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"Effect of aging on maintaining the hydration of the epidermal stratum corneum"

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1. Introduction

The maintenance of moisture in the stratum corneum (SC) of the epidermis is essential for healthy skin. Appropriate hydration of the epidermis helps to keep SC cells in good condition, resulting in soft, elastic, and healthy skin. Therefore, moisturizing care plays an important role. Various studies have been conducted to understand the moisture state of the SC. For example, Tagami *et al*. reported on the SC water state of normal and desquamated skin using *in vivo* water sorption-desorption test, setting SC hygroscopicity and water-holding capacity as parameters related to the water content of the SC [1]. In recent years, the water state of the SC has been measured using spectroscopic methods such as Raman spectroscopy and infrared (IR) Spectroscopy. In particular, confocal Raman microscopy enables direct measurement of the inside of the skin *in vivo*, e.g., investigation of the relationship between the keratin structure of the SC in the depth direction and the water binding state including water content in the SC [2]. Attenuated total reflection infrared (ATR-IR) spectroscopic imaging can visualize changes in SC moisture state over time to advances in analysis. Using these spectroscopic methods, differences in SC moisture content before and after water uptake and moisture distribution in the depth direction of the SC have been reported [3, 4].

On the other hand, it has been known that the moisturizing function of the SC changes with age. For example, the amount of sebum, intercellular lipids, and natural moisturizing factor (NMF) decrease with age, suggesting that these factors contribute to skin dryness [5]. In addition, focusing on the changes in skin appearance with aging, it has been shown that significant skin changes occur around age 40s and in the mid-50s [6]. As described above, many reports have been made on the physiological and appearance changes associated with aging, and consumers are becoming more and more aware of these changes.

Under these circumstances, we have received many responses from healthy people in their

40s who feel that their skin is dry and not moisturized even after skincare in our consumer surveys and interviews. This suggests that some changes may have occurred in relation to skin moisturization since around the 40s. However, there have been no reports examining the differences in SC moisture state between healthy subjects in their 40s and 20s and the causes of these differences, bearing in mind that the feeling of moisture retention becomes less pronounced with age.

In this study, we conducted the water sorption-desorption test on the skin of healthy subjects in their 40s and 20s to examine water retention capacity. Additionally, keratin, which makes up most of the SC, is not only known as a major factor for maintaining the mechanical skeleton of the corneocytes but also the retain water. Since most of the water is present in the corneocytes, rather than in the lamellas of intercellular lipids, and corneocytes largely consist of keratin filaments and hygroscopic NMF [2]. Therefore, we measured and analyzed the moisture state of SC, where keratin is present, in detail using spectroscopy, and investigated the differences of the moisture state in SC between healthy subjects in their 40s and 20s and the causes.

2. Materials and Methods

2-1. Subjects

Healthy female Japanese subjects who in their 20s (n=16, age = 26.1 ± 2.3) and 40s (n=21, age = 43.5 ± 2.8) were recruited with informed consent. Although some subjects had a history of conditions such as dyshidrotic eczema, seborrheic dermatitis, and atopic dermatitis, none of them had any skin symptoms at the time of the study. The study was approved by the Shiba Palace Clinic Ethics Review Committee (Approval number: 158453_m39804) and conducted according to the Declaration of Helsinki principles.

2-2. Measurement environment and measurement site

Prior to the measurement, subjects cleaned their face with a chosen make-up remover and cleansing foam. Their faces were wiped with a sterile disposable paper towel. Then, they were acclimatized for 15 min in a controlled atmosphere (21± 1°C room temperature and 50~55% relative humidity), in order to equilibrate to room conditions and to minimize perspiration. A series of measurements were performed on the same subject, on the same day, at the measurement sites shown in Figure 1, in the following order. 1: Measurements of skin parameters were performed on the defined area (1.5 x 1.5 cm) of their right cheek (Figure1-(a)). 2: Water sorption-desorption test was performed on three adjacent points on the subject's left cheek (Figure1-(b)). 3: SC cells were obtained by tape-stripping from the subject's right cheek (Figure1-(c)).

