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Association between hydrophilization of stratum corneum due to residual chlorine in tap water and loss of barrier function

Issei Koga [1], Takeyuki Yamamoto [1] and Hirohito Nakajima [1]

[1] Faucet Division, LIXIL, Aichi, Japan

1. Introduction

Chlorination is a disinfection method used to keep tap water sanitary. Some standards have been established around the world for water chlorination. For example, the WHO guidelines and the U.S. Environmental Protection Agency's water quality standards have set 5 mg/L and 4.0 mg/L as maximum chlorine levels, respectively. Chlorine level-based sanitary water management may also be required for public facilities such as swimming pools. The swimming pool management guidelines of the U.S. Centers for Disease Control and Prevention and the E.U. European Centre for Disease Prevention and Control recommend chlorine levels of 1-4 mg/L and 3-5 mg/L, respectively. One of the main chlorine disinfection methods uses sodium hypochlorite. Since the pKa of sodium hypochlorite is 7.5, both hypochlorous acid and hypochlorite ion are present in near-neutral solutions [1]. These constitute free residual chlorine in tap water (hereinafter referred to as residual chlorine). Hypochlorous acid and hypochlorite ions are also produced in vivo by the action of myeloperoxidase in neutrophils. Their high oxidation power is used as a defense mechanism against bacteria that have invaded the body [2]. However, excessive hypochlorous acid has been reported to cause inflammation and protein denaturation [3-5]. Meanwhile, it remains largely unknown how the skin is affected by residual chlorine derived from hypochlorous acid added for disinfection to tap water, which is used repeatedly for face washing, bathing, cooking, dishwashing, and other purposes in daily life. This study aimed to examine the effects of residual chlorine in tap water on the skin and clarify the benefits of purified water containing no residual chlorine in daily life.

2. Materials and Methods

Preparation of residual chlorine water

Residual chlorine water was prepared by adding sodium hypochlorite (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) to purified water (AS ONE, Osaka, Japan) conforming to the Japanese Pharmacopoeia to afford a concentration of 1 mg/L. The properties of the water preparations used in this study are shown in Table 1.

Table 1. Properties of water preparations used for exposure.

Sodium hypochlorite (mg/L)	pH	Hardness (mg/L)
1.0	7.8	<5
0	7.6	<5

Exposure of a three-dimensional cultured epidermis model to residual chlorine water

Prior to exposure to residual chlorine water, a three-dimensional cultured epidermal model (RHEM) (LabCyte Epi-Model 24, Japan Tissue Engineering, Aichi, Japan) was cultured for 8 days to achieve full differentiation into stratum corneum. Two peristaltic pumps (AS ONE, Osaka, Japan) were used to add and collect residual chlorine water at the same flow rate to reproduce the conditions of exposure to running water from the tap water (Fig. 1). To reproduce daily tap water use, the stratum corneum of RHEM was exposed to residual chlorine water at 40°C at a flow rate of 7.7 mL/min for one hour per day (1 h/d) or two hours per day (2 h/d) for three consecutive days.

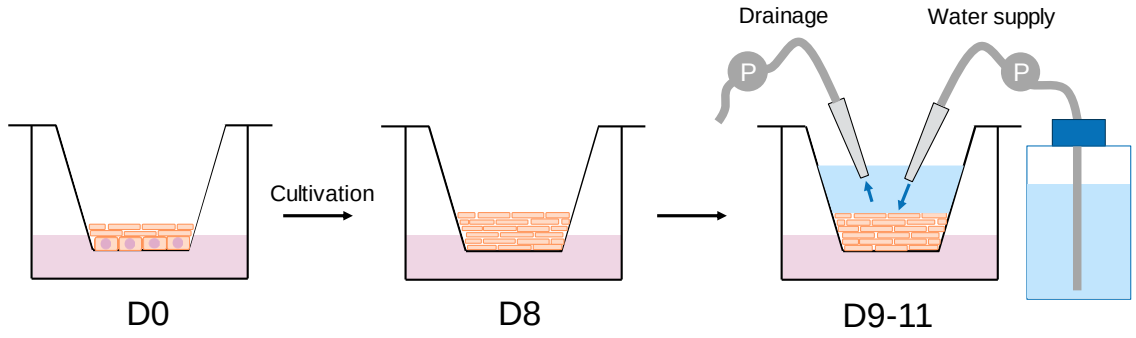

Figure 1. Test of residual chlorine exposure of RHEM.

