Received: 30 May 2018 | Revised: 13 September 2018 | Accepted: 19 September 2018

DOI: 10.1111/jocd.12798

ORIGINAL CONTRIBUTION

WILEY JCD Journal of Cosmetic Dermatology

The increase of interfollicular epidermal stem cells and regulation of aryl hydrocarbon receptor and its repressors in the skin through hydroporation with anti-aging cocktail

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Funding information
Korea Healthcare Technology R&D Project, Ministry of Health & Welfare, Republic of Korea., Grant/Award Number: HN14C0094

Abstract

Backgrounds: Hydroporation is a procedure that involves a subsonic flow of air and microdroplets into the skin. We previously reported that hydroporation treatment with a cocktail solution containing copper-glycyl-L-histidyl-L-lysyl, oligo hyaluronic acid, rhodiola extract, tranexamic acid, and β-glucan yielded positive effects on skin aging.

Objectives: The aim of this study was to evaluate the effects of hydroporation with anti-aging cocktail on interfollicular epidermal stem cells (IFESCs) and expression of aryl hydrocarbon receptor (AhR)/AhR repressor (AhRR) in the skin.

Methods: Skin samples from six volunteers who were treated with hydroporation were analyzed via confocal microscopic examination.

Results: Markers for dermal matrix (procollagen type I and fibrillin-1) and basement membrane (type IV collagen and integrin α6) were increased after treatment. Moreover, there was a significant increase in the expression level of histone deacetylase 1-positive/p63-negative basal cells, which we previously reported as interfollicular epidermal stem cells. The expression level of AhR was significantly decreased, whereas that of AhRR was increased. This indicates an alteration in the interaction between the skin and environment posttreatment.

Conclusion: Anti-aging hydroporation treatment recovered the stem cell potential of basal cells. Moreover, this treatment decreased AhR and increased AhRR in the skin, which may protect the skin from the harmful environment.

KEYWORDS

aryl hydrocarbon receptor, aryl hydrocarbon receptor repressor, hydroporation, skin aging, stem cells

1 | INTRODUCTION

Hydroporation is a technique that incorporates a subsonic flow of air and delivers microdroplets into the skin. Previously, we reported that hydroporation with a mixed solution containing copper-glycyl-L-histidyl-L-lysyl (GHK), oligo hyaluronic acid, *rhodiola* extract, tranexamic acid, and β-glucan (GHR formulation) improves cutaneous aging features both clinically and histologically.[1] In another study, we investigated the effects of this procedure on melasma, and found that it may be associated with depigmentation and

a reduction of erythema by enhancing the microenvironment of the skin.[2]

Decreased elasticity and wrinkle formation are characteristic features of skin aging, known to be caused by collagen and elastic fiber degeneration in the dermis.[3] However, there is still not enough information about the fate of adult skin stem cells and the regenerative ability of skin cells with aging. We previously reported that the regenerative potential of skin cells decreases with age: the degree of stem cell depletion correlates with aging.[4] However, it remains unclear whether anti-aging treatment can in fact restore stem cell decrease. To analyze the number of skin stem cells, a specific skin stem cell marker is necessary. Recently, we reported that combined staining with HDAC1 and p63 is specific in localizing the interfollicular epidermal stem cells (IFESCs) in the skin.[5]

Moreover, there are various causes of skin aging, including ultraviolet radiation, air pollution, smoking, toxic chemicals and so on.[6] The aryl hydrocarbon receptor (AhR)—a sensor of environmental chemicals—is expressed by skin cells, and recent research provides new insights into the role of AhR in skin aging.[7]

In this study, we investigated the changes in the fate of IFESCs and the expression of AhR after hydroporation with anti-aging cocktail.

2 | METHODS

2.1 | Treatment

Six female volunteers with melasma (mean age 51.5 ± 4.2 years) were enrolled and informed consent was obtained from all participants. They were treated by a hydroporation system such as Jetpeel3V (Tav-Tech, Yehod, Israel) with GHR formulation once a week for a total of 8 weeks. The treatment was done in two steps. After delivering saline to the face for superficial epidermal peeling, total face was treated with GHR formulation (handpiece was moving at 2 cm/s and the spurt pressure was 95psi).[2]

2.2 | Immunohistochemical study

To evaluate the histological changes, 2 mm punch biopsy specimens were obtained from six volunteers before and 8 weeks after the treatment. Immunohistochemical analyses were also performed using formalin-fixed tissues. The tissue sections were deparaffinized in a HistoChoice Clearing Agent (Amresco, Solon, OH, USA) and rehydrated. Antigen was retrieved using Trilogy solution (Cell Marque, Rocklin, CA, USA) and pressure cooker. After antigen retrieval, blocking was performed using normal chicken serum (#S-3000, Vector Laboratories, Burlingame, CA), normal donkey serum (#ab7475, Abcam, Cambridge, UK), or normal goat serum (#ab7481, Abcam).

