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Original article

Relationship between glycative stress markers and skin stiffness

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Abstarct

***Purpose*:** The purpose of this study was to verify the relationship between glycative stress and skin stiffness and fluorescence derived from advanced glycation end products (AGEs), N^{ε}-(carboxymethyl) lysine (CML) content in corneum or blood oxidative stress (OS), and the skin stiffness were measured in healthy men and women.

***Methods*:** The subjects were 58 healthy men and women in their 20s to 80s. The intensity of skin AGEs fluorescence (SAF) was measured using three devices, AGE Reader su, AGE Reader mu, and AGEs sensor. The measuring site tested by theAGE Reader su was the inside of the upper arm, and that by the AGE Reader mu were on the inside of the forearm, and that by the AGEs sensor was the middle finger. The CML content in corneum, which was from the inside of the upper arm using the tape stripping method, was measured by ELISA. Skin stiffness was evaluated on the inside of the upper arm using MyotonPRO, and the biomechanical parameters, Tone, Stiffness, D value (logarithmic decrement), were obtained. The oxidative stress (OS) was evaluated using a Spotchem i-Pack Oxystress test. The correlation analysis of the measured values were verified between the biomechanical parameters for skin stiffness and the SAF, the CML content in corneum or OS.This study was carried out with the approval of the Ethics Review Committee of the Society of Glycative Stress Research.

***Results*:** A significant positive correlation was noted between the subjects' age and the biomechanical parameters of Tone, Stiffness and D value. The SAF of the forearm measured by AGE Reader mu were significantly correlated with Tone and Stiffness, and had a correlation tendancy with D value. No correlation was observed between the SAF of the middle finger measured by AGEs sensor and the biomechanical parameters. CML content in the corneum was negatively correlated with Tone and Stiffness, while not correlated with the D value. The OS did not show any correlation with the biomechanical parameters.

***Conclusion*:** An elevation of the skin AGEs as well as aging were associated with higher Tone and Stiffness. In contrast, the increase of the CML content in the corneum was associated with lower Tone and Stiffness. The OS of the blood, an index of oxidative stress, was not associated with the biomechanical properties. These findings suggested that the skin AGEs and the CML content in the corneum accumulation due to glycative stress may influence the elasticity and viscoelasticity of skin.

***KEY WORDS*:** skin AGEs, N^{ε}-(carboxymethyl) lysine), skin tissue, stiffness, glycative stress

Introduction

Glycative stress is a concept that comprehensively captures the biological stress caused by the load of reducing sugars, i.e. glucose, and aldehydes, and the associated influence induced by the formation and accumulation of advanced glycation end products (AGEs)[1, 2]. AGEs include a variety of substances, i.e. pentosidine, N^{ε}-carboxymethyl lysine (CML), and crossline. With aging, AGEs accumulate in various tissues and organs of the body and affect the decline of physiological functions. It is a factor in the onset and progression of lifestyle-related diseases such as diabetic complications, osteoporosis[3], Alzheimer's dementia[4], and arteriosclerosis[5].

Accumulation of skin AGEs due to elevation of glycative stress is involved in skin elasticity[6] and the appearance of aging[7], thus becoming factors that acceleratethe progression of skin aging. Skin tissue is composed of dermis, epidermis and stratum corneum. The dermis, a connective tissue with dense fiber components, consists of three coponennts; collagen fiber which serves as a supporting tissue of the dermis and provides mechanical strength to the entire skin,

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elastin fiber which generates skin elasticity, and a highly viscous ground substance that fills the space between the constituents. It has been reported that pentosidine and CML, which are AGEs, accumulate in collagen and elastin fibers [8, 9], and AGEs are said to tightly bind collagen due to its cross-linking and browning properties [10], resulting in reduced elasticity, wrinkles, sagging and dullness [11]. Besides, CML is accumulated in the keratin in the epidermis [12]. It is speculated that the corneum, rich in keratin as a constituent protein, is greatly involved in the biomechanical properties of the skin along with collagen fibers and elastin fibers.