Transepithelial electrical resistance (TEER) measurement

RHEM culture cups containing RHEM and PBS(-) were placed in PBS(-)-containing wells of a 24-well plate, and the TEER meter EVM-MT-03-01 (World Precision Instruments, Sarasota, USA) was used to measure electrical resistance values.

Measurement of contact angles and evaluation of penetration rates

A drop (0.3 µL) of distilled water was placed on the stratum corneum of RHEM collected using a φ6 mm biopsy punch, and the contact angle of the droplet was measured using DMs-301 (Kyowa Interface Science, Saitama, Japan) immediately, 30 seconds, and 60 seconds after the droplet was placed. The θ/2 method was used to analyze the contact angles. Furthermore, the droplet volume was determined from the images obtained during the contact angle measurement. The penetration rate of a water droplet was calculated from the ratio of the droplet volume at 30 or 60 seconds to the droplet volume immediately after the droplet was placed (Fig. 2).

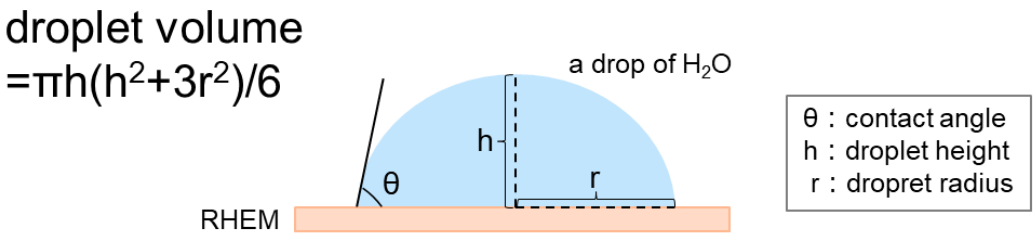

Figure 2. Contact angle and volume of a water droplet on RHEM.

Preparation of frozen sections of RHEM

The collected RHEM samples were embedded in OCT Compound (Sakura Finetek Japan, Tokyo, Japan) to prepare 5-µm sections using a cryostat.

Hematoxylin-eosin (HE) staining

A hematoxylin-eosin staining kit (ScyTek, Utah, USA) was used for HE staining of frozen sections in the prescribed manner.

Determination of hydrophobicity of RHEM

Five-µm frozen sections were immersed in ultrapure water for 10 min at room temperature and then in a 100 µM 1-anilinonaphthalene-8-sulfonic acid (1,8-ANS) solution (Sigma-Aldrich, Darmstadt, Germany) in ultrapure water for 10 min at room temperature and in the dark. Fluorescence images of the samples were obtained with the fluorescence microscope EVOS M5000 (Thermo Fisher Scientific, Waltham, USA) and analyzed using the image analysis software Corneocytometry 2 (CIEL, Tokyo, Japan) to determine fluorescence intensities [6].

Visualization of RHEM protein functional groups by fluorescent labeling

Frozen sections were used for visualization of the functional groups of RHEM proteins. Aldehyde groups were fluorescently labeled with fluorescein-5-thiosemicarbazide (FTSC) (Sigma-Aldrich, Darmstadt, Germany) [7]. Frozen sections were immersed sequentially in 0.1 M 2-(N-morpholino) ethanesulfonic acid sodium salt buffer (MES-Na buffer) (pH 5.5) for 10 min at room temperature and in 0.1 M MES-Na buffer (pH 5.5) containing 20 µM FTSC for 1 h at room temperature in the dark. Also, carboxyl groups were fluorescently labeled with dansyl cadaverine (DC) (Sigma-Aldrich, Darmstadt, Germany) [6]. Frozen sections were immersed in 0.1 M MES-Na buffer (pH 5.5) for 10 min at room temperature and then in 0.1 M MES-Na buffer containing 1 mM 1-(3-dimethylaminopropyl)-3-ethylcarbodiimide hydrochloride (Tokyo Chemical Industry, Tokyo, Japan) and 100 µM DC for 2 h at room temperature under light shielding. In addition, thiol groups and disulfide bonds were fluorescently labeled with Alexa Fluor 555 C2 Maleimide (Thermo Fisher Scientific, Waltham, USA) and N-(7-Dimethylamino-4-methylcoumarin-3-yl) maleimide (DACM) (Anaspec, Fremont, USA), respectively. To label thiol groups, frozen sections were immersed in 0.1 M

