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Original article

The relation of the OPH activity in the corneum, and skin AGEs

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Abstract

***Subjective*:** In this study, the relationship between oxidized protein hydrolase (OPH) activity in the corneum and advanced glycation end products (AGEs) in the skin is examined from the viewpoint of evaluating the effects of glycative stress on the skin.

***Methods*:** The subjects were 124 healthy Japanese men and women. The corneum specimens were obtained from the inside of the right upper arm by tape stripping, followed by measurement of OPH activity and N^{ε}-(carboxymethyl) lysine (CML) content. This study was conducted with the approval of the Ethics Review Committee.

***Results*:** No correlation was observed between the age of the subjects and OPH activity in the corneum, while noted a weak negative correlation between age and CML content. A positive correlation was found between age and the content of accumulated skin AGEs. The OPH activity had a weak negative correlation with the CML content. Between the OPH activity and skin AGEs content, there was no correlation in the analysys with total subjects, but had a positive correlation in the subclass analysis with the female subjects who were 39 years old or younger. Additionally, a weak negative correlation was noted between CML and skin AGEs content.

***Conclusion*:** The high OPH activity may be involved in the reduction of CML content in the corneum. The skin AGEs content increased with age, while the CML content decreased. The OPH activity did not correlate with age. It is considered that the CML content and OPH activity may not be directly affected by age-related changes.

***KEY WORDS*:** oxidized protein hydrolase (OPH), skin aging, N^{ε}-(carboxymethyl) lysine, glycative stress, advanced glycation end products (AGEs)

Introducton

The production and accumulation of advanced glycation end products (AGEs) due to glycative stress induces browning of proteins, sclerosis due to cross-linking, and inflammation due to RAGE (receptor for AGEs), that affects the deterioration of function [1]. Accumulation of AGEs *in vivo* becomes a cause of onset and progression of diabetic complications, Alzheimer's disease, osteoporosis, arteriosclerosis, and the regenerative diseases. It has been reported that pentosidine and N^{ε}-(carboxymethyl) lysine (CML), which are AGEs, accumulate in collagen and elastin fibers of the dermis [2, 3]. Furthermore, CML has also been found to accumulate in epidermal keratin, which has a relatively short turnover time [4]. Skin with a large accumulation of AGEs in the stratum corneum is said to lose its texture and look like an old face [5]. Elevation of glycative stress and accumulation of skin AGEs are involved in skin elasticity [6] and appearance aging [7], thus becoming factors that accelerate the progression of skin aging.

The corneum is located in the outermost layer of the skin and has a barrier function and a moisturizing function to protect our body from the outside [8]. The corneum is the final product of epidermal keratinocytes and has a structure where dead keratinocytes are stacked in 10 to 20 layers with a thickness of 10 to 20 μm. The corneum constantly repeats

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production by epidermal turnover and exfoliation of the outermost layer. Therefore, it is easy, without causing pain to the subjects, to collect specimens of the corneum (tape stripping) using the release sheet coated with adhesive or else. The samples obtained by the tape stripping are widely applied to the evaluation of morphological changes [9], changes in constituent components [10], and biochemical indices such as constituent components and enzyme activites [11, 12].

Oxidized protein hydrolase (OPH) is a type of serine protease present in tissues of swine liver, rat brain, and human blood [13]. OPH has the action of releasing the N-terminal acylated amino acid of protein, thus is also called an acylpeptide hydrolase (APEH) [14] or an acylamino acid-releasing enzyme (AARE, EC 3.4.19.1) [15]. Recently, we reported the AGE elimination activity of OPH in the glyacation model with human serum albumin (HSA)-glucose, the presence of OPH in the human corneum [16], and the presence of natural products that enhance OPH activity [17].

In this study, we evaluated OPH activity and CML content in the corneum and measured skin AGEs accumulation in healthy Japanese men and women, which relationships were further verified.

Methods

Subjects

The subjects in this study included healthy men and women aged 20 to under 100 years. The subjects were those involved in research at Anti-Aging Medical Research Center and Glycative Stress Research Center, Graduate School of Life and Medical Sciences, Doshisha University, and persons who participated in a study briefing session held in advance and agreed in writing to participate in this study. The subjects were 124 individuals who understood the contents of this trial and agreed to participate in the trial in writing.