Since skin covering the outermost layer of the body can be easily palpated and visually inspected, studies on changes in the skin itself, diagnosis of the inside of the living body through the skin have been actively conducted. In the study on healthy skin, elucidation of changes due to the skin aging, investigation of external risk factors on the skin deterioration, and examination of the effect on the skin by application and continuous interventions of drug base materials have been carried out. The evaluation method has changed from palpation to quantitative, non-invasive measurement that evaluates skin characteristics *in vivo* as a part of human organs, and the development of measurement equipment is being progressed [13-17]. The representatives are as follows; one is a fluorescence intensity measuring device measuring skin fluorescence derived for AGEs and the other is an evaluation device for measuring the biomechanical properties of the skin. In a previous study, we have verified, using several types of non-invasive measuring instruments [18-23], the usefulness of measuring the amount of skin AGEs in assessing glycative stress [24]. As with the behavior of the amount of substances that serve as biomarkers, capturing physical changes also is an important research subject.

The purpose of this study was to elucidate the relationship between glycative stress and skin stiffness. For healthy men and women, a correlation analysis was performed between the parameters representing the stiffness for the skin tissue of the inside of the upper arm and the amount of skin AGEs measured on the upper arm, forearm or fingertips. Concurrently, its correlation with blood oxidative stress (OS) was measured and analyzed.

Methods

Subjects

This study targeted healthy males and females between the ages of 20 and 100, people with the connection to Urata Clinic/Sqol Kanazawa (Kanazawa, Ishikawa, Japan) were recruited as subjects. The subjects were 58 people who participated in the briefing session of this test and understood the contents, and agreed to participate in the test in writing beforehand.

Examination design

This study was an in-group observation study. The subjects continued their usual lifestyle until the day of the test, came to the testing place by themselves, and underwent all tests. The tests were conducted at Urata Clinic/Sqol Kanazawa, March 2018.

Measurement of skin AGEs value

The intensity of skin AGEs fluorescence was measured by three devices: AGE Reader su, AGE Reader mu (DiagnOptics, Groningen, Nederland), and AGEs sensor (Sharp, Osaka, Japan). The measuring site tested by AGE Reader su was the inside of the right upper arm, and that by AGE reader mu was the inside of the right forearm, and that by AGEs sensor was the left middle finger.

Measurement of CML content of corneum

The CML was extracted from the corneum collected by the tape stripping method [25] on the inside of the right upper arm. The CML concentration of the extract was measured using CircuLex CML / N^{ε}-(Calboxymethyl) Lysine ELISA Kit (MBL, Nagoya, Aichi, Japan). The CML content in the corneum was a value obtained by converting this CML concentration per 1 mg of corneum protein in the extract (µg/mg).

Measuerement of skin stiffness

Skin stiffness was measured by MyotonPRO (Myoton AS, Tallinn, Estonia) [26, 27]. The measurement site was the same as the inside of the right upper arm from which the corneum samples were collected for measuring the CML content of the corneum. The measurement was done prior to collecting corneum samples. MyotonPRO is a hand-held non-invasive device that measures the biomechanical properties of muscle or soft tissue, *i.e.* Tone, Stiffness, and D (Logarithmic decrement). It can be applied for soft tissues such as tendons, joints, ligaments, and skin. When the probe at the tip of the device is placed vertically on the skin surface, MyotonPRO mechanically applies a quick impulse (time15 sec, force 0.4N) under constant preload (0.18N) to the contacted skin. Damped natural oscillation induced by this impulse is recorded, and three biomechanical properties are instantly calculated from the form of the acceleration signal. These biomechanical properties were used as measured values in this study.

Tone [Hz] means the tension of tissue such as muscles at rest. The higher the natural oscillation frequency, the greater the tension. The value indicated by F in the device corresponds to Tone.

Stiffness [N/m] is a measure of a tissue resistance to a contraction or an external force that deforms its initial shape. The higher the value, the higher the stiffness. The value indicated by S in the device corresponds to Stiffness.

The D value (Logarithmic decrement) indicates the logarithmic decrease of the damping free vibration of the tissue and is defined as elasticity. Elasticity is the ability to recover its initial shape after a contraction or removal of an external force. The D value is an arbitrary calculated value, thus there is no unit. Since the D value is the logarithmic value of the damping motion, the higher the value, the lower the elasticity. The value indicated by D in the device corresponds to the D value.

