Glycative Stress Research

Online edition : ISSN 2188-3610
Print edition : ISSN 2188-3602
Received : November 29, 2022
Accepted : February 21, 2023
Published online : March 31, 2023
doi:10.24659/gsr.10.1_27

Original article

Verification of improvement of periorbital wrinkles by using Asakado Skin Care

Yoshikazu Yonei, Masayuki Yagi

Anti-Aging Medical Research Center / Glycative Stress Research Center,
Faculty of Life and Medical Sciences, Doshisha University, Kyoto, Japan

Abstract

The present study conducted a trial for a skin application of a test cosmetic product with 34 healthy women aged between 35 to 55 years of age (43.4 ± 0.6) who satisfied the classification of 1, 2, and 3 in the standards for the wrinkle grade assessment in accordance with "Guidelines for Evaluation of Cosmetic Functions". In a half face method for 8 weeks, the test cosmetic sample was applied to one lateral periorbital lines of a face (applied side) twice a day and no treatment was applied over the other periorbital lines (non-applied side). The test product was a skin-care cosmetic that contained diverse constituents such as *Ceratonia siliqua* L. fruit extract, *Polygonum tinctorium* leaf/stem extract, and ribosomal constituents. In test results after 8 weeks for the primary endpoint of grade assessments of periorbital wrinkles, the grade on the applied side indicated significant improvements through a visual assessment during a medical examination and a visual assessment of observing case photographic images by medical specialists. However, results of the primary endpoint on the non-applied side also indicated significant improvements. There was no significant difference between two sides. Replica analysis examining periorbital wrinkles did not suggest any significant changes between on applied or non-applied side as well as between before and after test product application. Secondary endpoint of skin viscoelasticity (R2) significantly improved on the applied and non-applied sides after 8 weeks. There was no significant difference of changes in quantity. In the process of the trial, no adverse event was reported and the safety of the test product was confirmed. We conducted a further investigation to explore possibilities that diverse functional ingredients contained in this product were absorbed through percutaneous absorption with an assistance of ribosomal constituents, and exerted effects toward the other side of the face. To verify this hypothesis, further examinations are required to confirm blood concentrations of active components after tests.

***KEY WORDS*:** cosmetic products, periorbital wrinkles, skin elasticity, half face method, *Ceratonia siliqua*

Introduction

Skin wrinkles start to appear on the face around 30 years of age. The major causative factor is recognized to be quantitative and qualitative alternations in proteins, elastic fibers, and collagen, which are produced by dermal fibroblasts [1, 2]. Another causative factor for shallow facial wrinkles is structural alternations in epidermis due to epidermal dryness. Faces and necks, which are constantly exposed to the sunlight, have deep wrinkles. Many women are concerned about periorbital wrinkles (crow's feet wrinkles), whose appearance is noticeable. There is an expectation for developments in effective periorbital wrinkle amelioration of cosmetics in the cosmetic dermatology field.

The purpose of the present study is to verify improvement effects on wrinkles at the corners of the eyes by using the test cosmetic product (Asakado skin care cosmetics, Nabocul Cosmetics, Tokyo, Japan). Research participants were healthy women aged between 35 to 55 years of age who satisfied the classification of 1, 2, and 3 in the standards for the wrinkle grades [3]. A comparative verification was conducted to verify improvement effectiveness on periorbital wrinkles of the test cosmetic product, employing a half face method between the test product-applied side and non-applied side.

Contact Address: Professor Yoshikazu Yonei, MD, PhD
Anti-Aging Medical Research Center/Glycative Stress Research Center,
Graduate School of Life and Medical Sciences, Doshisha University
1-3, Tatara Miyakodani, Kyotanabe, Kyoto, 610-0394 Japan
TEL&FAX: +81-774-65-6394 e-mail: yyonei@mail.doshisha.ac.jp
Co-authors; Yagi M, myagi@mail.doshisha.ac.jp

Methods

Target

Potential participants in research were recruited: 63 healthy women aged between 35 to 55 years of age, who satisfied classifications in pictures of 1, 2, and 3 in the standards for the wrinkle grades in accordance with "Guidelines for Evaluation of Cosmetic Functions" (http://www.jcss.jp/journal/contents_guideline5.pdf)[3]. These potential participants underwent screening tests and pre-consumption tests (SCR & Visit-1). Among them, 34 participants who satisfied the inclusion criteria, didn't match with the exclusion criteria, and were judged as appropriate to this study by a responsible doctor, were selected as subjects for this test according subject selection standards.

Key inclusion criteria were as follows:

1) Japanese females aged 35-54 years, when they agreed to participate in the test
2) Individuals who do not have chronic physical illnesses, including skin disorders
3) Individuals whose wrinkles at the corners of the eyes are judged to be wrinkle grade 1-3
4) Individuals who have wrinkles at the corners of the eyes that are almost symmetrical
5) Individuals who were fully informed regarding the purpose and contents of the test, has an ability of consent, voluntarily applied for participation with a full understanding, and agreed to participate in the test with written informed consent
6) Individuals who can come on the designated examination date and receive the examination
7) Individuals judged appropriate for the study by the principal

Key exclusion criteria were as follows:
Individuals (who)

1) using medical products for diseases
2) under treatment or with history of mental disorders, sleep disorders, hypertension, diabetes, lipid metabolism abnormality, or other serious disorders
3) have a history of serious diseases (hepatic, renal, cardiovascular, respiratory, hematologic, *etc*.)
4) have a serious history and/or contract digestive disease and comorbidities
5) with a history of cosmetic troubles or current medical history
6) with skin symptoms such as atopic dermatitis, perioral dermatitis, and rosacea-like dermatitis
7) with experience in cosmetology and aesthetic medicine that affect the measuring area
8) have performed or received the following acts within the past 3 months
   a. Hormone replacement therapy
   b. Use of cosmetics (eye cream, sealer, patch, *etc*.) that have strong moisturizing effect and wrinkle-related effect
   c. Regular use of medicines, quasi-drugs, foods and drinks for the purpose of improving the skin
9) have an inflammation or a scar on a measuring area
10) receiving/received medical drug treatment for the past one month except for temporary relief medication for headache, menstrual pain, common cold, *etc*.
11) drink more than 60 g/day on average in terms of pure alcohol
12) with possible changes of life style during test periods (night work, long-term trip, *etc*.)
13) will develop seasonal allergy symptoms, such as pollinosis, use an anti-allergic drug
14) cannot avoid excessive direct sunlight exposure, such as sunburn, during test periods.
15) are currently or are possibly pregnant, or are breastfeeding
16) are participating and/or had participated in other clinical studies within the last 3 months
17 are judged as not appropriate to this study by a responsible doctor

Trial Design

This trial conducted a parallel-group trial with a shielding test, employing a half face test, to examine improvement effects on wrinkles at the corners of the eyes by using the test cosmetic product between the applied side of the face and the non-applied side.

Research participants, after morning and evening skin care for 8 weeks, dispensed one push of the test product (approximately 0.5 mL) into the palm of their hand from a container and applied it around the corner of their eye, which was assigned, allowing it to blend into the skin of the half of face. The other side is left untreated. The number of observations was three times: before the test for usage, 4 weeks after, and 8 weeks after the commencement of the test. The following was performed: visual assessment of medical examinations for grades in wrinkles of periorbital lines by medical specialists, visual assessment for grades in periorbital wrinkles using photographic images by specialized doctors, photographic images of periorbital wrinkle parts (Visia, Canfield Scientific, Parsippany, NJ, USA), replica analysis of periorbital wrinkle, measurements of skin viscoelasticity, medical interview by doctors, and judgments for the presence or absence of occurrence of adverse reactions/adverse events. For photographic images at periorbital wrinkle parts (Visia), analysis of periorbital wrinkle replicas, and measurements of skin viscoelasticity, the test was performed in an environmental test chamber with 21 ± 1 °C of temperature and 50 ± 5 % of moisture. Research participants recorded a living diary during the test period with usage conditions of the test cosmetic product, alternations in skin conditions and physical conditions (an investigation of adverse events), the presence or absence of alternations in living conditions, conditions of medication usage, conditions of dietary supplementation, and the presence or absence of menstruation.

In this trial, 34 research participants started to undergo the test and all the participants completed the test. Age of 34 female subjects was 43.4 ± 0.6 and visually inspected assessment of periorbital wrinkle was 2.265 ± 0.122 by a specialized doctor at the time of the assignment.

Test Product

The test product is produced by Nabocul Cosmetics co., ltd. as a brand-new product of Asakado Project (Nabocul Cosmetics). This company with an approximate 40 years old

history, has developed cosmetics of high performance and high quality based on cutting-edge dermatology focusing on the effectiveness of natural constituents existing in nature, based on their success in the usage of natural materials with excellent effectiveness, established Asakado Project, as a collaboration between an in-house research team and an outside research development institution.

Compounding ingredients are as follows: *Ceratonia siliqua* L. fruit extract, *polygonum tinctorium* leaf/stem extract, chamomilla recutita flower extract (*Anthemis nobilis*), glucosyl hesperidin, glucosyl rutin, herb Robert extract, hydroxyproline, coenzyme A, chondroitin sulfate sodium, saccharomyces lysate extract, sodium hyaluronate, beta glucan, sodium polyaspartate, octyldodecyl myristate, ubiquinone (oxidized coenzyme Q10), hydroxyethyl cellulose, adenosine triphosphate disodium, arginine, aloe vera leaf extract, glycosyl trehalose, glucosylglycerol, dipotassium glycyrrhizinate, hydroxyphenyl propamidobenzoic acid, ascorbyl palmitate, niacinamide, retinol palmitate, corn oil, hydrogenated starch hydrolysate, licorice extract, hydrogenated lecithin, xanthan gum, carbomer, BG, EDTA-2Na, glycerin, PEG-60 hydrogenated castor oil, hydroxyacetophenone, pentylene glycol, and aqua. The main ingredient is a new compound with a molecular formula of C_{23} H_{24} O_{13} that was separated and identified from a shell of *Ceratonia siliqua*; *Ceratonia siliqua* is derived from the Mediterranean coast. The structural formula is shown in ***Fig. 1*** [4].