Collection of corneum specimens

The corneum specimens were obtained from the inside of the upper right arm of the subject using a corneum peeling sheet (Corneum checker, AST-01, Asch Japan, Tokyo, Japan). Specimens were collected from one subject using three sheets. The attachment position was marked when the first sheet was attached to the skin, then the first sheet was peeled off, followed by the collection of a total of three samples from the same location using the second and third sheets. The second and third sheets were used for the measurement of the content of protein, CML and the OPH activity in the corneum, while the first sheet was not used.

Extraction of protein from corneum release sheet

As the protein extract, 1.5 mL of 50 mol/L Tris-HCl buffer (pH 7.4) containing 0.1% Triton-X100 was used. The collected second and third sheets (two sheets in total) were placed in a bottle containing 1.5 mL of the protein extract, and corneum proteins were extracted by ultrasonic treatment in an ultrasonic cleaner. The ultrasonic treatment for 3 minutes was repeated 3 times with a 30-second interval. From the resulting extract, 360 µL was collected for measurement of OPH activity (Extract A). The remaining extract was subjected to ultrasonic treatment for 5 minutes and repeated 4 times with a 30-second interval. The extract was replaced with 50 mol/L Tirs-HCl pH 7.2, and then, using Ultrafiltration Filter (Amicon Ultra Ultracel-3K; Merk, Darmstadt, Germany), concentrated 8 times (Extract B).

Measurement of protein contentt

DC protein assay (Bio-rad, CA, USA) was used to measure the protein concentration of the extract.

Measurement of OPH activity

The above Extract A was used for the measurement of the OPH activity in the corneum. *N*-acetyl-L-alanine *p*-nitroanilide (AAPA) was used as the enzyme substrate for OPH. After mixing 100 µL of 0.25 mol/L Tris-HCl (pH 7.4), 130 µL of Extract A, and 20 µL of 25 mmol/L AAPA, the mixture was reacted at 37°C for 24 hours, followed by absorbance measurement at 405 nm, as OPH activity, of the amount of *p*-nitroaniline (*p*NA) decomposed and separated by OPH from AAPA. OPH activity was defined as one unit (U) of *p*NA concentration (mol/L) released from AAPA at 37°C for 1 hour. The OPH activity was calculated as the OPH activity value per 1 mg of protein (U/mg) by dividing the amount of *p*NA released in the reaction solution by Extract A by the protein concentration of Extract A (mg/mL).

OPH activity (U/mg) = (S24 – S0)/24/P
S: *p*NA concentration in a sample extract (mol/L)
P: Protein concentration in a sample extract (mg/mL)
24: 24 hours
0: Immediately after the reaction (0 hour)

Measurement of CML content

Extract B was used for measurement of the CML content of the corneum extract. The CML concentration was measured using CircuLex CML/N^{ε}-(Carboxymethyl) lysine ELISA Kit (MBL, Nagoya, Aichi, Japan). For the CML content of Extract B, the CML concentration was divided by the protein concentration to calculate the amount of CML per mg of protein (µg/mg).

Measurement of skin AGEs accumulation in skin

Regarding the content of AGEs accumulated in skin, the intensity of skin autofluorescence (AF) resulting from AGEs was measured using AGE Reader mu (DiagnOptics, Groningen, Netherlands) on the inside of the upper-right arm.

Statistical analysis

The OPH activity and CML content in the corneum are shown as the average value ± standard deviation. Statistical analysis software BellCurve for Excel (Social Information Service, Tokyo, Japan) was used to verify the analysis results.

The correlation analysis was evaluated using the Pearson product moment correlation coefficient. Correlation was defined as $0.4 < |r| \leq 1.0$ and $0.2 < |r| \leq 0.4$ as weak correlation. Analysis results were considered significant when the risk rate (p value) was less than 5%.

Ethical standards

This study was conducted in compliance with the Declaration of Helsinki (revised at the 2013 WMA Fortaleza General Assembly) and the ethical guidelines for human-based medical research (notification by Ministry of Education, Culture, Sports, Science and Technology [MEXT] and Ministry of Health, Labour and Welfare [MHLW]). Regarding the collection of the corneum specimens and the measurement of skin AGEs content, the test contents were fully explained to the subjects in advance. The test was conducted after the applicant requested participation in the test and received a voluntary consent form. This research obtained the approval of the Ethical Committee of Doshisha University (Application Number #180003) and Society for Glycative Stress Research (GSE 2018-003), and was based on the deliberation and approval.

