillard reaction after the chemist who discovered the reaction, is a non-enzymatic irreversible reaction between reducing sugars and protein, finally generating advanced glycation end products (AGEs). AGEs are associated with the progression of diabetes and aging, and have been recently considered as a risk factor for age-related diseases and aging [1,2)] .

In our previous study on the inhibitory effects of various fruits, a high anti-glycation activity was found in pomegranate (*Punica granatum* L.) extract (PE)[3)]. Using the glycation model kit between collagen and glucose, the activity of PE was proven to be 3 fold that in other fruits which are reported to have anti-glycative activity [4)]. Since it will be important to examine the anti-glycation effect in humans, we undertook the present study to elucidate the anti-glycative effect of PE in an open clinical pilot study without control subjects.

Methods

Subjects

Subjects were 10 post-menopausal healthy females (aged 30 to 60 years old, 58.5 ± 3.9 years), with body mass index (BMI) of 22.3 ± 3.2 from who informed consent and written permission was obtained before participation. Persons with the following criteria were excluded:

1) Persons who have diabetes, renal dysfunction or a history of gastrointestinal surgery.
2) Persons taking anti-oxidative drugs or medications.
3) Persons not considered to be qualified by the responsible medical doctor.

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Study design

In this study, an open pilot study without control subjects, the subjects orally took 2 capsules per day, once a day for 12 weeks for a daily PE amount of 100 mg.

Before the study (0W), at 8 weeks (8W) and 12 weeks after the study (12W), the subjects underwent the following examinations: Anti-Aging QOL (quality of life) Common Questionnaire (AAQol) test, physical tests (blood pressure, pulse count, body weight and BMI), blood biochemistry tests [fasting plasma glucose (FPG), immune reactive insulin (IRI), HbA1c, glycoalbumin, 3-deoxyglucosone (3DG), N^{ε}-(carboxymethyl)lysine (CML) and pentosidine], and measurement of skin fluorescence from AGEs.

For the evaluation of safety, the subjects underwent the following examinations: peripheral blood tests (white blood cell count, red blood cell count, hemoglobin, hematocrit, platelet count, MCV, MCH, MCHC and white blood cell profile) and serum biochemistry tests [total protein,albumin, AST, ALT, LDH, total bilirubin, ALP, γ-GTP, BUN, creatinine, uric acid, sodium, chloride, potassium, calcium, total cholesterol (TC), low-density-lipid-cholesterol (LDL-C), high-density-lipid-cholesterol (HDL-C), and triglyceride (TG)]. The safety analyses will be documented in a subsequent report.

The subjects recorded, written in notes, about their lifestyles, adverse effects, and compliance of test sample intake. The study was conducted at Medical Corporation Shinkokai C'est La Vie Shinbashi Clinic (Minato-ku, Tokyo, Japan) during the period from November 1, 2012 to May 31, 2013.

Test product

The profile of the test product is presented in ***Table 1*** and components in one capsule in ***Table 2***. The test products were provided by Morishita Jintan Co., Ltd. (Chuo-ku, Osaka, Japan).

Table 1. Test product profile.

Test product	Pomegranate extract-containing capsule
Lot number	Lot.No.20121017
Production date	2012/10/12
Active ingredients per capsule	50 mg
Active ingredients per day	100 mg/2capsile
Type	Hard capsules
Amount	199.5 mg/capsule
Reserve condition	Room temperature
Date limit	1 year

Table 2. Ingredients in the test product per one capsule.

Ingredients	Test product	
	Amount (mg/capsule)	Ratio (%)
Pomegranate extract	50.0	25.1
Cellulose	94.0	47.1
Silicon dioxide	3.0	1.5
Calcium stearate	3.0	1.5
Gelatin	48.0	24.1
Caramel color	1.5	0.7
Total	199.5	100.0

Examination items

Subjective symptoms and anthropometry

Physical and mental symptoms were recorded and rated on a 5-grade scale using the Anti-Aging QOL Common Questionnaire (AAQol)[5,6]. The following anthropometric parameters were recorded: height (cm), body weight (kg), blood pressure (systolic/diastolic: mmHg), pulse rate (pulse/min).