Tris Acetate Sodium buffer (TAS buffer) (pH 6.8) for 10 min at room temperature and then in 0.1 M TAS buffer (pH 6.8) containing 1.6 mM Alexa Fluor 555 C2 Maleimide for 2 h at room temperature under light shielding. For labeling disulfide bonds, frozen sections washed with ultrapure water were immersed in 0.1 M TAS buffer (pH 6.8) containing 40 mM dithiothreitol (DTT) (Nacalai Tesque, Kyoto, Japan) and 0.5 mM EDTA for 10 min at 37°C to reduce disulfide bonds to thiol groups; after washing with ultrapure water, the reduced samples were immersed in TAS buffer (pH 6.8) containing 10 µM DACM for 3 min at room temperature under light shielding. Fluorescence-labeled RHEM was observed by fluorescence microscopy, and the acquired images were analyzed to determine fluorescence intensities in the same manner as described for 1,8-ANS.

Statistical analysis

Statistical significance tests comparing the 0 mg/L and 1 mg/L residual chlorine level groups were performed using the Student t-test. Differences with a p-value of less than 5% were considered significant.

3. Results

TEER

TEER values were measured to evaluate the effects of residual chlorine water exposure on the barrier function of RHEM (Fig. 3). No effects of residual chlorine on TEER values were observed after 1 h/d exposure. However, after 2 h/d exposure, the TEER value in the 1 mg/L group tended to be lower.

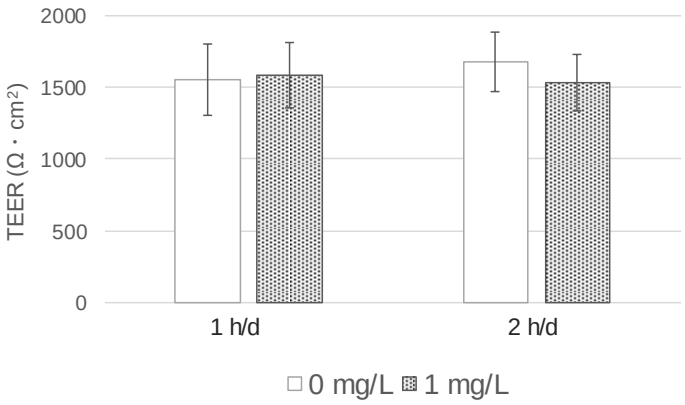

Figure 3. TEER values of RHEM after exposure to residual chlorine. Data are expressed as mean ± SD. n = 6 (1 h/d), n = 3 (2 h/d).

Contact angles and penetration rates

To evaluate the effects of residual chlorine exposure on the stratum corneum, the contact angles of water droplets and the rates of penetration of water droplets into the stratum corneum were determined. While contact angle values with and without exposure to residual chlorine did not differ significantly, the values after residual chlorine exposure at 1 mg/L tended to be generally lower. This result indicates that the stratum corneum of RHEM tended to become more hydrophilic after exposure to residual chlorine (Fig. 4(a)). Meanwhile, the penetration rates after residual chlorine exposure at 1 mg/L tended to be generally higher, with the rate at 30 seconds in the 1 h/d group being statistically significantly different from the rate without residual chlorine exposure.