Results

Subjects

There were 124 subjects (32 men, 92 women) in this study. The age and number of subjects are 14 in their 20s (women), 21 in their 30s (2 men and 19 women), 28 in their 40s (2 men and 26 women), 15 in their 50s (7 men, 8 women), 10 in their 60s (4 men, 6 women), 17 in their 70s (7 men, 10 women), 18 in their 80s (10 men , 8 women), and one woman in her 90s the average age of which was 57.6 ± 17.8 years (mean ± standard deviation).

Relationship between age and OPH activity and between CML and skin AGEs content

No correlation was noted between the subject's age and the OPH activity (***Fig. 1-a***). A weak negative correlation ($y = -0.075x + 12.062$, $r = -0.250$, $p < 0.05$) was recognized between age and the CML content (***Fig. 1-b***). Meanwhile, a positive correlation ($y = 0.0165x + 1.4514$, $r = 0.587$, $p < 0.05$) was found between age and skin AGEs content (***Fig. 1-c***).

A weak negative correlation ($y = -0.0023x + 0.1122$, $r = -0.242$, $p < 0.05$) was observed between the OPH activity and the CML content (***Fig. 2***). There was no correlation between OPH activity and skin AGEs content (***Fig. 3***), while a weak negative correlation ($y = -2.9209x + 14.8392$, $r = -0.273$, $p < 0.05$) was found between CML and skin AGEs content (***Fig. 4***).

Subclass analysis

The subjects were divided into age groups (39 years old or younger/40 years old or older) and sex (male/female),