Blood chemistry

The glycative stress markers measured by blood biochemistry included fasting plasma glucose (FPG), immune reactive insulin (IRI), HbA1c, glycoalbumin, 3-deoxyglucosone (3DG), CML and pentosidine. CML and 3DG were measured at Fushimi Pharmaceutical Co., Ltd. (Marugame, Kagawa, Japan). The rests were measured at LSI Medience Corporation (Chiyoda-ku, Tokyo, Japan).

Skin AGEs auto-fluorescence

The amount of AGEs in a subject's skin was measured using an AGE Reader™ (DiagnOptics, Groningen, Netherlands)[7,8]. This is a non-invasive method detecting auto-fluorescence (AF) specific for fluorescent AGEs accumulated in the skin exited by ultra-violet ray[9,10]. According to examinations of skin biopsy specimens from diabetic patients and hemodialysis patients with chronic renal failure, skin AF has been confirmed to be correlated with representative AGEs, *i.e.*, pentosidine, a fluorescent AGE, and CML, a non-fluorescent AGE[9,10]. In the present study, subjects were asked to rest their elbow on the AGE Reader. Measurements were taken at the outer upper right arm, 10 cm away from the edge of the elbow. After wiping the measuring area using a cotton swaband alcohol, AF intensity was measured three times at the same point, and the results were expressed as the mean of three values.

Skin elasticity test

Skin elasticity was evaluated using a Cutometer (MPA580; Courage & Khazaka Electronic, GmbH, Cologne, Germany)[11,12]. Briefly, a probe was placed on the skin surface of the inner side of the right arm at 10 cm from the elbow, and an area of skin was drawn up into the probe using negative pressure; the length of skin drawn into the probe was then measured using a glass prism. The R2 index is the ratio of skin length recovery after elongation and constriction (Ua1/Uf1), indicating improvement in elasticity as the ratio approaches 1.00; an ideal elastic material has an R2 value of 1.00, and normal skin has values in the range of 0.3 ~ 0.5. The R7 index is a ratio of skin elasticity during constriction (Ur1/Uf1); the most elastic skin has an R7 value close to 1.00. The R2 and R7 indices are the most reliable indices derived from a Cutometer; a previous study found that these indices decrease with aging, with the curve shifted down and forward in patients with diabetes mellitus[13]. Skin elasticity was measured four times to reduce error, with the highest and lowest values removed before averaging calculations.

Ethical standards

The trial followed the Japanese Ministry of Health and Welfare Ordinance No. 28 “Standards of implementation of the clinical trial of a pharmaceutical" (March 27, Heisei 9)” and was completed at a third-party medical institution (Medical Corporation Shinkokai C'est La Vie Shinbashi Clinic, Minato-ku, Tokyo, Japan). The trial was approved by the Ethics Committee of the clinic and KSO Co., Ltd. (Minato-ku, Tokyo, Japan).

Statistical analysis

Test results are expressed as the mean ± standard deviation (SD) of absolute values. Differences in variables between the start (0W), and at eight (8W) and twelve weeks after (12W), were tested by Wilcoxon rank sum test or by Dunnett’s test. Statistics were calculated with Dr. SPSSII (IBM Japan, Chuo-ku, Tokyo), and the level of significance was set at < 5% (two-tailed test).

Results

Mental and physical subjective symptoms in AAQol

All 10 subjects were qualified for the test protocol; no subject dropped out, and all were analyzed. In 34 items of the physical symptoms, no score was significantly improved at 12 weeks after PE intake (***Table 3***). In contrast, the “blurry eyes” score changed from 0W; 1.6 ± 0.7 to 12w;2.5 ± 0.5 ($p = 0.016$), “palpitation” score from 0W; 1.4 ± 0.5 to 12W; 2.0 ± 0.5 ($p = 0.031$). Precise examination of the individual cases revealed that no subject claimed the symptoms of “blurry eye” or “palpitation” as adverse effects. In the 21 items of mental symptoms, significant changes were not noted in the score (***Table 4***).