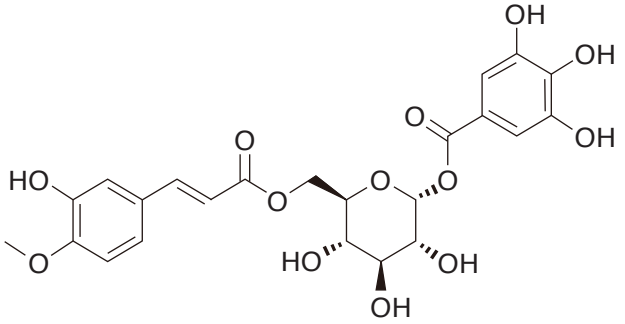

Fig. 1. **Novel compound that is extracted from *Ceratonia siliqua*.**

Assessment Items for Effectiveness

Visual inspection assessment of periorbital wrinkle grades by specialized doctors

Scoring for periorbital wrinkles was performed at the beginning of the trial based on the wrinkle grade assessment [3] in accordance with "Guideline for Evaluation of Anti-wrinkle Products for acquisition of new effectiveness", which was stipulated by Japanese Cosmetic Science Society (http://www.jcss.jp/).

At the time of each observation for each measurement point, referring to photographs of the beginning and the previous measurement point, scoring was performed based on the wrinkle grade assessment. Values of 50% and 25% were employed for scoring such as 2.5 or 2.25 when there was no standard picture grade for assessment.

Visual Assessment of Periorbital Wrinkle Grades by Specialized Doctors Using Case Photographic Images

At the beginning of the trial, the scoring for periorbital wrinkles using case photographic images of outer canthus was performed in accordance with the wrinkle grade assessment [3].

At the time of each observation for each measurement point, referring to photographs at the beginning and the previous measurement point, scoring was performed based on the wrinkle grade assessment. Values of 50% and 25% were employed for scoring such as 2.5 or 2.25 when there was no standard picture grade for assessment.

Replica Analysis of Periorbital Wrinkle

Replicas were prepared from the corners of eyes, from the area 5 mm away from the edges of the left and right eyes to the area of fine wrinkles with reticulations. The sample was analyzed by using a reflective replica analysis system, the 3D Skin Roughness Analysis Measurement System ASA-03RXD (Asahi Biomed Co., Tokyo, Japan). The range of sample analysis was 1) area of 1.0 cm × 1.0 cm, approximately 5–7 mm away from the corner of the eye, and 2) area of 1.0 cm × 1.0 cm, approximately 10–12 mm away from the corner of the eye. Analysis was performed in terms of both 0 degrees and 180 degrees, and the mean values were adopted. Assessment items were as follows:

1) 5–7 mm away from the corner of the eye
   area ratio of wrinkles/volume fraction of wrinkles/average depth of total wrinkles/average depth of maximum wrinkles/maximum depth of maximum wrinkle/the number of wrinkles
2) 10–12 mm away from the corner of the eye
   area ratio of wrinkles/volume fraction of wrinkles/average depth of total wrinkles/maximum depth of total wrinkles/the number of wrinkles

Secondary Endpoint

Measurement of Skin Viscoelasticity

Employing a cutometer, Cutometer MPA580® (Courage-Khazaka, Köln, Germany), the following area of the left and right faces was measured five times: the center part of connected lines from the bottom of earlobe and the edge of the lip. With the standard R2 values, maximum and minimum values were eliminated and the mean value of measurements, which were performed three times, was obtained.

Other Assessment Item

Photographic Images of Periorbital Wrinkle Area (VISIA)

Employing VISIA-Evo (Canfield Scientific), the left and right faces were photographed and periorbital wrinkle areas were trimmed to extract.

Safety Assessment Item

Medical Interview by a Medical Specialist for the Presence or Absence of Occurrence of Adverse Reactions/Adverse Events

At screening tests and pre-consumption tests, background questionnaires were performed. At the time of observation after the commencement of tests, subjective symptoms of research participants and occurrence of adverse events were checked, and medical recoding, interviews, and appropriate treatments were conducted.

Statistical Analysis

Items of target analysis were visual inspection assessment of periorbital wrinkle grades by specialized doctors, visual assessment of periorbital wrinkle grades by specialized doctors using case photographic images, replica analysis of periorbital wrinkles and skin viscoelasticity measurements. Values of examination results were compiled to a grand total table using Microsoft Office Excel 2016 (Microsoft Corp., Redmond, WA, USA). As fundamental statistics, the mean values, standard deviation, standard error, maximum values, and minimum values were calculated. Values used in the statistical analysis were measured values and amount of change from the commencement of usage. All examination items obtained as category data were compiled at each examination point. Statistical analysis was performed using appropriate statistical software such as SAS (SAS 9.4, SAS Institute Japan, Tokyo) or SPSS (Statistics 26, IBM Japan, Tokyo). For all tests, significance level was 5 % in two-tailed tests.

Statistical data of each examination point were analyzed in a paired t-test (two-tailed test) with comparison between groups, the test cosmetic product application side and the non-treatment side. Scores which were obtained by visual inspection assessment of periorbital wrinkle grades by specialized doctors were treated as non-parametric, and Wilcoxon signed-rank sum test was conducted for comparison between groups.

Statistical data of each examination were analyzed in a paired t-test (two-tailed test) with comparison among groups (time comparison), the time before the commencement of usage and each observation time after the commencement of usage. Scores which were obtained by visual inspection assessment of periorbital wrinkle grades by specialized doctors was treated as non-parametric, and Wilcoxon signed-rank sum test was conducted for time comparison.

Ethical Examination

The present study was conducted in compliance with ethical principles based on the Declaration of Helsinki (Amendment, Fortaleza, October 2013) and "Ethical principles for medical research involving human subjects (Partial amendment, 2017). Ethical approval was obtained from the committee of medical research involving human subjects of "general incorporated association: Society for Glycative Stress Research (GSE #2022-001). Pre-registration for a clinical trial was conducted (UMIN #000046645).

Results

Effectiveness Assessment Items

*Visual inspection assessment of periorbital wrinkle grades by specialized doctors (**Table 1**)*

On the applied side, before usage (2.27 ± 0.12) → 4 weeks after the commencement of usage (2.20 ± 0.12) → 8 weeks after the commencement of usage (2.10 ± 0.12). Wrinkle grade decreased. Compared with before usage, 4 weeks after the commencement of usage ($p = 0.005$) and 8 weeks after the commencement of usage ($p < 0.001$). Wrinkle grade significantly improved (***Fig. 2***).

On the non-applied side, before (2.27± 0.12) → 4 weeks (2.21 ± 0.12) → 8 weeks (2.10 ± 0.12). Wrinkle grade decreased. Compared with before usage, 4 weeks ($p = 0.029$) and 8 weeks ($p < 0.001$). Wrinkle grade significantly improved.

There was no significant difference between the two sides.

*Visual assessment of periorbital wrinkle grades by specialized doctors using case photographic images (**Table 1**)*

On the applied side, before (2.27 ± 0.12) → 4 weeks (2.18 ± 0.12) → 8 weeks (2.08 ± 0.12). Wrinkle grade decreased. Compared with before usage, 4 weeks ($p < 0.001$) and 8 weeks ($p < 0.001$). Wrinkle grade significantly improved (***Fig. 3***).

On the non-applied side, before (2.27 ± 0.12) → 4 weeks (2.19 ± 0.12) → 8 weeks (2.06 ± 0.12). Wrinkle grade decreased. In chronological comparison by Wilcoxon signed-rank sum test, compared with before usage, 4 weeks ($p = 0.003$) and 8 weeks ($p < 0.001$). Wrinkle grade significantly improved.

There was no significant difference between two sides.

*Replica Analysis of Periorbital Wrinkles (**Table 2**)*

No items regarding the following suggested any significant improvement on the applied side: wrinkles 5 mm from the corner of the eye in area ratio of wrinkles/volume fraction of wrinkles/average depth of total wrinkles/average depth of maximum wrinkles/maximum depth of maximum wrinkle/the number of wrinkles, and wrinkles 10–12 mm from the corner of the eye in area ratio of wrinkles/volume fraction of wrinkles/average depth of total wrinkles/maximum depth of total wrinkles/the number of wrinkles. There was no significant difference between both sides.

Secondary Endpoint Items

*Skin Viscoelasticity Measurement (**Table 3**)*

R2 on the test-product-applied side changed as follows: before usage (0.788 ± 0.012) → after 4 weeks (0.780 ± 0.011) → after 8 weeks (0.809 ± 0.009). The chronological comparison by the paired t-test indicated that wrinkles significantly improved at 8 weeks after the commencement of usage ($p = 0.004$) in comparison with before usage (***Fig. 4***).

R2 on the non-applied side changed as follows: before

usage (0.792 ± 0.010) → after 4 weeks (0.803 ± 0.009) → after 8 weeks (0.815 ± 0.007). The chronological comparison by the paired t-test indicated that wrinkles significantly improved at 8 weeks after usage ($p < 0.001$) in comparison with before usage.

Furthermore, in a comparison among groups by a paired t-test, the applied side was significantly lower than the non-applied side at the point of 4 weeks after usage ($p = 0.027$). There was no significant difference between two sides at 8 weeks.

Table 1. Periorbital wrinkle grades assessed by specialized doctors.

Periorbital wrinkle grades		Time Comparison					Comparison between groups	
		Before	4 W	P value	8 W	P value	p value (4W)	p value (8W)
Visual	Applied	2.27 ± 0.12	2.20 ± 0.12	**0.005**	2.10 ± 0.12	**<0.001**	0.527	1.000
	Non-applied	2.27 ± 0.12	2.21 ± 0.12	**0.029**	2.10 ± 0.12	**<0.001**		
Photographic images	Applied	2.27 ± 0.12	2.18 ± 0.12	**0.001**	2.08 ± 0.12	**<0.001**	0.782	0.597
	Non-applied	2.27 ± 0.12	2.19 ± 0.12	**0.003**	2.06 ± 0.12	**<0.001**		

Periorbital wrinkle grades are assessed by visual inspection and photographic images. Results are expressed as mean values ± SEM, n = 34. Wilcoxon signed-rank sum test is used for analysis of the time comparison with before usage and the comparison between applied side and non-applied side. SEM, standard error mean.