To evaluate the extracellular matrix and basement membrane, procollagen type I and fibrillin-1, type IV collagen, and integrin α6 staining were done. Moreover, p63 and HDAC1 combined staining was done to identify the interfollicular epidermal stem cells.[5] Finally, AhR and AhRR staining was done to investigate the susceptibility to harmful environments. Lists of primary and secondary antibodies were listed as follows: procollagen type I (SP1.D8) anti-mouse monoclonal antibody (Developmental Studies Hybridoma Bank, East Iowa City, Iowa, USA); Fibrillin-1 anti-mouse monoclonal antibody (11C1.3, ab3090, Abcam, Cambridge, UK); type IV collagen anti-rabbit antibody (ab6586, Abcam); integrin α6 anti-goat polyclonal antibody (N-19, sc-6597, Santa Cruz Biotechnology, Santa Cruz, CA, USA); p63(4A4) anti-mouse monoclonal antibody (ab735, Abcam); HDAC1 anti-rabbit polyclonal antibody (ab19845, Abcam); AhR anti-mouse monoclonal antibody (sc-133088, Santa Cruz Biotechnology); AhRR anti-rabbit polyclonal antibody (D-14, sc138745, Santa Cruz Biotechnology); Alexa Fluor® 488 goat anti-mouse IgG(H + L) antibody (A11001, Molecular Probes® Invitrogen, Carlsbad, CA); Alexa Fluor® 488 goat anti-rabbit IgG(H + L) antibody (A11008, Molecular Probes® Invitrogen); Alexa Fluor® 488 chicken anti-mouse IgG(H + L) antibody (A21200, Molecular Probes® Invitrogen); Alexa Fluor® 488 chicken anti-rabbit IgG(H + L) antibody (A21441, Molecular Probes® Invitrogen);Alexa Fluor® 488 chicken anti-goat IgG(H + L) antibody (A21467, Molecular Probes® Invitrogen); Alexa Fluor® 555 donkey anti-mouse IgG(H + L) antibody (A31570, Molecular Probes® Invitrogen); Alexa Fluor® 555 donkey anti-rabbit IgG(H + L) antibody (A31572, Molecular Probes® Invitrogen); and Alexa Fluor® 555 donkey anti-goat IgG(H + L) antibody (A21432, Molecular Probes® Invitrogen). In addition, to demonstrate an evidence for the specificity of AhRR staining, negative control staining was performed with an isotype-specific immunoglobulin (rabbit IgG isotype control, 31235, ThermoFisher Scientific, Rockford, IL) as a substitute for the primary antibody.

After staining with DAPI (1 μg/mL, 10236276001, Roche, Indianapolis, Indiana), images were obtained by Confocal Laser Scanning Microscope (CARL ZEISS, #LSM710, Jena, Germany) and analyzed using ZEN 2011 microscope software (CARL ZEISS).

2.3 | Image analysis

For evaluation of the expression level of procollagen type 1, fibrillin-1, type IV collagen, integrin α6, and AhR/AhRR, the intensity of immunohistochemical staining was scored from 0 to 5; 0 indicating no staining and 5 indicating most intense staining. The scoring was done by four independent investigators and the mean value was obtained.

To evaluate the epidermal stem cells, a count of all basal cells (DAPI-stained) was performed, and the percentages of epidermal cells with p63-positive/HDAC1-negative staining pattern were calculated.

2.4 | Statistical analysis

Wilcoxon signed rank test was performed to analyze the difference between before and after the treatment. All analyses were performed using IBM SPSS Statistics 22 (IBM Corporation, Armonk, NY). *P*-values of less than 0.05 were considered statistically significant.

3 | RESULTS

3.1 | Changes of Extracellular Matrix

The expression of procollagen type 1 and fibrillin was notably increased after the treatment (Figure 1A). The intensity score of procollagen type 1 was increased in all volunteers (Figure 1B, mean score before: 1.792 ± 0.431, after 3.167 ± 1.455, $P = 0.043$). Fibrillin was also increased in 4 out of 6 volunteers (Figure 1B, mean score before: 1.792 ± 0.941, after 2.792 ± 1.346, $P = 0.168$).