Blood biochemistry

The results of blood glycative stress markers are presented in ***Table 5***. During the 12-week observation period with oral PE

Table 3. Physical subjective symptom score in AAQol.

Parameter	0 W			8 W				12 W			
	mean	±	SD	mean	±	SD	*p* value	mean	±	SD	*p* value
Tired eyes	2.20	±	1.03	2.20	±	0.79	1.000	2.50	±	0.71	0.563
Blurry eyes	1.60	±	0.70	2.10	±	0.99	0.313	2.50	±	0.53	0.016*
Eye pain	1.20	±	0.42	1.50	±	0.53	0.250	1.70	±	0.48	0.063
Stiff shoulders	2.50	±	1.35	2.60	±	1.35	1.000	2.70	±	1.34	0.625
Muscular pain/stiffness	1.80	±	0.79	2.10	±	0.99	0.500	2.30	±	0.95	0.250
Palpitations	1.40	±	0.52	1.70	±	0.48	0.250	2.00	±	0.47	0.031*
Shortness of breath	1.70	±	0.82	1.90	±	0.57	0.750	2.10	±	0.57	0.344
Tendency to gain weight	2.30	±	1.42	2.30	±	1.42	1.000	2.60	±	1.17	0.375
Weight loss; thin	1.40	±	0.52	1.50	±	0.53	1.000	1.90	±	0.99	0.375
Lethargy	2.00	±	1.05	2.10	±	0.99	1.000	2.00	±	0.67	1.000
No feeling of good health	1.80	±	0.92	2.00	±	0.47	0.727	2.10	±	0.88	0.375
Thirst	1.60	±	0.84	2.10	±	0.99	0.250	2.30	±	0.82	0.109
Skin problems	1.80	±	0.42	2.00	±	0.67	0.500	2.00	±	0.82	0.688
Anorexia	1.50	±	0.53	1.80	±	0.63	0.375	1.60	±	0.52	1.000
Early satiety	1.70	±	0.67	1.80	±	0.79	1.000	2.00	±	0.94	0.250
Epigastralgia	1.50	±	0.71	1.80	±	0.63	0.250	2.00	±	0.67	0.063
Liable to catch colds	1.80	±	0.79	2.20	±	0.79	0.125	2.00	±	0.67	0.500
Coughing and sputum	1.80	±	0.79	1.70	±	0.82	1.000	2.30	±	0.82	0.125
Diarrhea	1.50	±	0.53	1.60	±	0.52	1.000	1.70	±	0.48	0.500
Constipation	2.20	±	1.03	2.40	±	0.97	0.500	2.50	±	0.97	0.375
Hair loss	2.20	±	0.79	2.50	±	1.08	0.250	2.80	±	1.03	0.148
Gray hair	3.60	±	0.97	4.00	±	0.82	0.125	3.70	±	0.82	1.000
Headache	1.60	±	0.70	1.70	±	0.67	1.000	1.90	±	0.57	0.250
Dizziness	1.20	±	0.42	1.30	±	0.48	1.000	1.70	±	0.48	0.063
Tinnitus	1.70	±	1.06	1.90	±	1.29	0.625	2.20	±	1.32	0.063
Hearing difficulty	1.70	±	0.82	2.00	±	0.82	0.250	2.20	±	0.92	0.125
Lumbago	2.20	±	0.79	2.50	±	1.35	0.531	2.50	±	0.85	0.375
Arthralgia	1.80	±	0.92	1.70	±	0.67	1.000	2.10	±	0.88	0.453
Edematous	1.40	±	0.52	1.50	±	0.71	1.000	1.70	±	0.67	0.375
Easily breaking into a sweat	2.10	±	0.99	2.50	±	1.08	0.500	2.10	±	0.99	1.000
Frequent urination	2.10	±	0.88	2.50	±	0.97	0.289	2.70	±	0.95	0.109
Hot flash	1.40	±	0.52	1.50	±	0.53	1.000	1.70	±	0.48	0.250
Cold skin	2.50	±	0.85	2.90	±	1.37	0.313	3.00	±	1.25	0.180