Table 2. Replica analysis of periorbital wrinkles.

Replica analysis			Time Comparison					Comparison between groups	
			Before	4 W	P value	8 W	P value	p value (4W)	p value (8W)
Area ratio of wrinkles (5 mm)	$\mu m^2/mm^2/100$	Applied	2.73 ± 0.18	3.00 ± 0.19	**0.015**	3.10 ± 0.19	**0.013**	0.730	0.750
		Non-applied	2.66 ± 0.16	2.88 ± 0.18	**0.088**	2.98 ± 0.18	**0.008**		
Volume fraction of wrinkles (5 mm)	$\mu m^3/mm^2/100$	Applied	39.39 ± 2.70	43.51 ± 3.05	**0.019**	46.12 ± 3.43	**0.017**	0.746	0.564
		Non-applied	38.22 ± 2.37	41.56 ± 2.74	**0.097**	43.35 ± 2.70	**0.010**		
Average depth of total wrinkles (5 mm)	μm	Applied	149.90 ± 1.74	150.57 ± 1.63	0.661	152.79 ± 1.72	**0.051**	0.474	0.379
		Non-applied	150.17 ± 1.62	151.68 ± 1.56	0.255	151.77 ± 1.23	0.197		
Average depth of maximum wrinkles (5 mm)	μm	Applied	152.22 ± 2.10	153.44 ± 1.96	0.461	156.45 ± 2.50	**0.030**	0.706	0.629
		Non-applied	152.84 ± 1.70	154.47 ± 1.50	0.302	156.14 ± 1.47	**0.035**		
Maximum depth of maximum wrinkle (5 mm)	μm	Applied	401.12 ± 9.80	410.30 ± 14.13	0.315	432.84 ± 14.97	**0.006**	0.365	0.642
		Non-applied	402.40 ± 13.26	399.63 ± 10.06	0.718	426.13 ± 15.23	0.107		
Number of wrinkles (5 mm)	N/mm	Applied	0.55 ± 0.02	0.58 ± 0.02	**0.027**	0.58 ± 0.02	**0.021**	0.307	0.880
		Non-applied	0.54 ± 0.02	0.56 ± 0.02	0.374	0.57 ± 0.02	**0.016**		
Area ratio of wrinkles (10 mm)	$\mu m^2/mm^2/100$	Applied	2.79 ± 0.25	3.23 ± 0.29	**0.001**	3.10 ± 0.24	**0.017**	0.084	0.787
		Non-applied	2.70 ± 0.22	2.89 ± 0.25	0.226	2.98 ± 0.23	**0.027**		
Volume fraction of wrinkles (10 mm)	$\mu m^3/mm^2/100$	Applied	41.04 ± 3.98	47.97 ± 4.85	**0.002**	45.83 ± 4.05	**0.019**	0.090	0.745
		Non-applied	39.45 ± 3.41	42.51 ± 3.87	0.202	43.68 ± 3.67	**0.032**		
Average depth of total wrinkles (10 mm)	μm	Applied	155.19 ± 2.85	155.92 ± 2.82	0.682	156.09 ± 2.58	0.584	0.309	0.980
		Non-applied	155.12 ± 2.44	157.48 ± 2.73	0.056	155.97 ± 2.34	0.442		
Maximum depth of total wrinkle (10 mm)	μm	Applied	402.70 ± 11.18	409.28 ± 12.87	0.277	413.55 ± 14.60	0.185	0.343	**0.007**
		Non-applied	400.44 ± 8.46	399.15 ± 7.91	0.817	382.77 ± 7.16	**0.025**		
Number of wrinkles (10 mm)	N/mm	Applied	0.49 ± 0.03	0.53 ± 0.03	**0.011**	0.52 ± 0.02	**0.025**	**0.036**	0.992
		Non-applied	0.49 ± 0.03	0.49 ± 0.03	0.981	0.51 ± 0.03	**0.049**		

Periorbital wrinkle is analyzed at 5 mm and 10-12 mm from the corner of the eye. Results are expressed as mean values ± SEM, n = 34. Paired t test is used for analysis of the time comparison with before usage and the comparison between applied side and non-applied side. SEM, standard error mean.

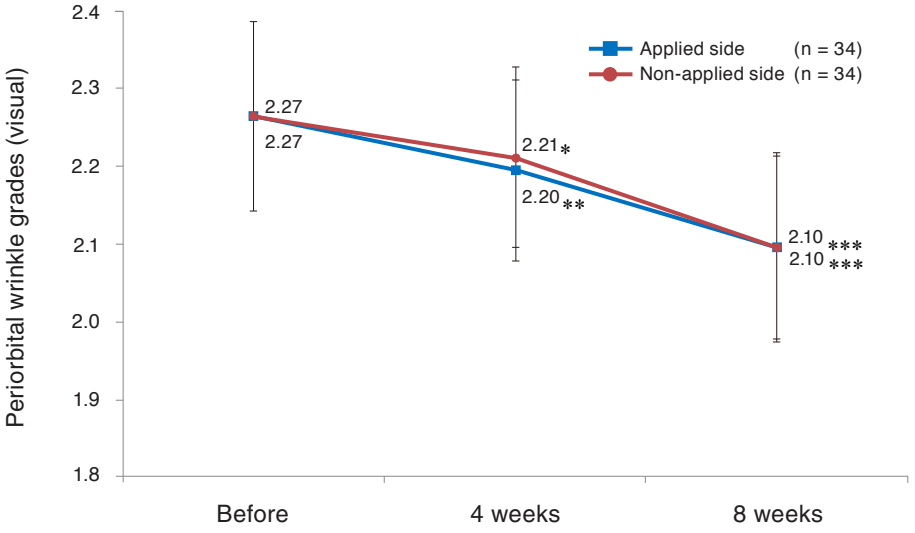

Fig. 2. **Visual inspection assessment of periorbital wrinkle grades by a specialized doctor.**

The mean value ± SEM, n = 34. Applied side, test-product topically applied for 8 weeks. *p < 0.05, **p < 0.01, ***p < 0.001 vs before usage, Wilcoxon signed-rank sum test. SEM, standard error mean.

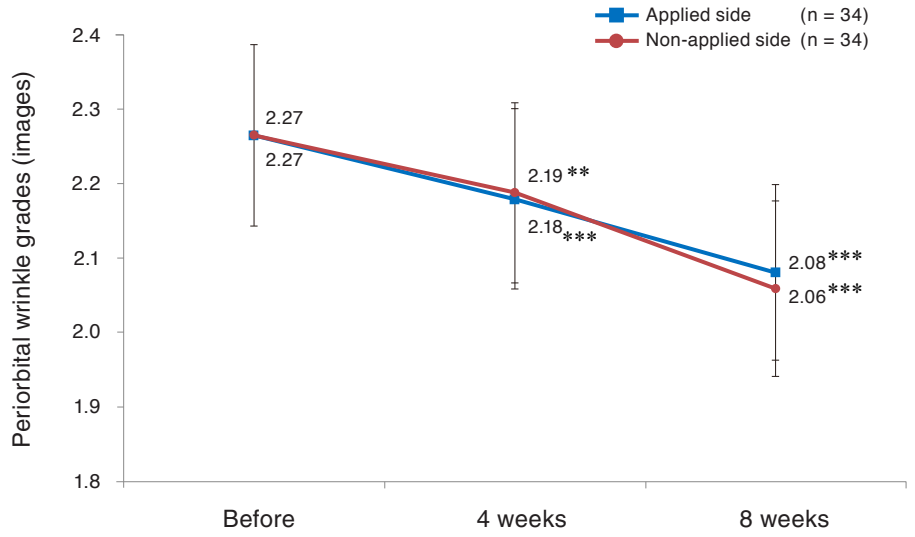

Fig. 3. **Visual assessment of periorbital wrinkle grades by specialized doctors using case photographic images.**

The mean value ± SEM, n = 34. Applied side, test-product topically applied for 8 weeks. **p < 0.01, ***p < 0.001 vs before usage, Wilcoxon signed-rank sum test. SEM, standard error mean.

Table 3. Skin Viscoelasticity Measurement.

Skin elastisity		Time Comparison					Comparison between groups	
		Before	4 W	P value	8 W	P value	p value (4W)	p value (8W)
R0	Applied	0.32 ± 0.01	0.34 ± 0.01	**0.001**	0.33 ± 0.01	**0.024**	0.479	0.552
	Non-applied	0.31 ± 0.01	0.33 ± 0.01	**0.004**	0.32 ± 0.01	0.165		
R2	Applied	0.79 ± 0.01	0.78 ± 0.01	0.320	0.81 ± 0.01	**0.004**	0.089	0.589
	Non-applied	0.79 ± 0.01	0.80 ± 0.01	0.179	0.82 ± 0.01	**0.001**		
R5	Applied	0.58 ± 0.02	0.51 ± 0.02	**0.000**	0.56 ± 0.02	0.163	**0.031**	0.805
	Non-applied	0.58 ± 0.02	0.55 ± 0.01	**0.001**	0.56 ± 0.01	0.055		
R6	Applied	0.37 ± 0.01	0.34 ± 0.01	**0.023**	0.35 ± 0.01	0.342	0.747	0.803
	Non-applied	0.37 ± 0.01	0.34 ± 0.01	**0.008**	0.35 ± 0.01	**0.021**		
R7	Applied	0.42 ± 0.01	0.39 ± 0.01	**0.000**	0.42 ± 0.01	0.351	0.052	0.948
	Non-applied	0.42 ± 0.01	0.41 ± 0.01	**0.022**	0.42 ± 0.01	0.264		

Skin elasticity is Measured by Cutometer. Results are expressed as mean values ± SEM, n = 34. Paired t test is used for analysis of the time comparison with before usage and the comparison between applied side and non-applied side. R0, Uf; R2, biological elasticity (Ua1/Uf1); R5, net elasticity (Ur/Ue); R6, viscoelasticity (Uv/Ue); R7: elastic recovery (Ur1/Uf1); SEM, standard error mean.