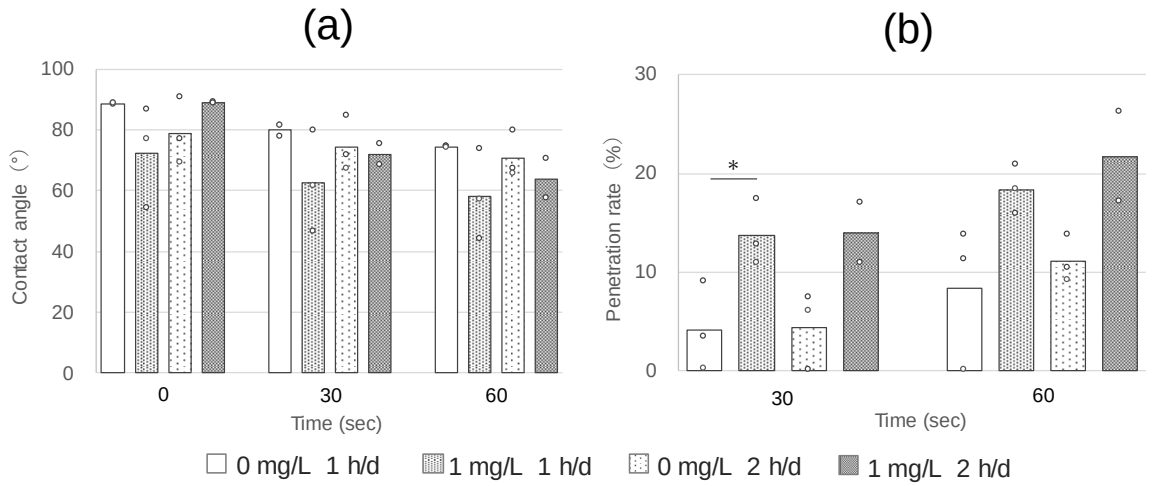

Figure 4. Contact angles (a) and penetration rates (b) of water droplets after exposure to residual chlorine. The dots denote the individual measurement data, and the bars denote the mean values. n = 3 (1 h/d), n = 2 (2 h/d). * $p<0.05$.

HE staining

To evaluate the effects of residual chlorine on the stratum corneum, the outermost layer of the epidermis and considered the skin layer most susceptible to residual chlorine, RHEM was cultured to afford the pure stratum corneum. HE staining was used to confirm that RHEM was composed exclusively of the stratum corneum (Fig. 5). While no major differences in the overall structure were observed between the stratum corneum samples with and without residual chlorine exposure, the tissue around the outermost part was disrupted in the stratum corneum exposed to residual chlorine, and the disruption was more noticeable in the stratum corneum exposed for 2 h/d than in that exposed for 1 h/d.

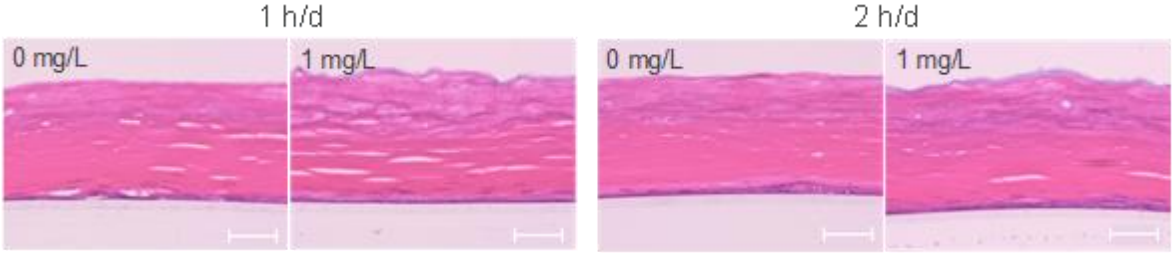

Figure 5. Photographs of HE-stained RHEM samples. Scale bar: 50 µm.

1,8-ANS staining

1,8-ANS is for the evaluation of the hydrophobicity/hydrophilicity of RHEM. The stratum corneum samples exposed to residual chlorine showed decreased fluorescence intensity in the near-surface part (Fig. 6). The fluorescence intensity of 1,8-ANS inversely correlates with the hydrophilicity of surrounding tissue. Thus, the result suggests that residual chlorine exposure increased hydrophilicity.