3.2 | Changes of Basement Membrane

Type IV collagen and integrin α6 are the main components of basement membrane. The expression of type IV collagen and integrin α6 was increased after the treatment (Figure 2A). All six volunteers showed an increased expression of type IV collagen (Figure 2B, before: 2.042 ± 0.579, after 3.417 ± 0.847, $P = 0.027$), and 5 out of 6 volunteers showed an increased expression of integrin α6 (Figure 2B, before: 1.833 ± 0.701, after 3.292 ± 1.461, $P = 0.207$).

3.3 | IFESCs

A combined staining of p63 and HDAC1 was performed to identify IFESCs. The outer differentiating cells were stained intensely, and a few cells in the basal layer were stained for HDAC1. In contrast, p63 was strongly expressed in the basal layer. In a merged image, there was a subpopulation, which was characterized by p63-positive/HDAC1-negative staining pattern in the basal layer (Figure 3A). The ratio of HDAC1-/p63+ cells was increased after anti-aging treatment in 5 out

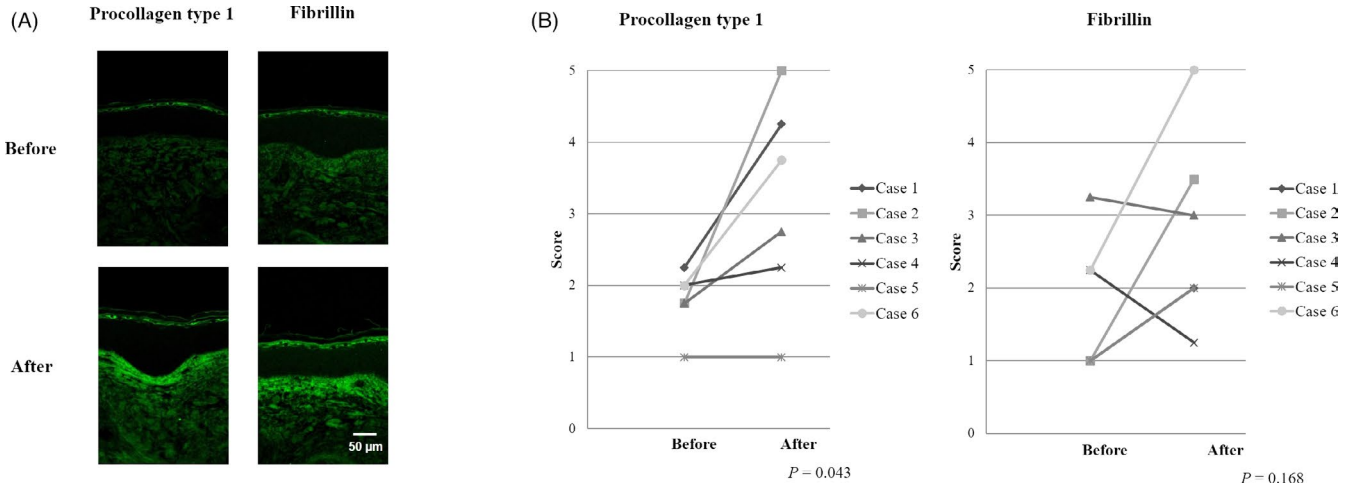

FIGURE 1 Changes of staining intensity of extracellular matrices, procollagen I and fibrillin-1 after the treatment. A, Immunohistochemical staining before and after treatment (green; procollagen I or fibrillin, x200). B, Changes of staining intensity of procollagen I and fibrillin-1