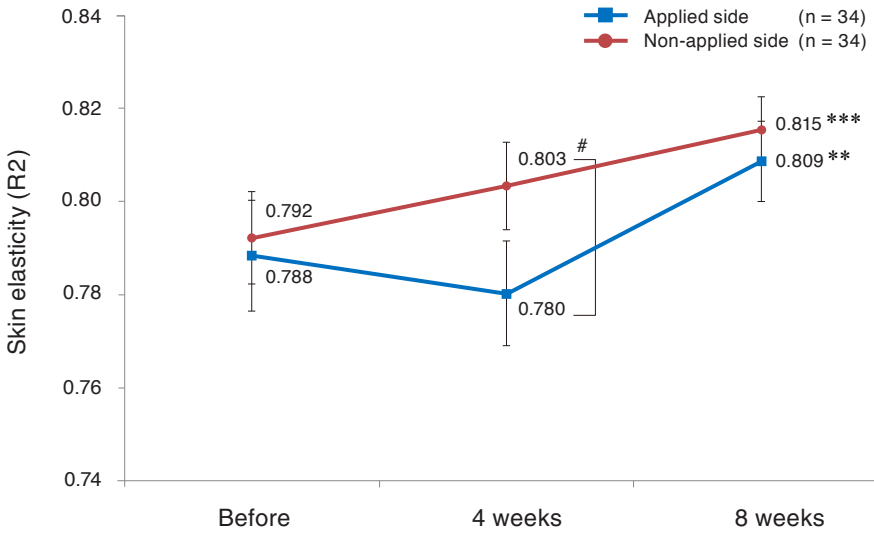

Fig. 4. **Skin elasticity changes.**

The mean value ± SEM, n = 34. Applied side, test-product topically applied for 8 weeks. **p < 0.01, ***p < 0.001 vs before usage, # p < 0.05 vs non-applied side, paired-t test. SEM, standard error mean.

Safety

No adverse reaction which was considered to be induced by the test product was reported by any research participants.

Discussion

Overview of Results

The present study verified improvement effectiveness of the test cosmetic product on periorbital wrinkles conducting a parallel-group comparison shield study (a half face method) with 34 healthy women aged 35 to 55 years of age whose periorbital wrinkles met the classification of 1, 2, and 3 in the standard photographs [3] for the wrinkle grade assessment.

Visual assessments on periorbital wrinkle graded by a medical examination of specialized doctors, and visual assessment by doctors observing case photographic images, which were effectiveness assessment items of this test, indicated significant improvements on the test-product-applied side. However, assessments on the non-applied side also indicated significant improvements. There were no significant differences between both sides. Regarding replica analysis, there was no difference. Though specialized doctors visually detected fine alternations in shallow wrinkles during before-after examinations, there was a possibility of some difficulties in detecting an alternation via replica analysis.

Skin viscoelasticity measurements, which were the secondary endpoint assessment item, indicated a significant improvement on the applied side at the point of 8 weeks. However, improvements were also observed on the non-applied side. There were no recognized significant differences between the two sides.

This test cosmetic product contained diverse types of functional ingredients as well as emulsifying agents and ribosomal constituents, which enhance skin permeability. In the half face method, a possibility cannot be eliminated that absorbed functional ingredients via skin application resulted in effects on the other side of the face.

For further investigations, we collected bibliographic information regarding the contained ingredients and explored possibilities of effects on wrinkles and percutaneous absorption. Furthermore, it was discussed how further research would verify if ingredients affected wrinkles on the other side via percutaneous absorption.

Components of the Test Product

To begin, we explain main components that are involved in the functions of this product.

• *Ceratonia siliqua* L. fruit extract

Ceratonia siliqua L. is in the legume family and native to the Mediterranean region. Legume of *Ceratonia siliqua*, in other words, pod and fruit, is designated as a carob. Carob has been used as food and as a food ingredient for generations. Ripe pods are approximately 10–25 cm long and taste mildly sweet. The carob contains a large amount of polysaccharide, cellulose and mineral and a small amount of non-hydrocarbon low molecular weight compounds, such as antioxidant polyphenols [5], and protein. Studies on new functions of carob have been developed in recent years. Extract of *Ceratonia siliqua* bark has an inhibitory effect on tyrosinase, whose substrate is L-tyrosine. In a clinical trial, application on the skin for 28 days improved skin tone by 3 % to even out skin with ultraviolet-induced hyperpigmentation [6]. Previous studies reported that pod extracts of Ceratonia *siliqua*, carob, had effects of the prevention against and the medical treatments for digestive diseases such as ulcerative colitis and gastric ulcer [7, 8]. Carob contains a chemical compound with ester linkages as a novel polyphenolic compound, where both isoferulic acid and gallic acid bind with glucose (***Fig.1***), as is reported. Carob seed extracts have inhibitory effects against α-glucosidase to suppress weight increase [4]. Possibilities based on effective assessments have reported that "OLANDU" promotes the expression of collagen *COL1A1* gene, enhances the formation of collagen, and ameliorates wrinkles [9].

Retinoid compounds (retinol palmitate) have been utilized as a topical medication effective for the treatment of photo-aging skin for decades. Niacinamide is a quasi-pharmaceutical product that has effectiveness for wrinkle improvement. A trial, which verified retinoid response gene of keratinocyte, indicated that activities of retinyl propionate, Vitamin A ester, were re-enforced by niacinamide (Nam) and *Ceratonia siliqua* L. fruit extract [10]. The test cosmetic product can be expected to have this type of synergy.

• *Polygonum tinctorium* leaf/stem extract

Polygonum tinctorium, which is designated as *inagomame* in Japan, is a plant in the knotweed family, polygonaceae. The kanji character of *ai* is "藍". This is also called *tadeai*, indigo plant. This plant has been mainly utilized in dyes. Other than that, effects of detoxication and anti-pyresis were known. Recent investigations discovered diverse functions and effects such as anti-tumor action, antioxidation, anti-inflammatory, anti-virus, anti-allergy [11], and anti-tumor action [12]. Tryptanthrine, which is one of the main components extracted from *Polygonum tinctorium*, strongly inhibits the formation of hepatocyte growth factor (HGF), which is stimulated by diverse inducers in human skin fibroblasts. It is confirmed that HGF not only arranges normal cell functions but also is involved in malignant-cell-related transformations and cancer-cell-related multiplications, invasions, and metastasis. It is supposed that the inhibition of HGF formation has a suppressive effect on the transformation of malignant cells and the progression of tumors [12]. An experimental study using mice, which had 2,4-dinitrofluorobenzene (DNFB)-induced atopic dermatitis, reported that extracts of *Polygonum tinctorium* suppressed serum concentration of IgE and interleukin-4, inhibited the expression of caspase-1 which was derived from mast cells of skin lesions, and improved clinical conditions [11]. It has been reported in cosmetic fields that Japanese indigo extracts have diverse effects such as moisturizing effects, anti-inflammatory effects, skin whitening effects, antioxidation effects and wrinkle improvement effects with enhanced skin resilience [13].

Our research lab reported that extracts of *Polygonum hydropiper* had an intensive inhibitory effect on the formation of advanced glycation end products (AGEs) with

in vitro testing, and an inhibitory effect of the formation of aldehydes (3-DG, GO, and MGO)[14]. To determine whether or not extracts from wild relatives of Japanese indigo (*Polygonum tinctorium*) also have anti-glycation abilities, further verifications have yet to be completed.

• Chamomile recutita flower extract *(Anthemis nobilis)*

Kamitsure (commonly used name: German chamomile, scientific name: *Anthemis nobilis*) is native to Europe. This plant started to be utilized in herbal remedies around the first century B.C. In the Middle Ages, it was documented that chamomile had effects for the mitigation of digestive disorders and abdomen enlarged feeling and effects that facilitate good sleep. Presently, chamomile is characterized as a typical European medical herb. Its evaluation covers diverse effects such as the mitigation and amelioration of anxiety, muscular paralysis, skin problems and digestive disorders for a broad range of age groups from infants to adults[15,16]. Flowers have a rich, mellow, fruity and apple-like aroma. This aroma provides relaxing effects and chamomile herb tea is consumed all over the world[17]. Main ingredients are sesquiterpene, α-bisabolol, chamazulene, which are in terpenoids, and apigenin, quercetin, patuletin, and luteolin, which are in flavonoids, as well as glucosides[18,19].

Clinical trials of mixed herbs such as *kamitusre* flower (*Anthemis nobilis*, Chamomile: flower, AN), *Crataegus oxyacantha* (Hawthor: berry, CO), *Houttuynia coradata* (Doku-dami: whole plants, HC), and *Vitis vinifera* (Grape: leaf, VV), suggested as follows: An open test indicated that HerbMix product[20] ingestion of 600 mg/day for 12 weeks led to the decreases of CML and 3DG in blood, and the improvement in skin viscoelasticity (elevation of skin elasticity, R2 and R7). A double-blind test indicated that through the product ingestion of 3,000 mg/day for 8 weeks, decreasing trends of CML and 3DG ($p < 0.1$) in blood, and increased improvement score of subjective symptoms are recognized[21]. Another double-blind test for 100 mg/day of the product ingestion for 12 weeks demonstrated the decrease of 3DG in blood and the reduction of melanin index and color difference b* (index for yellow) of upper arm skin[22]. Therefore, stable anti-glycative effects were exerted. There was a possibility that the ingredients of the test product contributed to the improvement of skin viscoelasticity.

• Hydroxyproline

Collagen, which is the most abundant protein in the human body, is an essential protein to maintain normal structure and strength of connective tissues such as bones, skin, cartilage, and blood vessels. Glycine, proline, and hydroxyproline account for 57 % of total amino acid (AA) of collagen[23]. Hydroxyproline, which is an abundant amino acid in type I collagen of the skin, is produced via a translational modification of proline by an enzyme prolyl hydroxylase, and this synthesis is irreversible. Hydroxyproline exists in the position of "Y" of a repetitive sequence (Gly-X-Y) in type I collagen, playing an important role in synthesis of collagen, and to thermodynamically stabilize the triple helical structures of collagen and related tissues[24,25]. Hydroxylation of proline requires ascorbic acid (vitamin C). Therefore, deficient vitamin C leads to lower ratio of hydroxyproline in collagen and consequently, stability deteriorates and scurvy is induced.