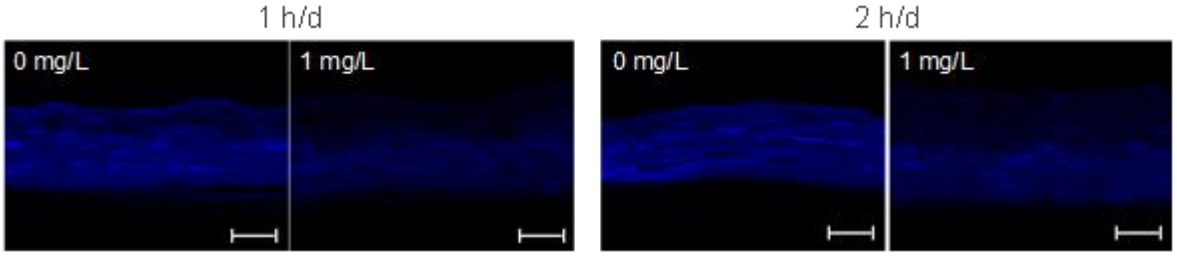

Figure 6. Fluorescence microscopy images of 1,8-ANS-stained RHEM samples. Scale bar: 50 µm.

Effects on functional groups

The effects of residual chlorine exposure on functional groups were evaluated (Fig. 7). Residual chlorine exposure increased the presence of aldehyde groups near the outermost layer. Similarly, carboxyl groups near the outermost layer were also found to increase after residual chlorine exposure. Thiol groups and disulfide bonds were present in the stratum corneum exposed to residual chlorine, with thiol groups decreasing in the near-surface area and disulfide bonds increasing near the outermost area. Furthermore, merged fluorescent images for thiol groups and disulfide bonds revealed that the ratio of those two in the near-surface region was altered in the residual chlorine exposure group. Results with different exposure durations indicated that the exposure time dependency was particularly noticeable for the carboxyl group.