*p < 0.05, vs.0W, n = 10, by Dunnet's Wilcoxon test. AAQol, Anti-Aging QOL Common Questionnaire; SD, standard deviation.

intake, IRI did not change significantly. HbA1c significantly decreased from 0W; 5.73 ± 0.27%, to 8W; 5.56 ± 0.16% (p = 0.001) and remained low to 12W; 5.53 ± 0.17% (p = 0.001, ***Fig. 1***). Glycoalbumin, 0W; 14.9 ± 0.7%, was significantly lower at 8W; 14.2 ± 0.6% (p = 0.005), however, it was not significant at 12W; 14.4 ± 0.5% (p = 0.054, ***Fig. 2***). Similarly, 3DG, 0W; 24.19 ± 4.80 ng/mL, was significantly lower at 8W; 16.92 ± 2.14 ng/mL (p < 0.001), but not significant at 12W; 22.79 ± 5.30 ng/mL (p = 0.485, ***Fig. 3***). Pentosidine showed 96.84 ± 18.25 pmol/mL at 0W and it was significantly lower at 8W; 83.66 ± 12.66 pmol/mL (p = 0.036), and then it returned to the baseline at 12W; 106.12 ± 15.40 pmol/mL (p = 0.154, ***Fig. 4***). CML, 0W; 4.27 ± 0.54 μg/ml, was significantly higher at 12W; 5.08 ± 0.98 μg/ml (p = 0.036, ***Fig. 5***).

Skin AGEs auto-fluorescence

The amount of skin fluorescent AGEs was not significantly changed by PE intake during the 12-week observation period (***Table 6***).

Skin elasticity test

PE showed no significant effect on index R2, R5, R6 and R7 of the skin elasticity test during the observation period (***Table 6***).

Table 4. Mental subjective symptom score in AAQol.

Parameter	0 W			8 W				12 W			
	mean	±	SD	mean	±	SD	*p* value	mean	±	SD	*p* value
Irritability	1.90	±	0.74	2.00	±	0.82	1.000	2.00	±	0.67	1.000
Easily angered	1.60	±	0.52	1.90	±	0.57	0.375	2.00	±	0.67	0.313
Loss of motivation	1.80	±	0.42	1.80	±	0.63	1.000	2.00	±	0.94	0.766
No feeling of happiness	1.50	±	0.53	1.50	±	0.71	1.000	1.60	±	0.70	1.000
Nothing to look forward to in life	1.50	±	0.53	1.50	±	0.53	1.000	1.80	±	0.92	0.750
Daily life is not enjoyable	1.70	±	0.48	1.40	±	0.52	0.250	1.50	±	0.53	0.625
Lose confidence	1.70	±	0.48	1.60	±	0.70	1.000	1.80	±	0.63	1.000
Reluctance to talk with others	1.50	±	0.71	1.70	±	0.95	0.500	1.80	±	0.63	0.250
Depressed	1.40	±	0.52	1.50	±	0.71	1.000	1.70	±	0.67	0.250
Feeling of uselessness	1.70	±	0.67	1.80	±	0.42	1.000	1.80	±	0.42	1.000
Shallow sleep	2.30	±	1.25	2.30	±	0.82	1.000	2.40	±	0.84	0.984
Difficulty in falling asleep	1.50	±	0.71	2.00	±	1.05	0.063	2.20	±	1.23	0.031
Pessimism	1.70	±	0.67	2.10	±	0.99	0.219	2.30	±	0.67	0.063
Lapse of memory	2.60	±	0.70	2.80	±	0.63	0.625	3.00	±	0.82	0.313
Inability to concentrate	1.80	±	0.63	2.30	±	0.48	0.125	2.40	±	0.52	0.125
Inability to solve problems	1.80	±	0.42	2.10	±	0.74	0.375	2.20	±	0.63	0.250
Inability to make judgments readily	2.10	±	0.74	2.20	±	0.79	1.000	2.30	±	0.82	0.625
Inability to sleep because of worries	1.90	±	0.74	2.20	±	0.79	0.375	2.00	±	0.67	1.000
A sense of tension	2.10	±	0.99	1.80	±	0.63	0.500	2.20	±	0.63	1.000
Feeling of anxiety for no special reason	1.20	±	0.42	1.70	±	0.48	0.063	1.50	±	0.53	0.250
Vague feeling of fear	1.10	±	0.32	1.20	±	0.42	1.000	1.30	±	0.48	0.500