It is reported that through ingestion of hydroxyproline, collagen synthesis of the skin increases and the proliferation of epidermal keratinized cells is promoted. Acetylhydroxyproline, which is a hydroxyproline derivative, is known to have a vulnerary promoting action and moisturizing effects with increased moisture content of horny cell layer[26].

This cosmetic test product, other than 5-hydroxyproline, contains lecithin, rutin derivative (glucosylrutin), and ascorbyl palmitate. Composite material, RonaCare VTA of Merck (Merck KGaA, Germany)[27,28] is designed to be produced as follows; Hyaluronic acid is protected from degrading enzyme and simultaneously, synthesis of glucosaminoglycan, which is an extracellular matrix, is promoted, where liposome, which contains hydroxyproline, rutin derivative, and magnesium ascorbyl phosphate, penetrate the skin. There is an expectation that the test product has similar effects.

• Niacinamide

Niacinamide (commonly used name: nicotinic acid amide, vitamin B3) is a type of vitamin B3, and is contained in fish, meat, beans, and grains. Niacinamide is approved as a quasi-pharmaceutical product.

The facilitation of ceramide synthesis *in vitro* is known for niacinamide. Different concentrations of niacinamide were added into normal human keratinocytes culture (keratinocytes), and ceramide synthesis was measured with uptake of 14-C serine to ceramide. Addition of niacinamide with concentrations over 1 μM significantly increased ceramide synthesis[29].

Niacinamide of 10 μM was added to a keratinocytes culture, and synthesis of ceramide, which is a structural component of intercellular lipid, fatty acid, and cholesterol, were measured with uptake of 14-C acetic acid to each lipid. The addition of niacinamide of 10 μM significantly promoted synthesis of not only ceramide but also fatty acid and cholesterol ($p < 0.01$)[29].

Kawada Akira, Professor of Department of Dermatology, Kinki University School of Medicine *et al*. verified usefulness of niacinamide on human skin wrinkles[30]. They conducted a clinical examination based on a double-blind trial, with 28 Japanese female subjects who had fine periorbitalwrinkles (31–49 year-old, the mean age: 39.4). Oil-in-Water (O/W) moisturizing agent of 4 % niacinamide mixture was applied on a half face and on the other side of the face, (O/W) moisturizing agent without niacinamide was applied for 8 weeks. According to guidelines of the Japan Cosmetic Industry Association, effectiveness was assessed at the points of 4 weeks and 8 weeks, through two researchers' observation and photographic images. Experimental results using five-grade evaluation, "remarkably significant", "significant", "somewhat significant", "no change" and "change for the worse", suggested that the application of moisturizing agent of 4 % niacinamide mixture exerted the middle or higher level of effects of wrinkle improvement to 18 out of 28 at 8 weeks. This result indicated that niacinamide was effective at wrinkle improvement. There are possibilities that permeability improvement of niacinamide by a cosmetic base could contribute to it[30].

Mechanism of anti-wrinkle effects in niacinamide has not yet to be identified. Judging the findings that the addition of niacinamide to keratinocytes cultures, which did not have sufficient collagen, significantly increased synthetic quantity of collagen[31]; however, there are peculiarities, to some extent, in the promotion of collagen synthesis. Contribution of this action could be a key role. Furthermore, there are other possibilities of complex actions such as cell activation that niacinamide possesses. We await further ongoing examinations.

• Mechanism of wrinkle formation

The major purpose of the present study is the prevention of wrinkle formation. We start by reviewing the mechanism of wrinkle formation.

Skin wrinkles start to appear on the face around 30 years of age. The major causative factor is recognized to be quantitative and qualitative alternations in proteins, elastic fibers, and collagen, which are produced by dermal fibroblasts[32-34]. Another causative factor for shallow facial wrinkles is structural alternations in epidermis due to epidermal dryness. Faces and necks, which are constantly exposed to the sunlight, have deep wrinkles. Farmers have chronic and severe sun exposure and thus, they have cutis rhomboidalis nuchae, which is deep triangle-shaped wrinkles on the neck, around 50 years of age. The skin surface is yellowish, grooved, and stiff. Histopathologically, on the cutis rhomboidalis nuchae, antibody against CML positive material is massively deposited in upper and middle layers of derma[35]. Briefly, a type of AGEs is accumulated on the skin. This alternation does not appear on the face skin before 20 years of age, and older people who are not exposed to ultraviolet radiation do not have cutis rhomboidalis nuchae. Cutis rhomboidalis nuchae is age-related alternation on the skin with chronic exposure to ultraviolet light. The relation to wrinkles is unknown.

Components of derma, which supports epiderma from below, are classified into two categories, cell constituent and interstitial component forming fibrillar tissues (extracellular matrix component). Interstitial components, principal components, consist of collagen fibers comprising collagen, elastic fibers elastin comprising elastine, and substrates fulfilling space between these. Fibroblasts, which produce these, are sparsely located as collagen fibers among them[1,36,37].

Collagen is configured in triple helical structure of three peptide chains, and the amino acid composition of these peptide chains have a repeated structure; a repetitive sequence consisting of three elements, which includes glycine, represents (glycine-amino acid X-amino acid Y). It is known that proline and hydroxyproline account for approximately 21%, and alanine accounts for approximately 11% of amino acid X and amino acid Y[38-41].

Collagen, which accounts for most of the interstitial component, is colloidal fibers, which are produced via a chemical association with a certain ratio of Type I collagen (80–85%) and Type III collagen (10–15%); a chemical association means an aggregation of atoms or molecules into larger units held together by relatively weak forces and behaves as if it is one molecule or one ion[42]. Type I collagen exists in the skin and bones in a large quantity and functions to enhance strength and resilience and to support structures of tissues. Type III collagen consists of thin fibers and has functions for resilience and flexibility. Both collagens maintain supple skins[1,36,43]. Furthermore, fibroblasts, cellular components, which are dispersed in the dermis, are cells where collagen fibers, elastic fibers, and mucopolysaccharide are produced. Fibroblasts are activated, when necessary, to form these compounds smoothly.

Impacts of ultraviolet exposure have been gradually elucidated on wrinkle-formation-related collagen fibers, protein in basal membranes of dermoepidermal junction. Ultraviolet B stimulates epidermal keratinocytes, and produces and releases inflammatory cytokines IL-1α, IL-6 and TNFα. These cytokines stimulate epidermal fibroblasts and keratinocytes in an autocrine manner, and increase levels of mRNA and proteins such as enzyme MMP-1, MMP-3, and MMP-9, which cleave collagen and elastic fibers, accelerating activities. It is considered that the cleavages of fibers progress, leading to wrinkles[44]. At an animal experiment level, it is suggested that by the inhibition of elastase activities, which cleave elastic fibers, ultraviolet-induced wrinkles are remarkably suppressed. Wrinkle formation is mainly induced by quantitative and qualitative alternation in elastic fibers, as is suggested[34]. However, there is a strong opinion that the decrease of collagen is a cause of wrinkle formation, as the expression of MMP-1 is apparent to cleave collagen fibers.

It is considered that Ultraviolet A directly acts on dermal fibroblasts, and induces the increased levels of mRNA and protein of enzyme MMP-1 and others, which cleave fiber proteins. Consequently, this results in wrinkle formation[45]. Involvements of active enzymes are a trigger of the acceleration of mRNA of MMMP-1 by Ultraviolet B and A. Recently, it is clarified that infrared light, like ultraviolet light, is involved, via active oxygen, in the acceleration of activities of MMP-1[46]. Mitogen-activated protein kinase (MAPK) is involved in its intracellular activation pathway[47].

In proportion to frequency of exposure to ultraviolet light, impacts on interstitial components become stronger. Aging phenomenon of the promotion of wrinkle formation and pigmentation gradually progress[48]. It is reported that the synthetic ability of collagen decreases due to ultraviolent exposure[49]. A cause of photoaging is an unbalance of production and degradation of extracellular matrix components induced by long-term ultraviolet exposure[50]. From these findings, it is significant for the suppression of ultraviolet-induced photoaging to promote the synthesis of collagens, whose synthetic quantity is decreased by ultraviolet exposure. Antioxidant agents is recognized to be effective on wrinkle formation.

In some fibroblasts of wound healing areas, p75 neurotrophin receptors (p75NTR), which are a marker of mesenchymal stem cells, are expressed. In a cellular culture system, fibroblasts, which are attached to collagen fibers, stop growth even under the existence of growth factors of proteins, and emulate the resting state of fibroblasts of normal tissues. Fibroblasts of wound healing areas proliferate

and are surrounded by collagen fibers. This demonstrates that in fibroblasts attached to collagen fibers of wound healing areas, new growth initiation factors exist, and prolyl-hydroxyproline (Pro-Hyp), which is a collagen-derived peptide, is produced. Pro-Hyp induces the proliferation of p75NTR positive fibroblasts, which are cultivated in a collagen gel culture, but does not induce the proliferation of p75NTR negative fibroblasts. Pro-Hyp is a growth initiation factor with a low molecular weight for a specific fibroblast, and is involved in the wound healing process [51].

Prolyl-hydroxyproline (Pro-Hyp) and hydroxyprolyl-Glycine (Hyp-Gly) appear in human blood by the ingestion of collagen hydrolysate, promote the proliferation of fibroblasts attached to collagen gel, and promote the formation of collagen. Therefore, Pro-Hyp and Hyp-Gly are related to the effectiveness of collagen hydrolysate ingestion such as improvements of symptoms of the skin and articulations [52]. Pro-Hyp is supplied to tissues via oral administration of gelatin and/or collagen hydrolysate. Therefore, through supply of gelatin and/or collagen hydrolysate, the therapeutic effect on chronic wounds can be expected. In animal experiments and human clinical trials, ingestion of gelatin and/or collagen hydrolysate promote therapeutic effects of treatments for decubitus of animals and humans, and improve protracted wound healing of animals with diabetes.