Measurement of blood oxidation

Oxidative stress (OS) of blood was measured by a Spot Chem i-Pack Oxystress Test (Arkray, Kyoto, Japan) using a venous blood sample obtained from a subject. The measurement was performed immediately after the blood was collected at the testing facility.

Statistical analysis

Fundamental statistics (average value, standard deviation) were calculated for each data point. A correration analysis was conducted between the biomechanical parameters for skin stiffness and the skin AGEs values measured by each skin AGEs measuring device or the OS. The data were analyzed with the statistical analysis software BellCurve for Excel (Social Information Service, Shinjuku, Tokyo, Japan), and the correlation 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. Statistical analysis results defined the risk rate of less than 5% as significant and less than 10% as a tendency.

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 the skin AGEs, the test contents were fully explained to the subjects followed by receiving a voluntary consent form, before the test was conducted. This research obtained the approval of the Ethical Committee of the Society for Glycative Stress Research (GSE 2018-003), which has discussed the ethics and validity of the study.

Results

Background of subjects

In the 58 subjects (5 males. 53 females), all items in the blood biochemistry test were out of the common reference interval of the Japanese Society of Laboratory Medicine, and did not fall under Judgment Category D (required medical treatment) of Japan Society of Ningen Dock, therefore all of them were healthy adults[24]. The subjects were 4 in their 20s, 11 in their 30s, 23 in their 40s, 8 in their 50s, 5 in their 60s, 6 in their 70s, one in their 80s, with an average age of 40.7 ± 11.0 years (mean ± standard deviation).

Relationship between skin stiffness properties and subjects' age

Correlation analysis was performed on each of the three biomechanical parameters for skin stiffness measured by MyotonPRO and the subjects' age. The subjects' age was significantly correlated with Tone ($y = 0.0653x + 9.63$, $r = 0.479$, $n = 58$, $p < 0.001$, ***Fig. 1-a***), Stiffness ($y = 2.018x + 110$, $r = 0.546$, $n = 58$, $p < 0.001$, ***Fig. 1-b***), and D value (Logarithmic decrement; $y = 0.0117x + 0.759$, $r = 0.520$, $n = 58$, $p < 0.001$, ***Fig. 1-c***). The skin tissue increased in stiffness and became less elastic with aging.

Relationship between skin stiffness properties and value of skin AGEs or blood oxidations

Correlation analysis was performed between the biomechanical parameters for skin stiffness and the value of skin AGEs or the degree of blood oxidation. ***Tabel 1*** shows the correlation coefficient between the skin AGEs value measured by three devices and the biomechanical parameters for skin stiffness. There was a significant correlation of the skin AGEs value of the upper arm measured by AGE Reder su with Tone ($y = 1.59x + 9.07$, $r = 0.323$, $n = 40$, $p < 0.05$) or Stiffness ($y = 46.6x + 98$, $r = 0.323$, $n = 40$, $p < 0.05$), while no correlation with the D value. Similarly, there was a significant correlation of the skin AGEs value of the forearm measured by AGE Reder mu with Tone ($y = 1.01x + 10.04$, $r = 0.287$, $n = 58$, $p < 0.05$, ***Fig. 2-a***) or Stiffness ($y = 29.7x + 126$, $r = 0.310$, $n = 58$, $p < 0.05$, ***Fig. 2-b***), and a correlation tendancy with the D value ($y = 0.130x + 0.949$, $r = 0.221$, $n = 58$, $p < 0.1$, ***Fig. 2-c***). On the other hand, the skin AGEs value of the middle finger measured by AGEs sensor was not significantly correlated with any biomechanical parameters. No correlation was observed between the OS and these biomechanical parameters.