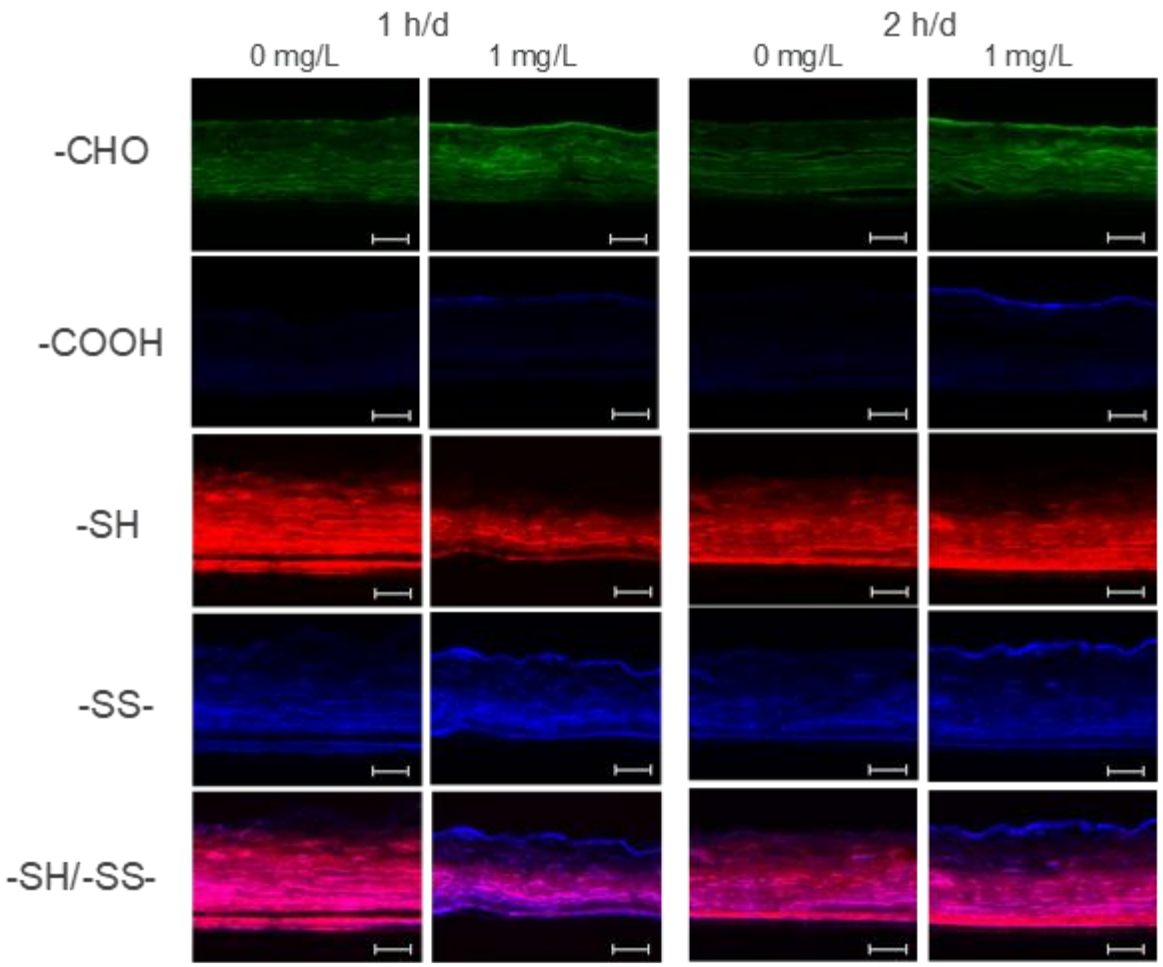

Figure 7. Fluorescence microscopy images of RHEM in which functional groups were fluorescently labeled. Scale bar: 50 µm.

4. Discussion

Effects of residual chlorine on barrier function of the stratum corneum

We exposed RHEM to water containing residual chlorine at a level possibly found in tap water to investigate its effects on the barrier function of RHEM. First, the presence of aldehyde groups in RHEM was confirmed to clarify whether residual chlorine in tap water maintained its oxidation power. Hypochlorous acid water is known to oxidize amino acid residues in proteins to aldehyde groups [8]. Fluorescence signals indicating the presence of aldehyde groups were observed in the surface part of the stratum corneum of RHEM exposed to residual chlorine water. This result suggests that hypochlorous acid in residual chlorine water is capable of oxidizing proteins (Fig. 7). Therefore, we measured the barrier function of RHEM exposed to residual chlorine water. TEER, an indicator of barrier function, tended to decrease after longer exposure, and the contact angle of water droplets on the surface tended to decrease in RHEM exposed to residual chlorine. Furthermore, the penetration rate of water droplets into RHEM tended to increase after exposure, with the difference being statistically significant under a certain experimental condition. When measured at the 60-second time point, a weak positive correlation ($r = 0.48$) was observed between TEER values and contact angles, while a negative correlation ($r = 0.69$) was found between penetration rates and TEER values (Fig. 8).

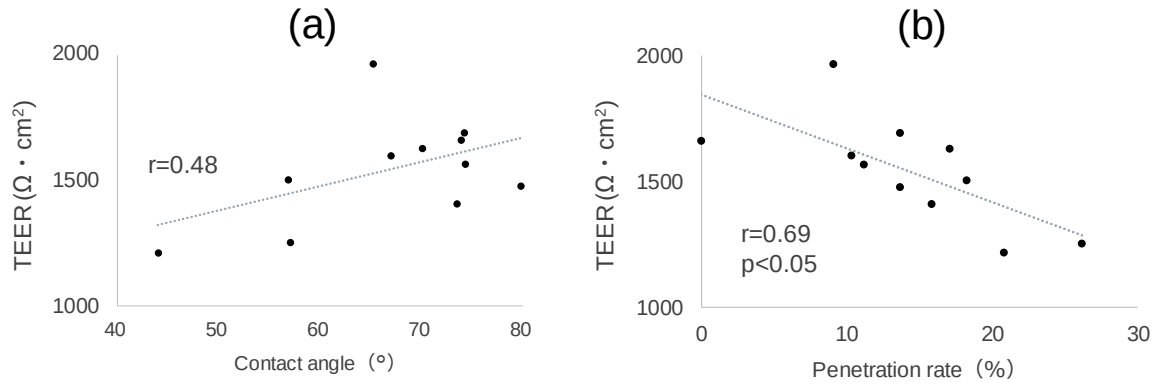

Figure 8. Relationships between contact angles and TEER values (a) and between penetration rates and TEER values (b) measured at 60 seconds.