• *Prevention of wrinkle formation*

The most significant causes of wrinkle are UV-exposure-induced photoaging and oxidative stress [1]. Thus, antioxidant materials are effective to the prevention of wrinkle formation. Compounding ingredients of the test product such as *Ceratonia siliqua* L. fruit extract [53], *Polygonum tinctorium* leaf/stem extract [54], flower extract of Roman chamomile (*Chamaemelum nobile*, former *Anthemis nobilis*) [55], *Geranium robertianum* L. extract [56, 57], and ubiquinone have antioxidant effects.

Niacinamide (it is also called nicotinamide) and nicotinic acid (it is commonly called niacin) are converted by each other, and thus, they have the same effects as a vitamin. These two materials are collectively called Vitamin B3. Niacinamide is a structural component of an important coenzyme related to the delivery and acceptance of hydrogen [58] Two codehydrogenases, nicotinamide adenine dinucleotide (NAD) and nicotinamide adenine dinucleotide phosphate (NADP), play a central and important role. External application of niacinamide has effects to stabilize the epidermal barrier functions including the decrease of transepidermal water loss and the improvement in water content of stratum corneum. Niacinamide induces an increase in synthesis of proteins such as keratin, the promotion of ceramide synthesis, the promotion of keratinocytes differentiation, and the increase of cellular NADP level. For aged skin, a topical application of niacinamide improves surface structures, and smooths wrinkles [58].

Rutin is a quercetin glycoside and a type of bioflavonoid, which is known for an antioxidative effect. A double-blind clinical trial, where cream containing rutin was applied on human skin for 4 weeks with 40 subjects between 30 to 50 years of age was conducted to examine effects of rutin in the human body. Experimental data were analyzed after 2 weeks and 4 weeks, with examinations of dermal density, skin elasticity, length and area of crow's feet wrinkles, and the number of periorbital wrinkles of a group with the application of the control cream and a group with the application of cream containing rutin. Results of assessments confirmed that rutin enhanced skin elasticity and decreased length, area, and the number of wrinkles. Effectiveness of the inhibition of wrinkle formation was confirmed [59]. It confirms that rutin increases the expression of mRNA of collagen type I and α1 in human skin fibroblasts and decreases the expression of mRNA of matrix metallopeptidase 1. Furthermore, it was verified that scavenging activities on active enzyme of rutin were stimulated in a dose-dependent manner. This finding confirmed that protective effects of rutin were exerted under the conditions of oxidative stress [59].

Hydroxyproline is involved in wrinkle formation. An experimental study on mice models with UVB-induced photoaging has suggested that UV radiation induces the increased activities of catalase and in particular, superoxide dismutase of the skin, and the decreased malondialdehyde content, the formation of matrix metalloproteinases (MMP-1 and MMP-3), and the decrease of hydroxyproline [60]. Histological evaluations confirm the infiltration of inflammatory cells, the degradation of collagen, and the abnormality of elastin. These findings are related to wrinkle formation. It is suggested that the decrease of hydroxyproline is involved in wrinkle formation.

Any research regarding wrinkle improvement effects of sole administration of hydroxyproline has not been reported yet. However, there are some reports regarding a peptide containing Hyp. With the purpose of verification of effective treatments of collagen peptide supply, a double-blind randomized placebo-controlled trial was conducted to assess effects on moisture, elasticity, and wrinkle improvement of human skin. Sixty-four subjects were randomly assigned to undergo the ingestion of 1,000 mg, once a day, of either placebo or low-molecular-weight collagen peptides (LMWCP) with tripeptide (Gly-X-Y) (content rate: 15 or higher percent), which contains Gly-Pro-Hyp (3%) for 12 weeks. Parameters of skin moisture, wrinkles, and elasticity were assessed at the base line, after 6 weeks, and 12 weeks. Compared with the placebo group, in the LMWCP group, hydration values of the skin were significantly high after 6 weeks and 12 weeks, and visual assessment scores and three parameters of wrinkles were significantly improved. Regarding skin elasticity in the LMWCP group; one item of three significantly improved from the base line to 12 weeks, and two items of three significantly improved after 12 weeks. These findings suggested possibilities of LMWCP usage as a health functional food material for moisture, elasticity, and wrinkles of the skin [61].

The present study infers that these compounds, which were contained in the test product, contributed to the improvement of wrinkle formation, creating synergy.

• *Involvement of glycative stress in skin aging*

Reducing sugar such as glucose forms aldehyde groups and ketone groups in alkaline solution. β-oxidation and hydrogen peroxide also form aldehyde groups and ketone

groups. Glycative stress is a state of excessive formations of aldehyde groups and ketone groups. Aldehydes are characterized by having a carbonyl group. Electrons of carbonyl groups shift towards oxygen atoms with a high electronegativity, which is polarization. Carbon atoms readily undergo nucleophilic attacks. These reactive groups uncontrollably react to a biological material, proteins, which induces post-modifications (protein denaturation), accumulations of waste materials, degradations of functional protein, functional disorders of TCA cycle, activations of intercellular signals, and tissue disorders. This is a major cause for aging-related degenerative change[62]. Maillard reaction, in a limited definition, is a non-enzymatic and irreversible reaction between reducing sugars and proteins. The process from the formation of Schiff bases, via Amadori rearrangements, through diverse intermediates, to the formation of AGEs, is Maillard reaction.

Glycative stress is a significant risk factor[62,63]. Glycative stress induces the denaturation of collagen and elastin, which comprise the skin, and nonelastic or sagging skins are caused. The accumulation of AGEs causes yellowish skin tone . Glycation of keratin and filaggrin (natural moisturizing ingredients) increases water evaporation from the skin, and decreases moisturizing function. The decrease of filaggrin triggers the destruction of skin barrier function, and raises the onset risk of atopic dermatitis[64]. Furthermore, glycative stress accelerates the melanin formation in melanocyte[65].

• *Improvement of skin viscoelasticity, Secondary endpoint*

Elasticity of the skin is reduced along with aging[2]. Dermal collagen type I is configured in a triple helix structure and plays a role with elastin fibers to maintain skin elasticity. The formation of extracellular matrix component such as fibronectin from fibroblasts decreases due to aging. Formations of collagen and elastin also decrease. Furthermore, via glycative stress, collagen fibers[66] are glycated to AGEs and undergo crosslinking. Consequently, flexibility of fibers reduces, which is a significant cause of decreased skin resilience. Lysine and arginine residues, which comprise collagen protein, readily undergo glycation reaction, and form crosslinking via AGE glycation. Thus, collagen loses mobility[66]. Aging-related alternation in human skin elasticity is shown in ***Fig. 5***[20]. In patients with diabetes, who have strong glycative stress, elasticity index curves of R2 and R7 shift downward in comparison with healthy persons. Skin AGE fluorescence volume measured by an AGE Reader has a positive correlation with skin stiffness[67].

A previous clinical trial regarding skin viscoelasticity with R2 index is introduced. The test, which verified improvement effectiveness of periorbital wrinkles and skin quality with usage of a serum containing multiple types of beauty ingredients, reported improvement effects on not only periorbital wrinkles but also skin elasticity[68]. Assessment of R5 by Cutometer (the elastic portion of the suction phase versus the elastic portion of the relaxation phase) showed an increasing (improving) trend on the test product applied side from before the application 0.51 ± 0.07 to after 4 weeks 0.56 ± 0.14 ($p = 0.076$). Assessment of R2 (the ratio of the skin resistance to the suction phase and ability to return during the relaxation step) showed on the applied side significantly improved from before 0.62 ± 0.07 to after 4 weeks 0.70 ± 0.09 ($p = 0.002$). Assessment of R7 (the ratio of elastic recovery to the total deformation) on the applied side significantly improved from before 0.31 ± 0.04 to after 4 weeks after 0.38 ± 0.14 ($p = 0.018$).

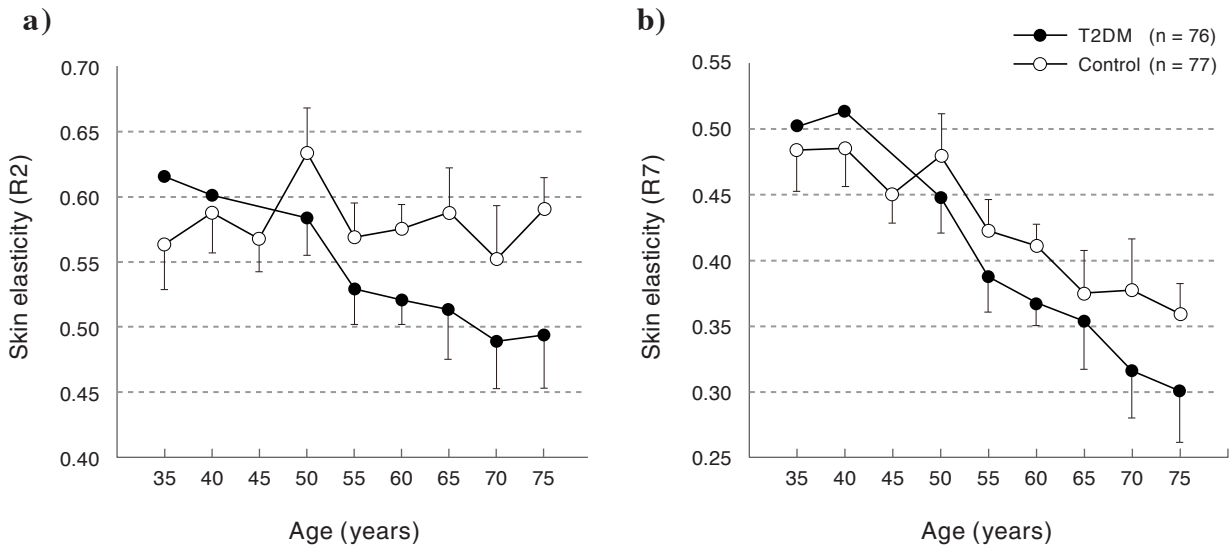

Fig. 5. **Transitional change by age of skin elasticity and impacts of glycative stress.**

a) R2, **b**) R7. The mean value ± SEM, n = 77, healthy persons; n = 76, patients with type 2 diabetes (T2DM). Measurement of the inside of an upper arm using Cutometer (Courage & Khazaka). R2, biological elasticity (Ua1/Uf1); R7, elastic recovery (Ur1/Uf1); SEM, standard error mean.