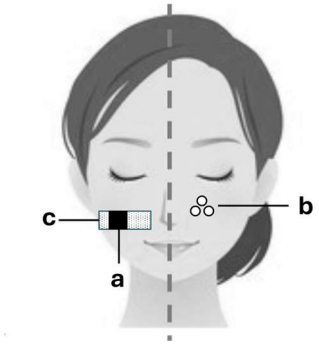

Figure 1. Measurement area: (a) Skin parameters; (b) Water sorption-desorption test; (c)Tape-stripping of the SC.

2-3. Measurement of skin parameters

The hydration state of the skin surface was evaluated by conductance measurements at 3.5 MHz high-frequency current using the SKICON-200EX-USB (Yayoi Co., Ltd., Japan). Trans epidermal water loss (TEWL) was measured by the Tewameter TM300 (Courage + Khazaka Electronic GmbH, Germany). L* a* b* level and skin reflectance (400-700 nm) were measured with the spectrophotometer CM-600d (Konica Minolta Co., Ltd., Japan). Each parameters were measured multiple times according to the characteristics of the instrument, and the average value was taken as the unique value.

2-4. Water sorption-desorption test

Water sorption-desorption test was conducted according to the method reported by Tagami *et al*. [1] with some modifications. First, conductance of bare skin (no water applied) was measured. Next, 75 µL of deionized water was dropped onto the same site and left for 2 minutes. Then, press the wiping paper against the skin for 5 seconds to thoroughly wipe away the water on the skin surface. The skin conductance was then measured continuously for 2 minutes from the measurement immediately after wiping off, and skin conductance values measured every 30 seconds were used for analysis. The test was performed three times at adjacent sites on the left cheek and the average value was used. Relative values were calculated based on bare skin conditions, and following values were defined.

Using the skin conductance measured just after the water removal σ_0 (maximum water sorption) and an average of those measured four times respectively after 30, 60, 90 and 120 seconds σ_m, water holding capacity C was defined as follows:

$$C\ (\%) = \frac{\sigma_m}{\sigma_0} \times 100$$

2-5. Collection of SC, water sorption test of SC tape, and IR measurement.

SC cells were obtained by tape-stripping. Adhesive tape (Nichiban Co., Ltd., Japan) was pressed onto the skin of right cheeks for about 10 seconds and then peeled off slowly. Adhered SC samples were stored at −20 °C until testing. Three days before the measurement, the SC samples were thoroughly dried using a vacuum dryer and then stored in a sealed container with silica gel. Water sorption test using SC tape was conducted under a controlled environment (room temperature of 23 °C, relative humidity of 20-25 %). First, 30 µL of deionized water was dropped onto the SC tape and left for 5 minutes to ensure sufficient water sorption. Next, wiping paper was pressed against the sample for 5 seconds to completely wipe off the water. Then, the IR spectra of the SC tape was obtained.

All the ATR-IR spectra were measured using a Fourier-transform infrared spectrometer (Bruker, ALPHA, Germany) coupled with a diamond ATR accessory. A total of 64 scans was co-added to obtain each spectrum at a wavenumber resolution of 4 cm^{-1}.

2-6. Spectral Data analysis by Multivariate Curve Resolution (MCR)

The IR spectra of the sample obtained as described above (SC-tape spectra) can be considered to have overlapping signals from the tape, SC and water. Therefore, signal decomposition was performed using Multivariate Curve Resolution (MCR) [7]. Initially, to remove the signal from the tape contained in the SC-tape spectra, MCR calculations were conducted assuming three components in the range of 3700-2500 cm^{-1}. The component signal that matched the waveform of the ATR-IR spectrum of tape was subtracted from the SC-tape spectra (SC spectra). Subsequently, in the OH stretching region of 3700-3000 cm^{-1}, MCR calculations assuming two or three components were carried out to obtain the signal

intensities of components attributed to the SC and presumed hydrated water. The results for each subject were averaged from the results obtained from three spectral measurements. Before the MCR calculation, data identified as outliers during measurements or analysis were excluded.