Then, we measured fluorescence intensities derived from 1,8-ANS to determine whether residual chlorine water actually induced changes in the hydrophobicity of the stratum corneum surface. The fluorescence intensity of 1,8-ANS has been reported to be dependent on the hydrophobicity of the surrounding environment [9]. Therefore, the intensity of 1,8-ANS fluorescence decreases as the hydrophobicity of RHEM decreases. In the RHEM samples exposed to residual chlorine water, decreased intensities of 1,8-ANS-derived fluorescence were observed in the near-surface part of the stratum corneum, indicating reduced hydrophobicity and increased hydrophilicity. In addition, we tested whether residual chlorine exposure affected the presence of carboxyl groups, thiol groups, and disulfide bonds of stratum corneum proteins, which are functional groups considered to be involved in the hydrophobicity of the stratum corneum, by visualizing these functional groups with fluorescent labels. The abundance of the carboxyl group, which is a hydrophilic functional group, was increased near the outermost layer of the stratum corneum exposed to residual chlorine. Meanwhile, residual chlorine exposure decreased the abundance of the thiol group, a hydrophilic functional group, and instead increased the abundance of disulfide bonds, which contributes to an increase in hydrophobicity. The oxidization power of residual chlorine was considered to underlie this effect [10]. Thus, among changes in the amount of functional groups that can explain the increased hydrophilicity of stratum corneum proteins, the results suggest that an increased abundance of carboxyl groups may contribute more than changes in the abundance of thiol group/disulfide bond. In addition to the formation of disulfide bonds through oxidation, thiol groups can also be oxidized to sulfite and sulfate groups [10]. Although we did not examine the oxidation of thiols to sulfite and sulfate groups in this study, increases in sulfite and sulfate groups are also possible contributors to the increased hydrophilicity of stratum corneum proteins.

Taken together, these findings experimentally verified that residual chlorine in tap water increases the hydrophilic functional groups of stratum corneum proteins in the surface part of the skin through oxidation reactions, thereby increasing the hydrophilicity of the stratum corneum; consequently, barrier function is likely to be affected as suggested by trends toward a decreased contact angle of water droplets, an increased penetration rate of water droplets into the stratum corneum, and a decreased TEER.

Estimation of human impact based on results in RHEM

HE staining and fluorescence microscopic observation of frozen sections confirmed that the effects of residual chlorine exposure did not reach the deeper part of the stratum corneum and were concentrated in the near-surface part. This may be attributed to the high reactivity of residual chlorine. The action of residual chlorine concentrated in the surface part of the stratum corneum may cause the following effects. First, we cannot rule out the possibility that residual chlorine oxidatively modifies corneodesmosome proteins, which are adhesion factors of corneocytes. If the oxidative modification imparts exfoliating enzyme resistance to the corneodesmosome proteins, corneocytes will be retained in the surface layer of the skin for a long time. As the retained corneocytes have highly carbonylated proteins, these cells may cause skin drabness [11]. Furthermore, we cannot rule out the possibility that the presence of abundant carbonyls in the stratum corneum may interfere with the barrier function of the epidermis through their biological effects. These effects currently remain hypothetical but should be addressed in future studies. Nevertheless, to prevent such risks, the routine use of purified water containing no residual chlorine is advisable, although evidence remains to be established.

5. Conclusion

The present study demonstrated that residual chlorine in water at a level possibly found in tap water retained reactivity sufficient to oxidize stratum corneum proteins in a three-dimensional cultured epidermis model, and increased hydrophilicity of the stratum corneum surface presumably through alteration of certain functional groups of the stratum corneum proteins. Such residual chlorine-mediated oxidative modifications of proteins were suggested to result in reduced barrier function of the stratum corneum. Therefore, it was confirmed that residual chlorine in tap water can be regarded as one of the external stimuli, such as dryness and ultraviolet rays, and should be considered as a factor affecting the skin health. Furthermore, purified water containing no residual chlorine could exclude this factor, its daily use is considered useful for the skin.

6. References

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