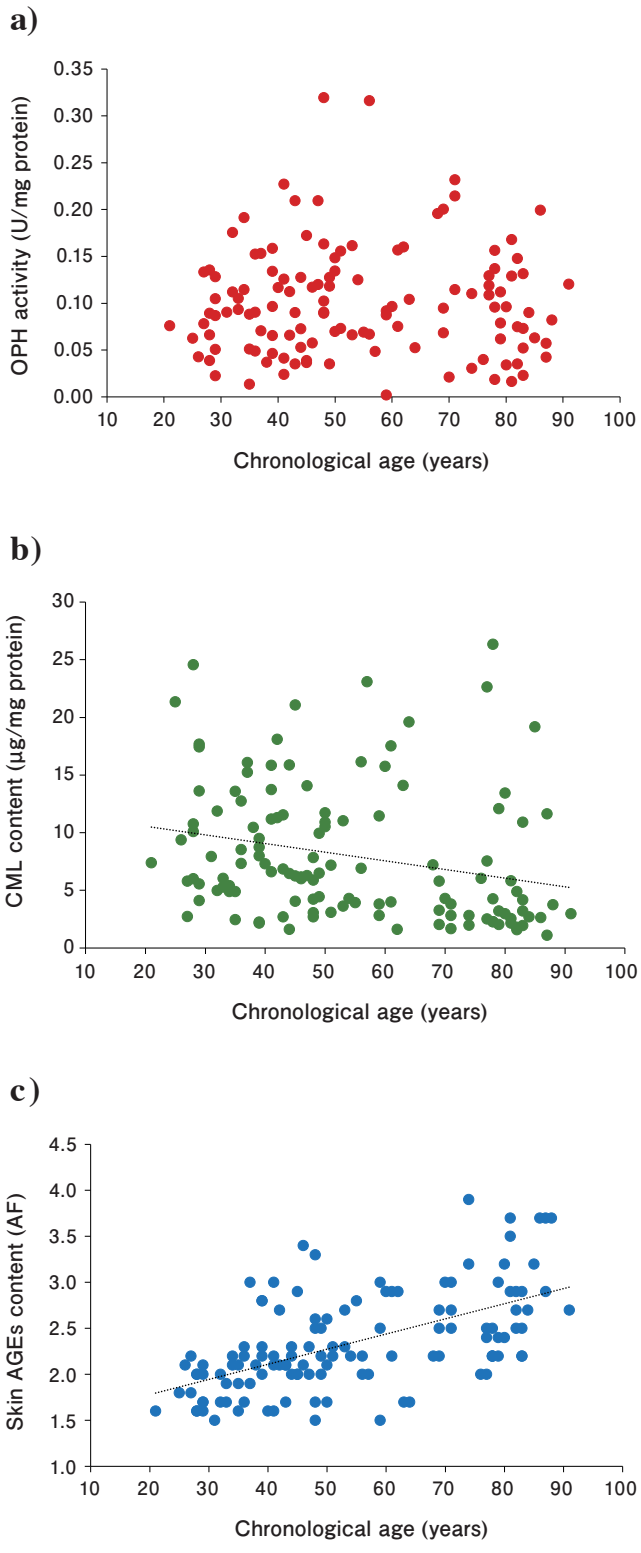

Fig. 1. **Correlation of mesurement values and chronological age.**

a) OPH activity: $y = 0.005x + 0.1003$, $r = 0.005$, p = N.S., **b)** CML content: $y = -0.075x + 12.062$, $r = -0.250$, $p < 0.05$, **c)** skin AGEs content: $y = 0.0165x + 1.4514$, $r = 0.587$, $p < 0.05$; $n = 124$, Statistical analysis, Pearson product-moment correlation coefficient; N.S., Not significant. OPH, oxidized protein hydrolase; CML, N^{ε}-(carboxymethyl) lysine; AGEs, advanced glycation end products; AF, autofluorescence.

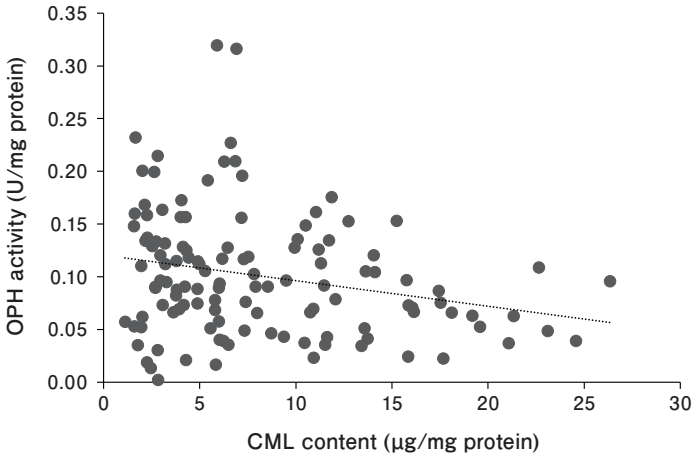

Fig. 2. **Correlation of OPH activity and CML content.**

Correlation, y = −0.0023x + 0.1122, r = −0.242, $p < 0.05$, n = 124; Statistical analysis, Pearson product-moment correlation coefficient. OPH, oxidized protein hydrolase; CML, N^{ε}-(carboxymethyl) lysine.

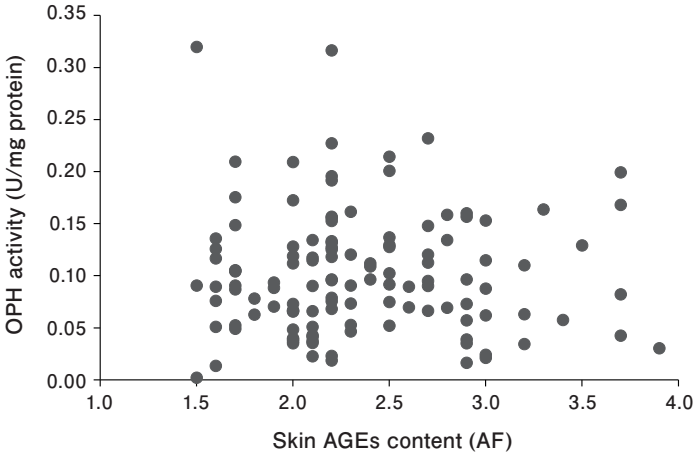

Fig. 3. **Correlation of OPH activity and skin AGEs content.**

Correlation, y = −0.0019x + 0.1056, r = −0.018, p = N.S., n = 124; Statistical analysis, Pearson product-moment correlation coefficient; N.S., Not significant. OPH, oxidized protein hydrolase; AGEs, advanced glycation end products; AF, autofluorescence.