p Value vs. 0W, n = 10,by Dunnett's Wilcoxon test. AAQol, Anti-Aging QOL Common Questionnaire; SD, standard deviation.

Table 5. Blood glycative stress markers.

Parameter	0 W			8 W				12 W			
	mean	±	SD	mean	±	SD	*p* value	mean	±	SD	*p* value
FPG (mg/dL)	86.3	±	6.5	83.8	±	5.9	0.130	87.6	±	6.4	0.523
HbA1c (%)	5.73	±	0.27	5.56	±	0.16	0.001**	5.53	±	0.17	<0.001**
IRI (mU/mL)	3.12	±	1.06	2.79	±	1.20	0.408	3.63	±	0.88	0.148
Glycoalbumin (%)	14.9	±	0.7	14.2	±	0.6	0.005**	14.4	±	0.5	0.054
CML (mg/mL)	4.27	±	0.54	4.87	±	0.76	0.133	5.08	±	0.98	0.036*
3DG (ng/mL)	24.19	±	4.80	16.92	±	2.14	<0.001**	22.79	±	5.30	0.485
Pentosidine (pmol/mL)	96.84	±	18.25	83.66	±	12.66	0.036*	106.12	±	15.40	0.154

*p < 0.05, **p < 0.01 vs. 0W, n = 10, by Dunnett's test.
FPG, fasting plasma glucose; IRI; immune reactive insulin; 3DG, 3-deoxyglucosone; CML, N^{ε}-(carboxymethyl)lysine; SD, standard deviation.

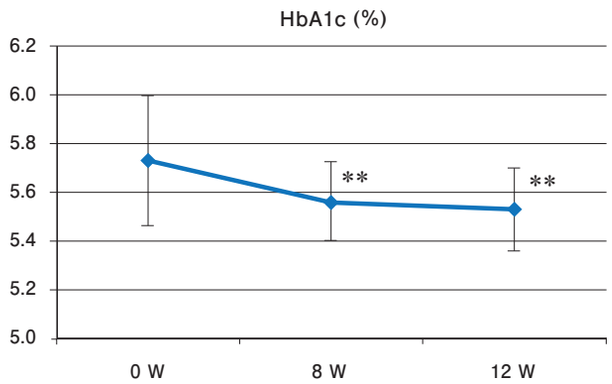

Fig.1. **Serum HbA1c**
**p < 0.01 vs. 0W, n = 10, by Dunnett's test.
Bar indicates standard deviation.

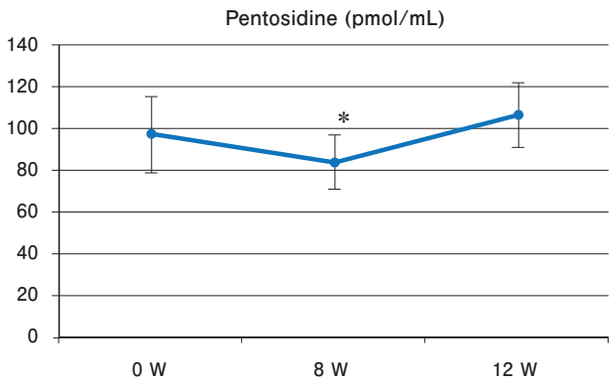

Fig.4. **Serum pentosidine**
*p < 0.05 vs. 0W, n = 10, by Dunnett's test.
Bar indicates standard deviation.