2-7. Statistical analysis

The Wilcoxon rank sum test was performed to compare the results of skin parameters and the water sorption-desorption test in the 20s and the 40s. Student's *t*-test was applied to evaluate the signal intensity values of each component obtained from the SC spectra using IR spectroscopy. Statistical significance was indicated as follows: **p*<0.05, ***p*<0.01, ****p*<0.001.

3. Results

3-1. Age-related differences in skin parameters

First, skin parameters were measured to understand the differences in skin conditions between the 40s and the 20s. The results are summarized in Table 1. Comparing the 40s and the 20s, no differences were found in skin conductance and TEWL, considered to be related to the moisturizing function. On the other hand, L* were lower in the 40s than in the 20s, and a* and b* were higher in the 40s. In addition, spectral reflectance (400-700 nm) was lower in the 40s than in the 20s (Table 1 and Figure 2).

Table 1. Skin parameters of bare skin. Data were shown as mean ± SD. (20s: n=16; 40s: n=21) **p*<0.05, ***p*<0.01, ****p*<0.001.

	20s mean (SD)	40s mean (SD)	*p*-Value
Conductance (μS)	279.1 (149.0)	280.8 (149.0)	N.S.
TEWL (g/h/m²)	16.75 (4.13)	17.21 (3.34)	N.S.
L*	66.93 (2.36)	64.21 (1.98)	＊＊＊
a*	10.39 (1.97)	11.37 (1.46)	＊
b*	14.28 (1.24)	16.14 (1.77)	＊＊＊
400 nm (%)	21.73 (2.08)	17.38 (2.28)	＊＊＊
500 nm (%)	33.54 (2.89)	29.24 (2.29)	＊＊＊
600 nm (%)	47.74 (2.99)	44.35 (2.58)	＊＊＊

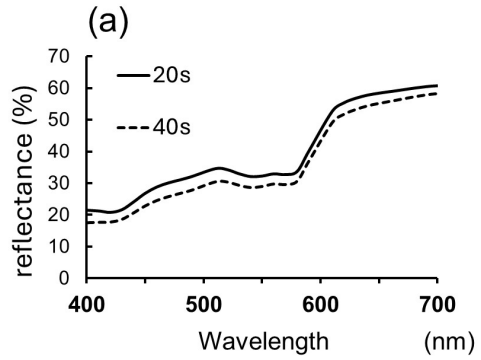

Figure 2. Spectral reflectance of bare skin. Data were shown as mean (20s: n=16, 40s: n=21)

3-2. Water sorption-desorption test

Because the reason for the lack of “moisturized feeling” in the 40s could not be found in the above measurement, the water sorption-desorption test was conducted. Skin conductance of bare skin (pre-hydration) tended to be lower in the 40s than in the 20s (Figure 3-(a)), but no significant difference was observed. Because there is a large individual difference in skin moisture content, in the subsequent analyses, the relative values based on the bare skin conductance of each individual was calculated. The average values of pre, 0 (immediately after water loading), 30, 60, 90, and 120 seconds after water loading were plotted in Figure 3-(b). Relative values at each time point were not significantly different between the 20s and the 40s, including immediately after water loading (σ_0). However, subjects in their 20s had a significantly higher water holding capacity than those in their 40s (Figure 3-(c)).