The present trial showed no significant differences in R5 and R7 examinations. R2 significantly decreased. No improvement effects on skin elasticity of this test product were confirmed.

Research reports have been published regarding effects of skin elasticity improvements by oral administration of mixed herb [69], mangosteen extracts [70], mangosteen pericarp extract-containing black vinegar drink [71], and trapa japonica extract [72].

Among components of the test cosmetic product, it was considered that *Kamitsure* (*Anthemis nobilis*) flower extract, glucosyl hesperidin, and glucosylrutin contain inhibitory effects of the formation of AGEs. However, it is considered that these did not reach to improvements of skin elasticity.

Reason that no differences were observed between the applied side and non-applied side

As the findings of the present study are described, from the perspectives of characteristics of compounding ingredients of the test product, effects of the ameliorations of wrinkle formation and the improvements of skin elasticity can be explained. However, the point is that no differences were observed between the applied side and non-applied side of the face.

Manner of process of aging are individually different [73-75]. There are diverse skin types: a person with dry skin, a person who is concerned about blemishes, wrinkles and yellowish tone, and a person with reduced elasticity. Visual parameters for assessment on skin have been developed as quantitative index; skin hydration measurement [63, 76] for skin age of moisture, image analysis [76] for skin age of stain and wrinkle, color-difference meter and skin AGE fluorescence measurement [77] for skin age of yellowing, and elasticity test [20, 63, 78] for soft skin age (supple).

Risk factors are common in the skin and the body. When an antioxidant supplement is ingested, not only the skin but also the body are affected. This is the same as anti-glycation supplements.

The test product used in the present study was a topical application on the skin. This product adopts emulsifying agents and liposomal components as well as natural ingredients. High permeability is expected. A possibility was suggested that this could result in influence appearances on not only the applied side but also the non-applied side. We consider that this finding would be verified by examining blood concentration changes of effective components after an application of the test product on the skin.

Safety

As the results of safety assessment, no adverse reaction effects induced by the test products were reported. Regarding the safety of the test product, we judged that there was no problem.

Conclusions

We conducted a trial to verify effects of the test product application on the skin with a target of women who were concerned with periorbital wrinkles. Due to collective actions of diverse functional components, improvement effects of periorbital wrinkles were confirmed by two types of assessment methods (medical examination of visual assessment in wrinkle grade and visual assessment in wrinkle grade using photographic images). However, improvements were observed on the non-applied side of the face and there was no statistically significant difference between the applied side and the non-applied side. During the trial, no adverse event was reported and the safety of the trial was confirmed. The test product contained diverse types of functional components. The test product is designed to enhance skin permeability. Therefore, after the percutaneous absorption of effective components, it is possible that the components could move to the other side of the face. However, the examination of the present study cannot confirm that this occurs. To verify this inference, further examinations are required to confirm that blood concentrations of effective components would increase by a percutaneous absorption after an application on the skin of the test product.

Conflict of Interest

The present study was supported by Nabocul Cosmetics Co., Ltd.

References

1) Ichihashi M, Ando H, Yoshida M, et al. Photoaging of the skin. *Anti-Aging Med*. 2009; 6: 46-59
2) Ichihashi M, Yagi M, Nomoto K, et al. Glycation stress and photo-aging in skin. *Ant-Aging Med*. 2011; 8: 23-29.
3) Japanese Cosmetic Science Society. Wrinkle grade standard photo of periorbital wrinkles. Guidelines for cosmetic function evaluation methods. (in Japanese) http://www.jcss.jp/journal/contents_guideline5.pdf
4) Tsim K, Yashiro T, Dong T, et al. New polyphenol compound. Patent JP6976014B1, 2021.
5) Zulim Botega D, Bastida S, Marmesat S, et al. Carob fruit polyphenols reduce tocopherol loss, triacylglycerol polymerization and oxidation in heated sunflower oil. *Journal of the American Oil Chemists' Society*. 2009; 86: 419-425.
6) Lall N, Kishore N, Momtaz S, et al. Extract from *Ceratonia siliqua* exhibits depigmentation properties. *Phytother Res*. 2015; 29: 1729-1736.
7) Rtibi K, Jabri MA, Selmi S, et al. Preventive effect of carob (*Ceratonia siliqua* L.) in dextran sulfate sodium-induced ulcerative colitis in rat. *RSC Advances*. 2016; 6: 19992-20000.
8) Rtibi K, Selmi S, Grami D, et al. Chemical constituents and pharmacological actions of carob pods and leaves (*Ceratonia siliqua* L.) on the gastrointestinal tract: A *review*. *Biomed Pharmacother*. 2017; 93: 522-528.
9) Nabocul Cosmetics Co., Ltd. Developed a new brand "Asakado" and announced "OLANDU", the latest technology chemical compound in the aging care field. Press Distribution, Jan 19, 2022. (in Japanese) https://kyodonewsprwire.jp/prwfile/release/M106981/202201196276/_prw_PR1fl_LS85a7Fd.pdf
10) Lam ECS, Li R, Rodrigues MR, et al. Enhanced retinoid response by a combination of the vitamin A ester retinyl propionate with niacinamide and a flavonoid containing *Ceratonia siliqua* extract in retinoid responsive *in vitro* models. *Int J Cosmet Sci*. 2021; 43: 102-106.
11) Han NR, Kang SW, Moon PD, et al. Genuine traditional Korean medicine, Naju Jjok (Chung-Dae, *Polygonum tinctorium*) improves 2,4-dinitrofluorobenzene-induced atopic dermatitis-like lesional skin. *Phytomedicine*. 2014; 21: 453-460.
12) Motoki T, Takami Y, Yagi Y, et al. Inhibition of hepatocyte growth factor induction in human dermal fibroblasts by tryptanthrin. *Biol Pharm Bull*. 2005; 28: 260-266.
13) Cella Cosmetics. *Persicaria tinctoria* leaf/stem extract. "Ingredient Dictionary," Cella Cosmetics. (in Japanese) https://cella.jp/ingredient/detail.php?p_id=1370 (Accessed at Feb 7, 2023)
14) Takabe W, Yamaguchi T, Hayashi H, et al. Identification of antiglycative compounds in Japanese red water pepper (red leaf variant of the *Persicaria hydropiper* Sprout). Molecules. 2018; 23: 2319.
15) Johnson LR, et. German chamomile. "Guide to Medicinal Herbs", Nikkei National Geographic Inc, Tokyo, pp145-147, 2014. (in Japanese)
16) Miraj S, Alesaeidi S. A systematic review study of therapeutic effects of *Matricaria recuitta* chamomile (chamomile). *Electron Physician*. 2016; 8: 3024-3031.
17) Nagashima T. German chamomile. "Herbal Tea: The Healing of Science", Flagrance Journal, Tokyo, pp42-43, 2010. (in Japanese)
18) McKay DL, Blumberg JB. A review of the bioactivity and potential health benefits of chamomile tea (*Matricaria recutita* L.). Phytother Res. 2006; 20: 519-530.
19) Hayashi S. German chamomile. "The Encyclopedia of Medical Herb", Tokyodo Shuppan Co. Ltd, Tokyo, pp74-75, 2016. (in Japanese)
20) Kubo M, Yagi M, Kawai H, et al. Anti-glycation effects of mixed-herb-extracts in diabetes and pre-diabetes. *J Clin Biochem Nutr*. 2008; 43(Suppl. 1): 66-69.
21) Yonei Y, Miyazaki R, Takahashi Y, et al. Anti-glycation effect of mixed herbal extract in individuals with pre-diabetes mellitus: A double-blind, placebo-controlled, parallel group study. *Anti-Aging Med*. 2010; 7: 26-35.
22) Kawai H, Shoshihara M, Kawakami H, et al. Anti-glycation and skin beautification properties from ingestion of mixed herb extract: A placebo-controlled, double-lind, randomized, parallel-group study. *Glycative Stress Res*. 2016; 3: 236-245.
23) Li P, Wu G. Roles of dietary glycine, proline, and hydroxyproline in collagen synthesis and animal growth. *Amino Acids*. 2018; 50: 29-38.
24) Gordon MK, Hahn RA. Collagens. *Cell Tissue Res*. 2010; 339: 247-257.
25) Srivastava AK, Khare P, Nagar HK, et al. Hydroxyproline: A potential biochemical marker and its role in the pathogenesis of different diseases. *Curr Protein Pept Sci*. 2016; 17: 596-602.
26) Cosmetic ingredients online. Basic information, purpose of formulation, and safety of hydroxyproline. Cosmetic Ingredient Journal, the latest update at July 11, 2022. (in Japanese) https://cosmetic-ingredients.org/skin-conditioning-miscellaneous/1271/
27) Matsumoto Trading Co., Ltd. Ronacare VTA. "Database of Cosmetic Ingredients," Sept 1, 2009. (in Japanese) https://matsumoto-trd.com/product/pdf/concept/h02.pdf
28) Matsumoto Trading Co.,Ltd. Ronacare ASCIII & Ronacare VTA. "Database of Cosmetic Ingredients," Oct 1, 2009. (in Japanese) https://matsumoto-trd.com/product/pdf/02/vo2/05.pdf
29) Tanno O. Improvement epidermal permeability barrier by increasing *de novo* synthesis of ceramides. *Fragrance Journal*. 1999; 27(10); 23-28. (in Japanese)
30) Kawada A, Konishi N, Oiso N, et al. Evaluation of anti-wrinkle effects of a novel cosmetic containing niacinamide. *J Dermatol*. 2008; 35: 637-642.
31) Matts PJ, Oblong JE, Bissett DL. A review of the range of effects of niacinamide in human skin. *International Federation of Societies of Cosmetic Chemists (IFCC) Magazine*. 2002; 5: 285-289.
32) Fisher GJ, Datta SC, Talwar HS, et al. Molecular basis of sun-induced premature skin ageing and retinoid antagonism. *Nature*. 1996; 379(6563): 335-339.
33) Chung JH, Seo JY, Choi HR, et al. Modulation of skin collagen metabolism in aged and photoaged human skin *in vivo*. *J Invest Dermatol*. 2001; 117: 1218-1224.