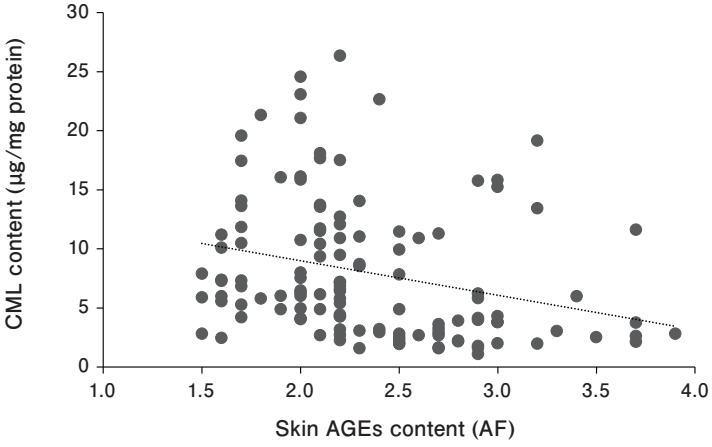

Fig. 4. **Correlation of CML content and skin AGEs content.**

Correlation, y = −2.9209x + 14.8392, r = −0.273, $p < 0.05$, n = 124; Statistical analysis, Pearson product-moment correlation coefficient. CML, N^{ε}-(carboxymethyl) lysine; AGEs, advanced glycation end products; AF, autofluorescence.

followed by the correlation analysis between OPH activity, CML, and skin AGEs content. In a group of women aged 39 years or younger ($y = 0.0406x + 0.0096$, $r = 0.362$, $n = 33$, $p < 0.05$), there was a positive correlation between the OPH activity and skin AGEs content. No correlation was found between the other groups.

Discussion

Relationship between aging and other factors: OPH activity, CML, and skin AGEs content.

The results showed a positive correlation between the subject's age and skin AGEs content.

A previous study, in which non-invasive AGEs measuring devices were used to measure skin AGEs content, in the forearm or upper arm of 58 healthy men and women in their 20s to 80s, confirmed a positive correlation between the age and skin AGEs content [18]. Besides this finding, various survey results have reported that skin AGEs content of the forearm and upper arm increases with age [19-21]. From the results of this test, it was considered that the age-related changes in the subjects were involved in the amount of skin AGEs accumulated.

Furthermore, a weak negative correlation was found between age and the CML content, while no correlation noted between age and OPH activity.

In 52 healthy women in their 20s to 70s, we measured the CML content and OPH activity in the same manner as in this study, and showed a positive correlation with the subject's age and CML content, and a negative correlation with the age and OPH activity, that had different results from this study [16]. CML is also produced by lipid oxidation and oxidative stress in the body [22]. Therefore, it is possible that the CML content measured in this study was affected by factors other than aging. To date, there have been no reports of age-related changes in OPH activity in men and women. Reportedly, the serine proteases present in the corneum include trypsin-like serine proteases, *i.e.* tratum corneum trypic enzyme (SCTE), kallikrein-related peptidase (KLK)-5, and chymotrypsin-like enzymes, *i.e.* stratum corneum chymotrypic enzyme (SCCE), KTK-7 [11,12]. SCTE decreases with age, however, KLK5 and KLK7 do not change with age [23]. Cathepsin D, which is involved in detachment of corneum, and caspase, which is involved in apoptosis, have no clear relationship with aging. Further verification is needed regarding the involvement of OPH in aging. The findings of this study suggest that aging may not be a factor that directly affects CML content and OPH activity in the corneum.

Relationship between OPH activity, CML, and skin AGEs content

In this study, a weak negative correlation was noted between OPH activity and CML content in the corneum. This result is consistent with the previous report [16]. In the report that OPH was added to the glycation model with HSA and glucose, OPH may reduce CML content [16]. Therefore, it is suggested that the decrease in OPH activity may be involved in the elevation in CML content.

No correlation was observed in this study between OPH activity and skin AGEs content. However, the subclass analysis showed, in female subjects aged 39 years or younger, that OPH activity was positively correlated with skin AGEs content. The accumulated amount of AGEs measured as SAF is fluorescent [24], but CML is a type of non-fluorescent AGEs [25]. The relationship between the OPH activity, CML, or skin AGEs content may involve the type and characteristics of AGEs with respect to the reactivity of OPH. Both fluorescent AGEs and CML are final products of the glycation reaction, and concurrently have different structures and physical properties. Therefore, further studies are needed to determine how much the reaction specificity and substrate specificity of OPH can be accepted as substrates. Similarly, regarding the results showing a negative correlation between the CML and skin AGEs content, it cannot be ruled out as having a possibility that physical properties of AGEs or differences in measurement methods had an influence. Another possibility is that the CML content may reflect a glycative stress state with a different meaning from the values of skin AGEs content.