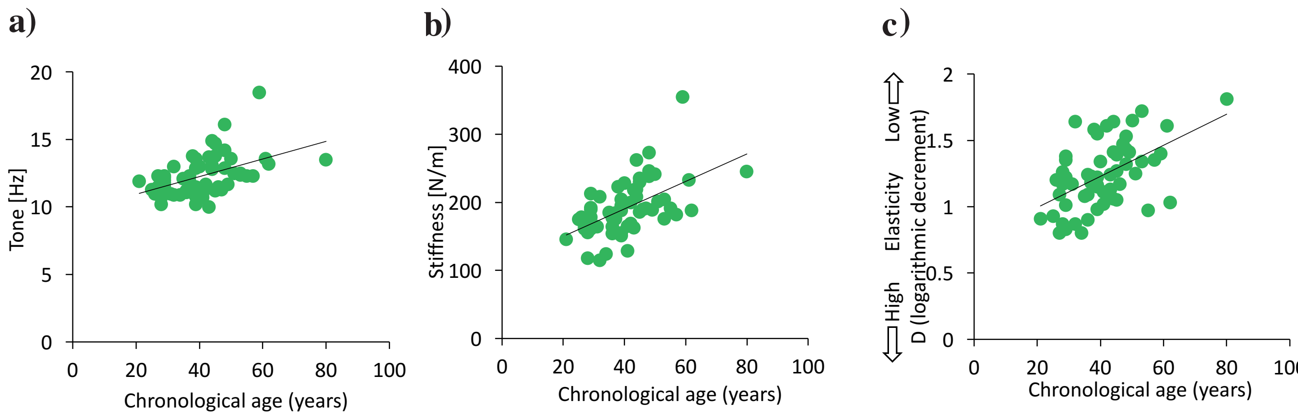

Fig. 1. **Correlations between chronological age and biomechanical properties measured by MyotonPRO.**

a) Tone: $y = 0.0653x + 9.63$, $r = 0.479$, $p < 0.001$. **b)** Stiffness: $y = 2.02x + 110$, $r = 0.546$, $p < 0.001$ **c)** D (log decrement): $y = 0.0117x + 0.759$, $r = 0.520$, $p < 0.001$. Measured region; upper-arm, n = 58.

Relationship between skin stiffness properties and CML content of corneum

A correlation analysis was performed between the biomechanical parameters for skin stiffness and the CML content in the corneum obtained by the tape stripping [25].

There was a significant negative correlation of the CML content in the corneum with Tone ($y = -0.0824x + 13.05$, $r = -0.323$, $n = 58$, $p < 0.05$, ***Fig. 3-a***) or Stiffness ($y = -2.031x + 211$, $r = -0.294$, $n = 58$, $p < 0.05$, ***Fig. 3-b***), while no correlation with D value (***Fig. 3-c***). The skin stiffness reduced with the increase in the CML content of the corneum.

Table 1. Correlation of Myoton parameters with various parameters.

		Clonorogical age	AGE Reader su Upperarm	AGE Reader mu Forearm	AGEs sensor Middle finger	OS (mg/dL)	CML (μg/mg)
Tone [Hz]	r	0.479	0.323	0.287	0.0430	0.0639	−0.323
	p value	<0.01	0.0395	0.0291	0.749	0.634	0.0133
Stiffness [N/m]	r	0.546	0.323	0.310	−0.0044	0.0904	−0.294
	p value	<0.01	0.0395	0.0180	0.974	0.500	0.0252
D (log decrement)	r	0.520	0.184	0.221	−0.0252	0.0627	−0.105
	p value	<0.01	0.253	0.0947	0.851	0.640	0.433

Total subjects, AGE Reader su; n = 40 (male: 2, female: 38), others; n = 58 (male: 5, female: 53)
OS, oxidative stress value in the plasma; CML, N^{ε}-(carboxymethyl)lysine in the corneum