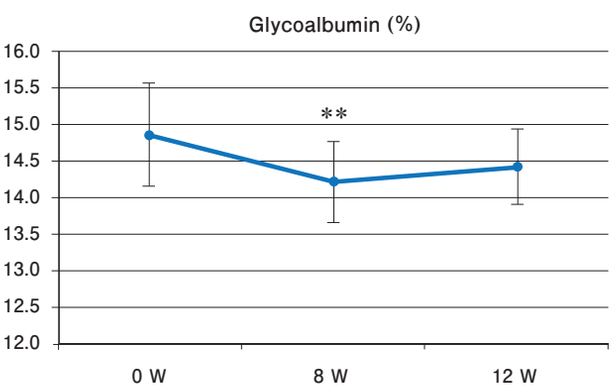

Fig.2. **Serum glycoalbumin**
**p < 0.01 vs. 0W, n = 10, by Dunnett's test.
Bar indicates standard deviation.

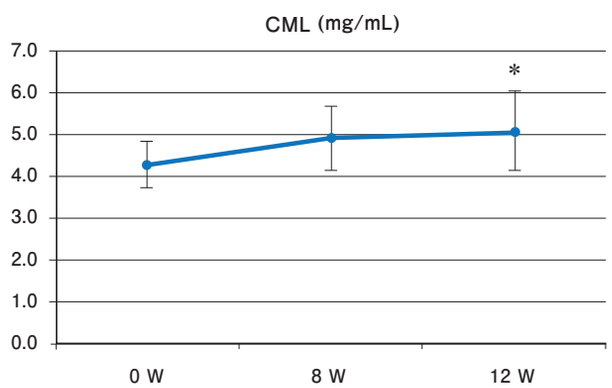

Fig.5. **Serum CML**
*p < 0.05 vs. 0W, n = 10, by Dunnett's test.
CML; N^{ε}-(carboxymethyl)lysine.
Bar indicates standard deviation.

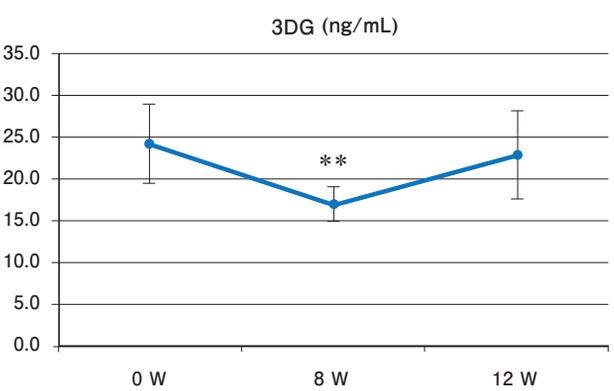

Fig.3. **Serum 3DG**
**p < 0.01 vs. 0W, n = 10, by Dunnett's test. 3DG; 3-deoxyglucosone.
Bar indicates standard deviation.

Table 6. Skin fluorescent AGEs and elasticity index.

Parameter	0 W			8 W				12 W			
	mean	±	SD	mean	±	SD	*p* value	mean	±	SD	*p* value
Skin fluorescent AGEs	2.37	±	0.39	2.33	±	0.31	0.811	2.35	±	0.20	0.965
Skin elasticity test (R2)	0.817	±	0.038	0.830	±	0.033	0.291	0.816	±	0.031	0.998
Skin elasticity test (R5)	0.609	±	0.073	0.643	±	0.055	0.151	0.636	±	0.050	0.278
Skin elasticity test (R6)	0.367	±	0.040	0.384	±	0.038	0.498	0.374	±	0.030	0.867
Skin elasticity test (R7)	0.446	±	0.055	0.465	±	0.044	0.251	0.463	±	0.034	0.334

p Value vs. 0W, n = 10, by Dunnett's test. AGEs, advanced glycation end products; SD, standard deviation.