OPH activity in the corneum and glycative stress

In this study, we examined the relationship between OPH activity, CML and skin AGEs content from the viewpoint of assessing the effects of glycative stress on the skin. The production and accumulation of AGEs due to glycative stress is a factor that progresses skin aging [5-7]. Accumulation of AGEs in the skin leads to browning, elasticity reduction due to disordered protein cross-link formation, and deteriorated function of tissue proteins. For example, pentosidine and CML are more accumulated in the skin collagen tissue of diabetic patients, who are under severe glycative stress, than in non-diabetic patients [2]. The skin elasticity of type 2 diabetic patients is lower than that of non-diabetic patients [6]. Furthermore, it has been reported that protein carbonylation is involved in skin yellowing [26]. Methods for assessing glycative stress on the skin include the measurement of skin AGEs content using a non-invasive AGEs measuring instrument [18] and the measurement of CML content using the tape strip method [27]. When assessing the influence of glycative stress on the skin, the amount of accumulated AGEs in the skin and the CML content in the corneum can be used as markers.

The corneum is located in the outermost layer of the skin, and mainly has a barrier function and a moisturizing function, as well as being involved in appearance. Although the corneum is composed of dead cells, there still remain enzymes involved in proteolysis, lipid metabolism, protein modification, and antioxidant. These enzymes are involved in skin turnover, moisturizing and maintaining barrier function. The skin tissue from the upper layer of the epidermis to the stratum corneum is rich in bleomycin hydrolase (BH) [28]. BH is a kind of aminopeptidase with a molecular weight of 45,000 and is an enzyme that further decomposes the N-terminal of peptides in which filaggrin is decomposed, thus releasing amino acids. The amino acid released by the action of BH functions as a natural moisturizing factor (NMF). As a result, skin with high BH activity in the corneum can maintain a high barrier function. The barrier function of the skin deteriorates due to aging, exposure to

ultraviolet rays, and disturbance of turnover. BH expression is suggested to be low in women aware of dry skin [29]. Similar to BH, the action of the enzyme existing in the corneum may be affected by the aging change of the subject as well as by the lifestyle.

In this study, a positive correlation was observed between the subject's age and skin AGEs content. This finding shows that glycative stress and skin AGEs accumulation are strongly related to each other as in the previous report [18-21]. On the other hand, in this study, there was no correlation between the subject's age and OPH activity. In addition, there was a weak negative correlation between age and CML content, which was different from the previous report [16]. However, as in the previous report, a negative correlation was observed between the OPH activity and CML content; the subclass analysis showed a positive correlation between OPH activity and the skin AGEs content in female subjects aged 39 and younger, while showing no significant correration with age. These results suggest that OPH may be more strongly associated with the amount of AGEs, caused by glycative stress, than with age. An increase in the skin AGEs content is associated with reduction of skin texture [5]. Therefore, it is considered that the OPH may act on the AGEs in the corneum and play a role in protecting from the changes in the skin condition induced by glycative stress.

Research limitations

OPH is shown in many reports to have the releasing action of the N-terminal amino acid from posttranslationally modified proteins. However, few reports have verified OPH as an enzyme that decomposes AGE-modified proteins due to glycative stress, and the existence and significance of AGE-degrading enzymes in the skin have not been fully elucidated. Comparing this study with a previous report in which subjects are different [16], the correlation between OPH activity and CML content was similar, while the correlation between OPH activity and age was different. It is necessary to examine the relationship between OPH activity and glycative stress in various subjects in the future.

Conclusions

The results showed that the higher the OPH activity, the smaller the CML content in the corneum, suggesting that OPH acts as a defense against glycative stress in the skin. Although the relevance was not observed between OPH activity and skin AGEs content, little is known about the reaction specificity or substrate specificity of OPH to a wide variety of AGEs, thus further verification is required.

Acknowledgement

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Conflict of Interest Statement

The skin AGEs measurement devices used in this study are provided by the courtesy of Selista inc. (Chiyoda-ku, Tokyo, Japan).

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