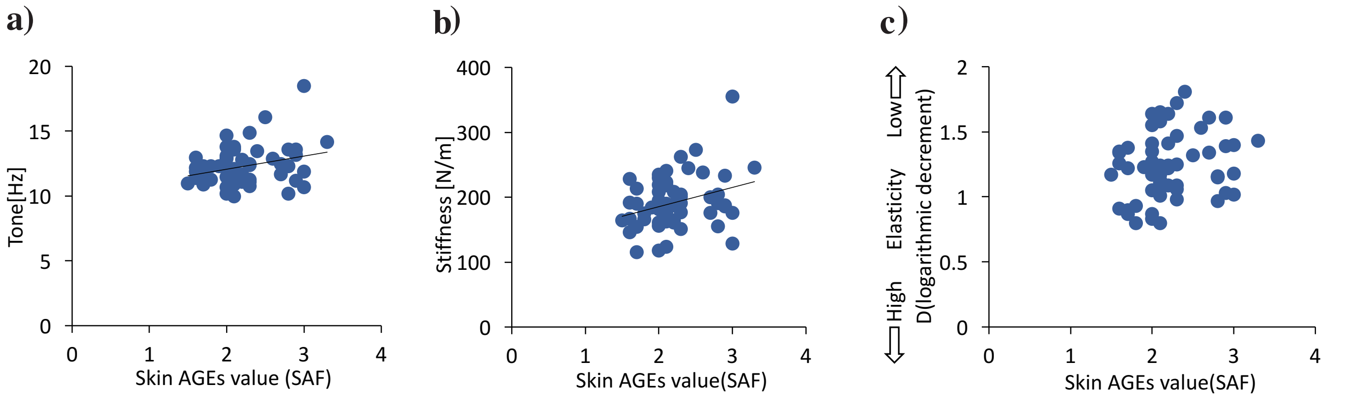

Fig. 2. **Correlations between skin AGEs value and biomechanical properties measured by MyotonPRO.**

a) Tone: $y = 1.01x + 10.04$, $r = 0.287$, $p < 0.05$. **b)** Stiffness: $y = 29.7x + 126$, $r = 0.310$, $p < 0.05$. **c)** D (log decrement): $y = 0.130x + 0.949$, $r = 0.221$, $p < 0.1$. Correlation of AGE Reader mu value. Measured region: skin AGEs value, forearm; Myoton parameter, upper-arm, n = 58.

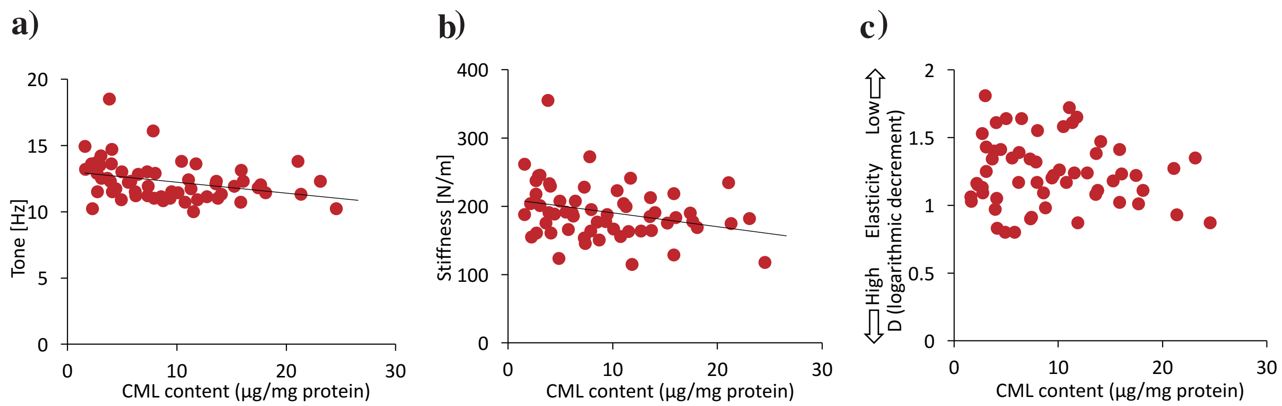

Fig. 3. **Correlations between skin CML content in corneum and biomechanical properties measured by MyotonPro.**

a) Tone: $y = -0.0824x + 13.05$, $r = -0.323$, $p < 0.05$. **b)** Stiffness: $y = -2.03x + 210$, $r = -0.294$, $p < 0.05$. **c)** D (log decrement): $r = -0.105$, $p = 0.433$. Measured region; Myoton parameter, upper-arm, n = 58.