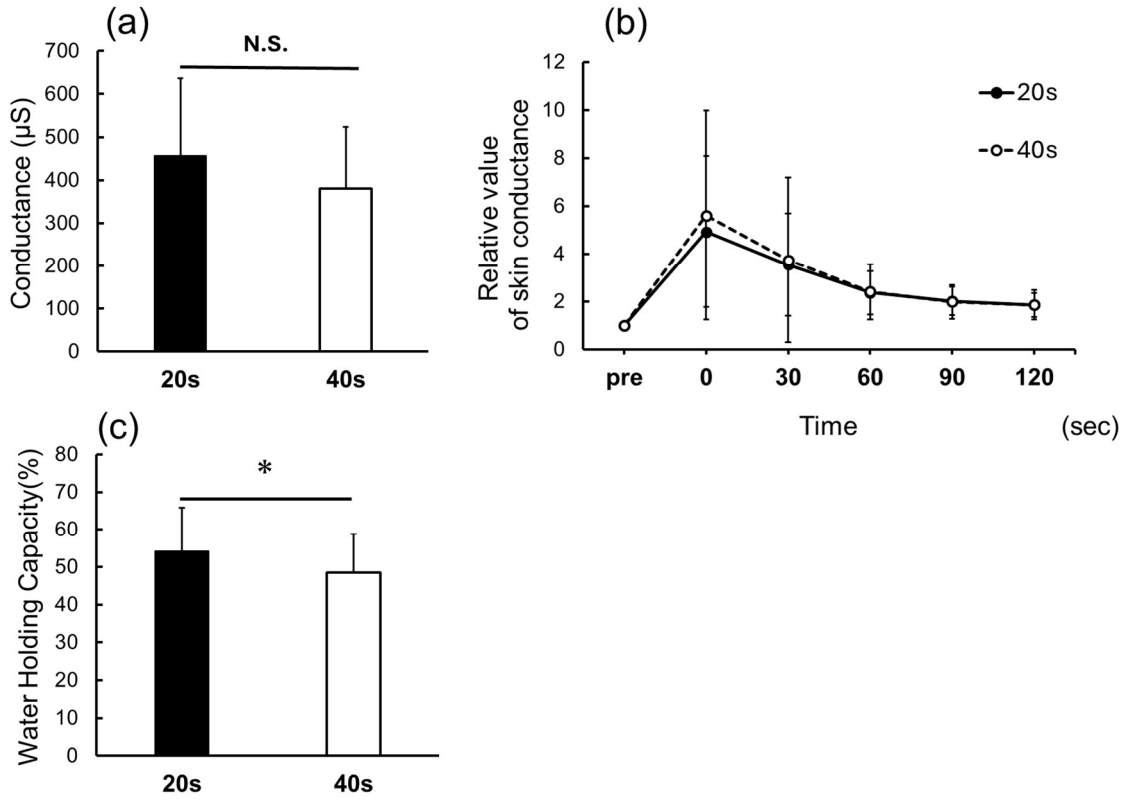

Figure 3. Water sorption-desorption test: (a) Bare Skin Conductance; (b) Changes in relative values of skin conductance after application of deionized water; (c) Water Holding Capacity; Data were shown as mean ± SD (20s: n=16, 40s: n=21) *p<0.05.

3-3. Age-related differences in SC state by spectral data analysis using MCR

It was assumed that most of the moisture retention capacity comes from SC. Therefore, we conducted a water sorption test of tape-stripped SC and analyzed it using IR spectroscopy.

Figure 4 shows (a) IR spectra, (b) Two component waveforms obtained by MCR in the range of 3700-3000 cm^{-1} (OH stretching region) of SC, respectively. The spectral features upon separation into two components resembled those of the spectra obtained from the SC itself and water. Thus, it is presumed that the first component represents the spectral component derived from the SC and the second represents that from water. Figure 4-(c) shows the percentage of the water intensity profiles in the total spectral intensity. The results indicated

that there was no significant difference in the intensity of water-derived components between individuals in their 40s and 20s.