Discussion

Effects of pomegranate extract (PE)

The active ingredient of test products is PE. The extracts of pomegranate seed, rind, flower are reported, in *in vitro* studies, experimental animals and culture cells, to show various activities including anti-oxidant capacity [14,15], anti-inflammation [16-19], anti-bacteria [20-23], anti-tumor [24-27], and anti-diabetes [28-30]. PE shows anti-bacterial actions against *Listeria monocytogenes* [21], *Pseudomonas stutzeri* [22], and *Staphylococcus aureus* [23]. Anti-tumor effect was shown against cell lines derived from mammalian gland cancer [24], ovarian cancer [24], endometrium cancer [24], osteosarcoma [25], colon cancer [26] and prostate cancer [27]. Furthermore, in rats PE is reported, to show a preventive effect against carvacrol on methotrexate-induced bone marrow toxicity [31], renal ischemia-reperfusion injury [32], hepatotoxicity induced by diethylnitrosamine and phenobarbital [33], and bleomycin-induced pulmonary fibrosis [34].

PE seems favorable towards sugar metabolism showing anti-diabetic actions [28-30]. Recent research has focused on the mechanism of PE actions and experiments using diabetic rats have demonstrated the stimulation of insulin secretion [35] and inhibition of α-amylase and α-glucosidase [36]. PPARγ activation by PE [37] may be involved in these actions. As a result, PE is expected to be a possible treatment for fatty liver [38] and hypertension care [39], wound healing enhancement [40,41] and cognitive function improvement [42].

Recent studies on anti-glycation activity by PE have shown that PE inhibits albumin glycation [43] and AGEs generation [3,44,45]. In these findings on anti-diabetic actions, the basi of PE actions may be its anti-glycation effect.

Data interpretation

In the present study, after the 12-week PE administration, glycative stress markers, FPG and IRI did not change. HbA1c, glycoalbumin, 3DG and pentosidine were significantly reduced at 8 weeks, and HbA1c stayed significantly low at 12 weeks. PE, as in the previously reported experiments, may fulfill its anti-glycation effect, thus reducing AGEs generation in the body. The reason why CML tended to increase still remains unclear. In order to obtain new findings, we have a study plan in the next trial to check skin corneal CML by the tape stripping method [46].

Since the purpose is focused on confirming safety and effect as a pilot open study, the subjects were limited only to healthy persons, those who include both persons with high glycation stress and persons with normal glycation stress. In the latter, markers such as skin fluorescent AGEs may be low or normal, and there would be no room for reducing these markers by PE intake. It is necessary to modify the design in a subsequent study, for an example, to include subjects with high skin fluorescent AGEs as assessed by screening.

In a previous clinical study of type 2 diabetic patients and healthy subjects, serum malondialdehyde (MDA) and hydroxynonenal (HN) were reduced only in the diabetic patients; they were not changed in the healthy subjects [47]. The reason may be that MDA and HN were almost normal in the healthy persons and there was no room for improvement. Similarly, in the present study the subjects were healthy persons and skin fluorescent AGEs were not reduced for the same reason.

There were no adverse effects claimed in this study, indicating that PE can be considered a safe product. The evaluation of safety will be described in the next report.

Conclusions

In this open study without control subjects to elucidate the anti-glycation activity of PE, the subjects were 10 post-menopausal females who received oral administration of PE 100 mg/day for 12 weeks, and as a result, some glycative stress markers were reduced. This finding indicates an equivocal effect of PE on glycative stress improvement. Further studies are necessary to elucidate the anti-glycation effect of PE, such as a double-blind randomized controlled trial with subjects with high glycation stress with skin fluorescent AGEs selected by screening.

Conflict of interest statement

The authors are indeted to the Fund "Research and development support program for regional revitalization in agriculture, forestry, fisheries and in the food industry sector'' of the New Business and Intellectual Property Division, Food Industry Affairs Bureau, from the Ministry of Agriculture, Forestry and Fisheries of Japan, 2013.

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