Discussion

Relationship between skin stiffness properties and subjects' age

MyotonPRO is a lightweight, handy size and simple measuring device, and can non-invasively measure the stiffness of joints, ligaments, and skin as well as muscles. It is expected to be used in fields such as sports, health, and beauty, while there are few reports on the skin of healthy individuals. Agyapong-Badu S *et al.*[28] divided 123 healthy men and women with ages of 10s to 90s into a young group (n = 61, 18 to 35 years) and an older group (n = 62, 65 to 90 years), and measured by MyotonPRO, biomechanical properties of the biceps, of which the relationship to the age and sex was investigated. This report showed that Tone, Stiffness, and D value were significantly higher in the older group than in the young group. Our present results are compatible with this report, and indicate that the skin tissue increased in stiffness and the elasticity decreased with aging.

Relation between measurement sites of skin AGEs value and skin stiffness properties

There is no report of the relation between the values of skin AGEs (the intensity of skin auto fluorescence) and the biomechanical properties of skin stiffness measured by MyotonPRO. In this study, the value of skin AGEs was measured using three types of skin AGEs measuring devices with different measuring sites, and the relationship with the biomechanical parameters of skin stiffness measured by MyotonPRO was investigated. Even though the principle of every skin AGEs measuring devices is to measure the fluorescence specific to AGEs (excitation wavelength 370 nm, fluorescence wavelength 440 nm), the measurement site differs depending on the devices such as forearm[18, 21, 23], upper arm[19, 20], and fingertip[22]. In a previous report[24], we showed that the value of the skin AGEs measured on the inside of the forearm and the upper arm had the same significance. In contrast, the measured skin stiffness parameters by MyotonPRO may be affected by the type and properties of the muscles under the skin surface[28].

Therefore, in this study, we confirmed the relationship between the mechanical parameters measured on the inside of the upper arm and the skin AGEs measured at the same site, and verified whether the relationship could be seen with the skin AGEs measured on the forearm or middle finger. The positive correlation between the skin AGEs measured by AGE Reader su and Tone or Stiffness was also confirmed by the skin AGEs measured by AGE Reader mu. Namely, even if the measurement site of skin AGEs value and the measurement site of skin stiffness were different, it was possible to capture the hardening phenomenon of the skin tissue as the amount of skin AGEs increased.

The AGEs sensor has many advantages since the skin AGEs value is measured with the middle finger, it is less susceptible to the effects of ultraviolet rays and melanin, and handling is also good. However, the AGEs sensor did not correlate with the skin AGEs value of other measuring devices[24], and this time, no correration was recognized with the biomechanical parameters of the skin tissue. The measurement value by AGEs sensor is said to correlate with the number of diabetic complications and blood MG-H1 (methyl-glyoxal-hydro-imidazolone) concentration[29], thus being useful as a device for evaluating glycative stress. Further verification is required to clarify the significance of AGEs sensor measurement in relation to other devices used for evaluation of glycative stress.

Relationship between oxidative stress and skin stiffness

OS was used as an index to evaluate the level of subjects' oxidative stress. There was no significant correlation between the OS and biomechanical parameters. It was suggested that the biomechanical properties measured by MyotonPRO may be weakly influenced by oxidative stress.