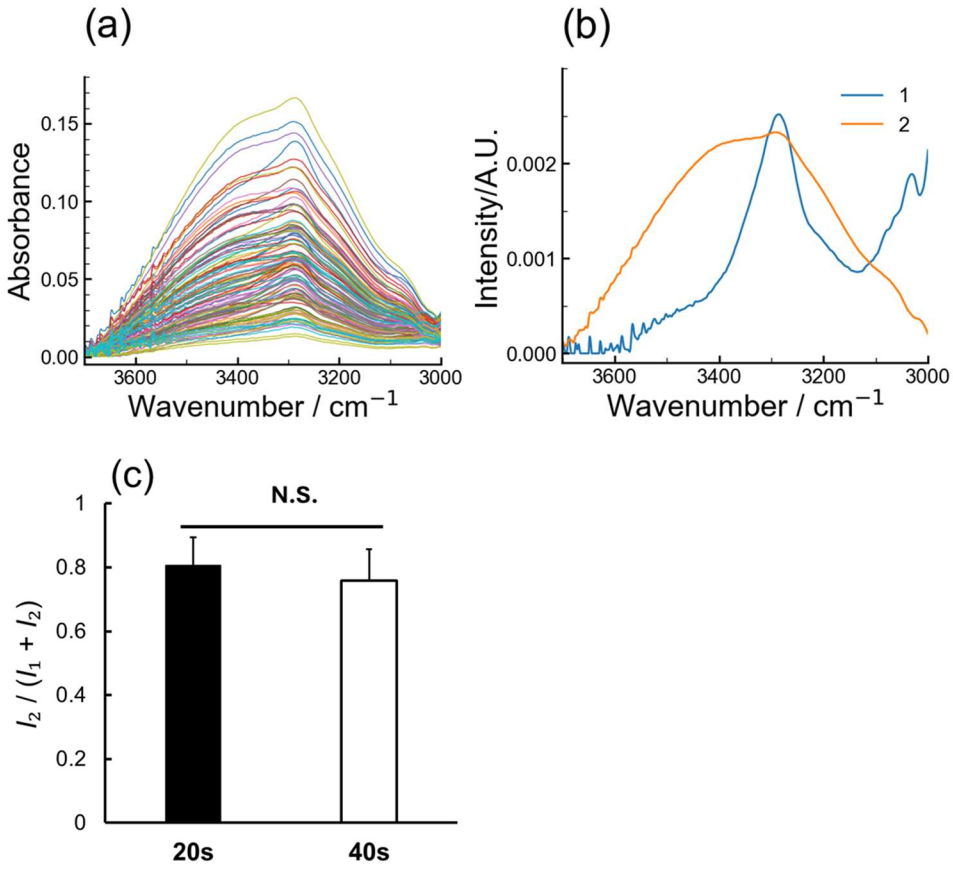

Figure 4. Two components MCR spectral data analysis: (a) IR spectra of SC in the 3700-3000 cm^{-1} region; (b) Component spectra separated into two components by MCR; (c) Percentage of OH region derived from water in the whole spectrum (I_1: the intensity profile of SC derived components, I_2: the intensity profile of water derived components). Data were shown as mean ± SD (20s: n=16, 40s: n=20) *p<0.05.

However, we predicted the water structure in SC might depend on age. Figure 5-(a) plots component spectra decomposed from the ATR-IR spectra assuming three components by MCR. Three different and independent waveforms were clearly identified. The broad features of the waveforms #1 (blue) and #2 (orange) are considered to be different water structures having higher and lower wavenumber contributions around 3400 and 3300 cm^{-1}, while relatively sharp peak at 3300 cm^{-1} assigned to N-H stretching was found for the waveform #3 (green). The waveform #3 was more or less similar to that of the ATR-IR spectrum of SC, representing the component of SC. According to the previous works by Morita *et al*. [8, 9], the waveforms #1 and #2 having the higher and lower contributions were attributed to the water tightly bound to SC and that loosely bound to SC and/or that having bulk like structure, i.e., free-water, respectively. In order to clarify the difference in the water structure, the ratio of the contribution of the waveform #1, which is assumed to the bound-water, I_1 to the sum of #1, #2 and #3, i.e., $I_1 + I_2 + I_3$, were plotted in Figure 5-(b). The ratio of the contribution of the waveform #2 I_2 arising from free-water to the sum of #1, #2 and #3 were also plotted in Figure 5-(c). These results reveal that the SC samples obtained from the 20s exhibit a

significantly higher proportion of bound-water, while those from the 40s have higher free-water. Additionally, the results clearly indicate that the MCR can be used to evaluate the moisture states within SC.