34) Hachiya A, Kobayashi A, Ohuchi A, et al. The paracrine role of stem cell factor/c-kit signaling in the activation of human melanocytes in ultraviolet-B-induced pigmentation. *J Invest Dermatol*. 2001; 116: 578-586.
35) Mizutari K, Ono T, Ikeda K, et al. Photo-enhanced modification of human skin elastin in actinic elastosis by N^{ε}-(carboxymethyl)lysine, one of the glycoxidation products of the Maillard reaction. *J Invest Dermatol*. 1997; 108: 797-802.
36) Asada Y. "Mechanism and functions of the dermis." The Encyclopedia of Cosmetic Dermatology. Chuo Shoin Co.,Ltd. Tokyo, pp28-33, 2022. (in Japanese)
37) Shimizu H. Dermis." Textbook of Modern Dermatology." Nakayama Shoten Co., Ltd. Tokyo, pp13-20, 2018. (in Japanese)
38) Hattori S, Kuwaba K. Cosmetics and Collagen. "Manufacturing, Application and Development of Collagens 2. CMC Publishing Co.,Ltd. Tokyo, pp143-166, 2020. (in Japanese)
39) Oki M, et. "collagen" Encyclopedic dictionary of chemistry, Tokyo Kagaku Dojin, 1989; 814. (in Japanese)
40) Okuyama K. "Molecular structure of collagen" Manufacturing, Application and Development of Collagens II," CMC Publishing Co.,Ltd. pp1-12, 2020. (in Japanese)
41) Seiwa Kasei Co Ltd. Hydrolyzed collagen derived from fish scale. Patent JP2007326869A. Dec 20, 2007.
42) Keene DR, Sakai LY, Bächinger HP, et al. Type III collagen can be present on banded collagen fibrils regardless of fibril diameter. *J Cell Biol*. 1987; 105: 2393-2402.
43) Murakami Y, Adachi H, Sakaida T, et al. The Reduction mechanism of the type III/I collagen ratio with aging: Age-related change in meprin, a type III collagen propeptide cleavage enzyme. *Journal the Society of Cosmetic Chemists of Japan*. 2013; 47: 278-284. (in Japanese)
44) Woodley DT, Kalebec T, Banes AJ, et al. Adult human keratinocytes migrationg over nonviable dermal collagen produce collagenolytic enzymes that degrade type I and type IV collagen. *J Invest Dermatol*. 1986; 86: 418-423.
45) Herrmann G, Walscheke M, Lange TS, et al. UVA irradiation stimulates the synthesis of various matrix-metalloproteinases (MMPs) in cultured human fibroblasts. *Exp Dermatol*. 1993; 2: 92-97.
46) Schieke SM, Schroeder P, Krutmann J. Cutaneous effects of infrared radiation: From clinical observations to molecular response mechanisms. *Photodermatol Photoimmunol Photomed*. 2003; 19: 228-234.
47) Calles C, Schneider M, Macaluso F, et al. Infrared A radiation influences the skin fibrobrast transcriptome: Mechanisms and consequences. *J Invest Dermatol*. 2010; 130: 1524-1536.
48) Asada Y. Acute and chronic skin lesions. "The Encyclopedia of Cosmetic Dermatology." Chuo Shoin Co., Ltd., Tokyo, pp195, 2002. (in Japanese)
49) Tanaka H, Okada T, Konishi H, et al. The effect of reactive oxygen species on the biosynthesis of collagen and glycosaminoglycans in cultured human dermal fibroblasts. *Arch Dermatol Res*. 1993; 285: 352-355.
50) Obayashi K, Kyotani D, Masuda K, et al. Inhibitory effects of the plant extracts on matrix proteinases. *Journal the Society of Cosmetic Chemists of Japan*. 1998; 32: 272-279. (in Japanese)
51) Sato K, Asai TT, Jimi S. Collagen-derived di-peptide, prolylhydroxyproline (Pro-Hyp): A new low molecular weight growth-initiating factor for specific fibroblasts associated with wound healing. *Front Cell Dev Biol*. 2020; 8: 548975.
52) Asai TT, Oikawa F, Yoshikawa K, et al. Food-derived collagen peptides, prolyl-hydroxyproline (Pro-Hyp), and hydroxyprolyl-glycine (Hyp-Gly) enhance growth of primary cultured mouse skin fibroblast using fetal bovine serum free from hydroxyprolyl peptide. *Int J Mol Sci*. 2019; 21: 229.
53) Ben Ayache S, Behija Saafi E, Emhemmed F, et al. Biological activities of aqueous extracts from carob plant (*Ceratonia siliqua* L.) by antioxidant, analgesic and proapoptotic properties evaluation. *Molecules*. 2020; 25: 3120.
54) Tokuyama-Nakai S, Kimura H, Hirabayashi Y, et al. Constituents of flavonol O-glycosides and antioxidant activities of extracts from seeds, sprouts, and aerial parts of *Polygonum tinctorium* Lour. *Heliyon*. 2019; 5: e01317.
55) Al-Dabbagh B, Elhaty IA, Elhaw M, et al. Antioxidant and anticancer activities of chamomile (*Matricaria recutita* L.). *BMC Res Notes*. 2019; 12: 3.
56) Ben Jemia M, Aidi Wannes W, Ouchikh O, et al. Antioxidant activity of Tunisian *Geranium robertianum* L. (Geraniaceae). *Nat Prod Res*. 2013; 27: 2076-2083.
57) Catarino MD, Silva AMS, Cruz MT, et al. Antioxidant and anti-inflammatory activities of *Geranium robertianum* L. decoctions. *Food Funct*. 2017; 8: 3355-3365.
58) Gehring W. Nicotinic acid/niacinamide and the skin. *J Cosmet Dermatol*. 2004; 3: 88-93.
59) Choi SJ, Lee SN, Kim K, et al. Biological effects of rutin on skin aging. *Int J Mol Med*. 2016; 38: 357-363.
60) Zhou Y, He L, Zhang N, et al. Photoprotective effect of *Artemisia sieversiana* Ehrhart essential oil against UVB-induced photoaging in mice. *Photochem Photobiol*. 2021 Nov 12.
61) Kim DU, Chung HC, Choi J, et al. Oral intake of low-molecular-weight collagen peptide improves hydration, elasticity, and wrinkling in human skin: A randomized, double-blind, placebo-controlled study. *Nutrients*. 2018; 10: 826.
62) Nagai R, Mori T, Yamamoto Y, et al. Significance of advanced glycation end products in aging-related disease. *Anti-Aging Med*. 2010; 7: 112-119.
63) Yonei Y, Uenaka S, Yagi M, et al. Effects on skin by dewaxed brown rice: An open label test. *Glycative Stress Res*. 2021; 8: 29-38.
64) Furue M, Chiba T, Tsuji G, et al. Atopic dermatitis: immune deviation, barrier dysfunction, IgE autoreactivity and new therapies. *Allergol Int*. 2017; 66: 398-403.
65) Abe Y, Takabe W, Yagi M, et al. Inhibition of AGE-induced melanogenesis in B16 melanoma cells by iridoid-containing plants. *Glycative Stress Res*. 2017; 4: 67-70.
66) Cerami A, Vlassara H, Brownlee M. Glucose and aging. *Sci Am*. 1987; 256: 90-96.
67) Ishizaki K, Yagi M, Morita Y, et al. Relationship between glycative stress markers and skin stiffness. *Glycative Stress Res*. 2020; 7: 204-210.
68) Kaneko T, Miyata A, Ishida N. Cosmetic essence makes both effects which looks younger than the actual age and skin improvement. *Medical Consultation & New Remedies*. 2016; 53: 807-814. (in Japanese)

69) Yonei Y, Yagi M, Hibino S, et al. Herbal extracts inhibit Maillard reaction, and reduce chronic diabetic complications risk in streptozotocin-induced diabetic rats. *Anti-Aging Med*. 2008; 5: 93-98.
70) Ohno R, Moroishi N, Sugawa H, et al. Mangosteen pericarp extract inhibits the formation of pentosidine and ameliorates skin elasticity. *J Clin Biochem Nutr*. 2015; 57: 27-32.
71) Takabe W, Yagi M, Ogura M, et al. Effect of mangosteen pericarp extract-containing black vinegar drink on skin quality through anti-glycative actions. *Glycative Stress Res*. 2017; 4: 158-171.
72) Takeshita S, Ishioka Y, Uemura T, et al. 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. *Glycative Stress Res*. 2017; 4: 299-316.
73) Yonei Y, Mizuno Y. The human dock of tomorrow: Annual health checkup for anti-aging. *Ningen Dock*. 2005; 19(6): 5-8.
74) Yonei Y, Takabe W. Aging assessment by anti-aging medical checkup. *Health Evaluation and Promotion*. 2015; 42: 459-464.
75) Yonei Y, Takabe W, et al. What does the Anti-Aging Medical Checkup show?: Data presentation. *Health Evaluation and Promotion*. 2017; 44: 600-605.
76) Miyamoto K, Inoue Y, Hsueh K, et al. Characterization of comprehensive appearances of skin ageing: An 11-year longitudinal study on facial skin ageing in Japanese females at Akita. *J Dermatol Sci*. 2011; 64: 229-236.
77) Nomoto K, Yagi M, Arita S, et al. A survey of fluorescence derived from advanced glycation end products in the skin of Japanese: Differences with age and measurement location. *Anti-Aging Med*. 2012; 9: 119-124.
78) Yo K. The relationship between aging-related changes in cheek appearance and skin properties. *Journal of the Japanese Society for Cutaneous Health*. 2012; 34: 108-112. (in Japanese)