Skin stiffness parameters measured by MyotonPRO

Devices for evaluating the skin stiffness include Cutometer (Courage + Khazaka, Cologne Germany)[16] and Venustron (Axiom, Koriyama, Fukushima, Japan)[17], which are widely used in the fields of dermatology and cosmetics. A Cutometer is based on the negative pressure suction method, stretches the skin under the constant vacuum pressure of the probe with a hole. Total elongation was related to extensibility. The immediate retraction to total elongation ratio after the final point of vacuum application was utilized as elasticity. In the cutometer, the Ur/Uf ratio (value after release of negative pressure/maximum extension value) is an elasticity index and the Uv/Ue ratio (difference between Uf and Ue/immediate extension value after suction) is a viscoelasticity index. A Venustron is a viscoelastic property measurement device that detemines changes in the resonant frequency (Δf) that occurs when a vibrating probe comes into contact with an object. The resonance frequency depends on the softness of the subjects, because the vibration mode is influenced by variation in the acoustic impedance of the object. Changes in the resonant frequency (Δf) is an elasticity index and relative frequency change (ΔΔf) when a load of 2 to 10 g is a viscoelasticity index. Although each device has different principles and methods, the values indicating elasticity, *i.e.* Ur/Uf (Cutometer), Δf (Venustron) or D value (MyotonPro), decrease with aging[13, 17, 30]. In contrast, in the viscoelasticity, although there was no report that Uv/Ue (Cutometer) or ΔΔf (5g) (Venustron) had a significant correlation with age[13, 17, 31], a significant correlation was found between Stiffness by MyotonPro and age in this study, which is consistent with the results of Agyapong-Badu S *et al.*[28]. These findings indicated that MyotonPRO could monitor changes in skin stiffness with aging.

Regarding the relationship between the amount of AGEs accumulated on the skin and elasticity, Tada A *et al.* reported that a significant correlation was found between the AGEs index, calculated by measuring the cheeks using Skin Skan (Horiba Ltd., Kyoto, Japan), and Ur/Uf (Cutemeter)[32, 33]. Their report also noted that there was no significant correlation between the AGEs index and Δf (5g) by Venustron.

Since Δf (5g) is closely related to the measurement values of other devices that indicate the skin condition of the epidermis, it is considered to be a value that more strongly reflects the skin condition of the epidermis than the dermis[17].

Therefore, they may have considered that Δf reflected more epidermal conditions and Ur/Uf reflected more dermal conditions, although Δf and Ur/Uf showed a good correlation. Similar to the AGEs index, the measured values of AGE Reader su and mu also reflect the amount of AGEs on the entire skin. Therefore, the finding that there was no relationship between those values and D value may suggest that D value more reflects the epidermal properties. Tone and Stiffness changing with the skin AGEs value may reflect the stiffness of the whole skin.

Tada A *et al.*[33] have also verified the correlation between the level of AGEs in the corneum and skin elasticity or viscoelasticity, and confirmed that the level of AGEs in the corneum do not correlate with dermal elasticity (Ur/Uf) or epidermal elasticity (Δf (5g)), but have a negative correlation with skin viscoelasticity (ΔΔf (5g)).

In this study, the CML content in the corneum did not show a correlation with the D value, which is the elasticity index of MyotonPro. The siffness indicated by the value of Tone and Stiffness, which showed a significant negative correlation with the CML content in the corneum, may have properties similar to skin viscoelasticity (ΔΔf (5g)) by Venustoron. Since the stiffness properties of MyotonPro changed depending on the skin AGEs and the CML content in the corneum, MyotonPro was shown to be applicable in the evaluation of the hardness of skin tissue due to glycative stress.

Research limitations

In this study, we investigated the relationship between the biomechanical properties of skin tissue measured by MyotonPRO and the value of skin AGEs measured on the inside of the upper arm and the forearm or the CML content in the corneum in 58 healthy Japanese men and women, and clarified the effects of glycative stress on the stiffness of skin. On the other hand, the value of skin AGEs measured by AGEs sensor at the fingertip did not correlate with the biomechanical parameters, however, we could not obtain any data that could predict its significance.

Conclusion

The value of skin AGEs in the inner part of the forearm or upper arm as well as subjects' age correlates with biomechanical properties measured by MyotonPRO, *i.e.* Tone, Stiffness of the skin, and it was observed that aging and glycative stress caused the skin tissue to increase in stiffness and the elasticity to reduce. In addition, the CML content in tne corneum was negatively correlated with Tone and Stiffness, while OS measured as an index of oxidative stress did not correlate with biomechanical properties of skin. It was suggested that the accumulation of skin AGEs and CML in the corneum due to glycative stress may influence the stiffness of the skin.

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), and MyotonPRO is provided by Medical Agent Co., Ltd (Kyoto, Japan). The agents for Spotchem i-Pack Oxystress test are provided by the courtesy of Arklay Marketing (Shinjuku-ku, Tokyo, Japan).

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