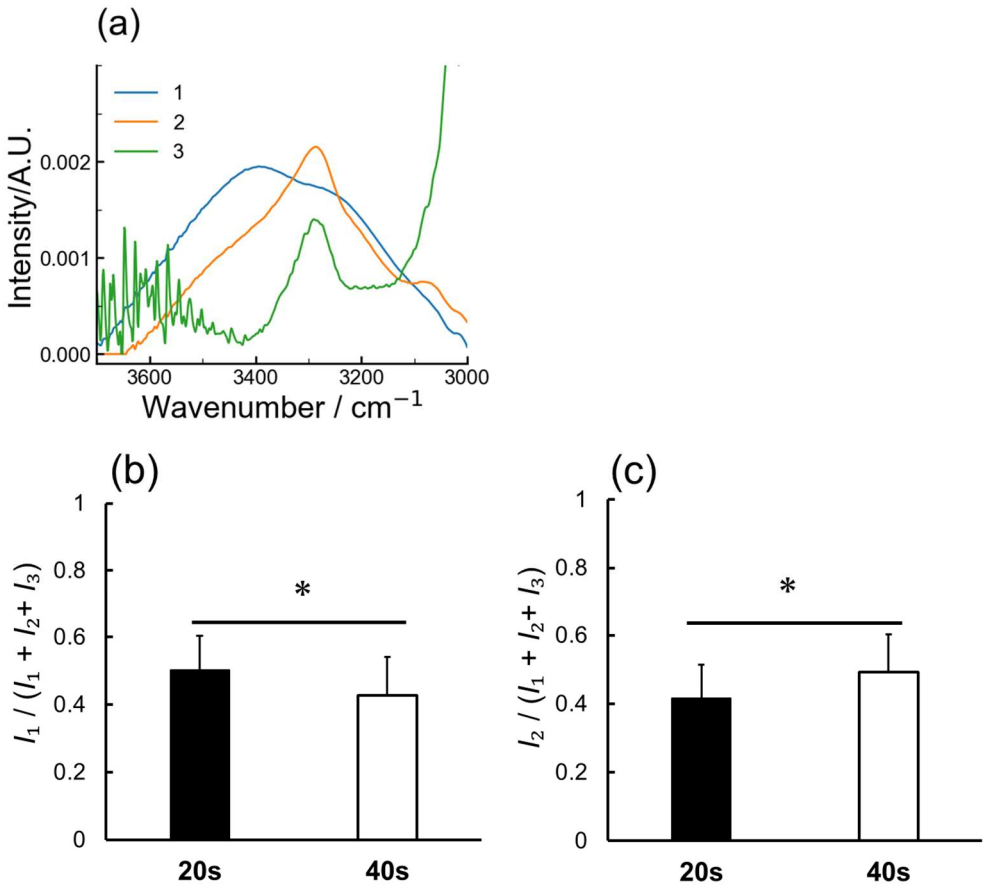

Figure 5. Three components MCR spectral data analysis: (a) Component spectra separated into three components by MCR; (b) Percentage of OH region derived from bound-water in the whole spectrum; (c) Percentage OH region derived from free-water in the whole spectrum (I_1: the intensity profile of bound-water derived components, I_2: the intensity profile of free-water derived components, I_3: the intensity profile of SC derived components). Data were shown as mean ± SD (20s: n=16, 40s: n=20) *p<0.05.

4. Discussion

We investigated the SC moisture state and related factors through the water sorption-desorption test and IR spectroscopy on healthy subjects of groups of the 40s and the 20s to clarify the reasons for consumers' perception that skin in the 40s feels dry and not moisturized even after skincare compared to skin in the 20s. First, the results of examining the hydration level of bare skin showed a decreasing trend with no significant difference between those in the 40s and the 20s, which was consistent with previous reports [10]. To clarify the reason why skin care does not provide a moisturizing sensation even though there is no significant difference in the water content with age in healthy skin, we next performed the water sorption-desorption test, which is equivalent to the act of moisturizing. The result indicated that the water sorption rate of the skin in the 40s did not differ from that in the 20s, but the subsequent water retention rate was significantly lower in the skin in the 40s. Furthermore, MCR analysis of the obtained IR spectra revealed that the proportion of bound-water in the

SC of the 40s after water loading was significantly lower than that of the 20s and the proportion of free-water was significantly higher. These results suggest that SC in the 40s have less area in keratin where water can bind, reducing their ability to retain moisture, and therefore may not retain as much moisture as SC in the 20s, even with skin care, and may not feel as moisturized.

In this study, we were thought by analyzing the skin condition under water-applying, which is equivalent to the act of moisturizing (application of cosmetics) and other products, we might be able to clarify part of the reason why people in their 40s and older feel that they do not feel moisturized enough even after skin care. In other words, this feeling may be due to age-related changes in the amount of bound and free water after water sorption, even though there is no difference in SC water content or TEWL between in their 40s and 20s as has been reported. In the MCR analysis, when the spectra of SC samples after water loading were evaluated using the two components of MCR (SC component and water component), no difference in SC water content could be found between the 40s and the 20s. However, further analysis about the OH region yielded the results shown above. Although we are still in the process of analyzing, we have also found differences in the amide I region (1700-1500 cm^{-1}) of the infrared spectra, which is derived from the secondary structure of keratin, between subjects in the 40s and the 20s. Therefore, we thought that a change of the secondary structure of the SC keratin with aging may affect the abundance of the bound and the free water.

In our research, we measured not only SC water content but also L* a* b* levels such as lightness index and spectral reflectance under bare skin conditions, and the results showed that L* level, b* level and spectral reflectance were lower in the 40s than in the 20s. All of the subjects in this study were women who performed daily skin care routines, and yet the reflectance remained low in the 40s. This might be a possibility that moisture is unable to remain in the SC as bound-water. Conversely, if the condition of keratin and side chains can be adjusted to replenish bound-water, it was thought that this could lead to an increase in the SC moisture retention ability, as well as increases in the L* value and reflectance, thereby maximizing the functionality of the cosmetic product. This is because the structural state of SC keratin is closely related to light scattering properties and light transmittance, which were reported to be affected by carbonylation [11]. It has been also known that SC catalase activity is reduced in exposed areas such as the cheeks [12]. Furthermore, it has been shown that the hydrogen bonding state of water molecules can only be explained by comprehensively considering both the molecular structure of keratin and the contribution of NMF [2]. Therefore, based on these reports, further research is needed to understand SC keratin and water.

Iwai *et al.* have reported that wetting and drying slowly the SC improve the state of SC [13]. That is, when SC is well hydrated, water penetrates between the keratin fiber bundles, causing a temporary change in the arrangement of keratin, and then as it slowly dries, the keratin fibers align properly, improving the SC condition. This may indicate that keratin state, including not only its secondary structure and side chain modifications but also its tertiary structure, is important for further understanding the moisture retention ability of the aged SC. Therefore, further studies related to these factors may be warranted to better understand the changes in SC that occur with age.

Finally, in this study, we were able to evaluate the SC moisture state in more detail by performing spectral separation of infrared spectra using the MCR method, rather than the curve fitting method that is often used. This study was conducted in a simple way that did not burden the subjects. It is hoped that this study will contribute to a better understanding of SC moisture state.

5. Conclusion

There were no differences in the skin moisture levels of healthy female subjects in the 40s and the 20s, and through the water sorption-desorption test, it was found that while there was no disparity in the SC's water absorption capacity in the 40s group, the water retention rate was significantly lower. Furthermore, the observation by IR spectroscopy revealed a decrease in bound-water and an increase in free-water in SC of subjects in their 40s, suggesting a link to age-related decline in water retention capacity.

6. Acknowledgements

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7. References

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