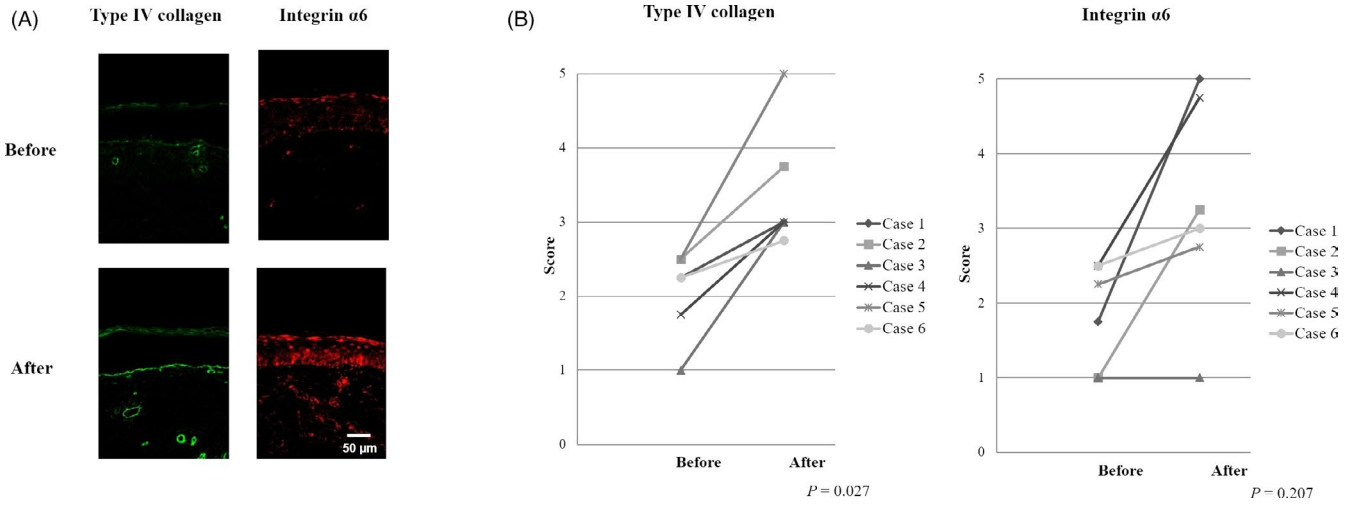

FIGURE 2 Changes of staining intensity of basement membrane components, type IV collagen and integrin α6 after the treatment. A, Immunohistochemical staining before and after treatment (green; type IV collagen, red; integrin α6, x200). B, Changes of staining intensity of type IV collagen and integrin α6

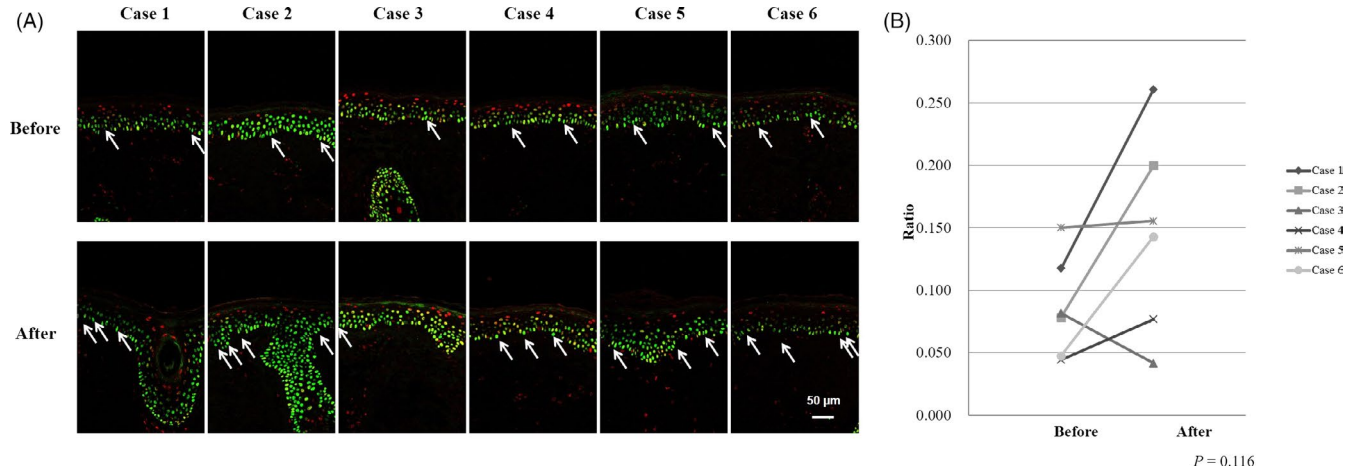

FIGURE 3 Changes of interfollicular epidermal stem cells after the treatment. A, Immunohistochemical staining before and after treatment (green; p63, red; HDAC1, white arrow; p63 positive/HDAC1 negative cells, x200). B, Changes of the ratio of p63 positive/HDAC1 negative cells to total basal cells in epidermis

of 6 volunteers (Figure 3B). The mean ratio was 0.087 ± 0.041 and 0.146 ± 0.080 before and after the treatment, respectively ($P = 0.116$).

3.4 | Changes of AhR and AhRR

In the skin, most of the cell types in the epidermis, as well as fibroblasts in the dermis, and immune cells are known to express AhR and AhRR.[8,9] In this study, AhR and AhRR expressions in the epidermis was evaluated because epidermis is directly exposed to the environment. Furthermore, the number of fibroblasts and immune cells are relatively small and variable. After the treatment, AhR expression was notably decreased in the skin (Figure 4A). The intensity score of AhR was decreased in all volunteers (Figure 4B, mean score before: 3.292 ± 1.336, after 2.083 ± 1.310, $P = 0.046$). Whereas, the staining intensity of AhRR was dramatically increased in the skin after the treatment (Figure 5A). The intensity score of AhRR was decreased in

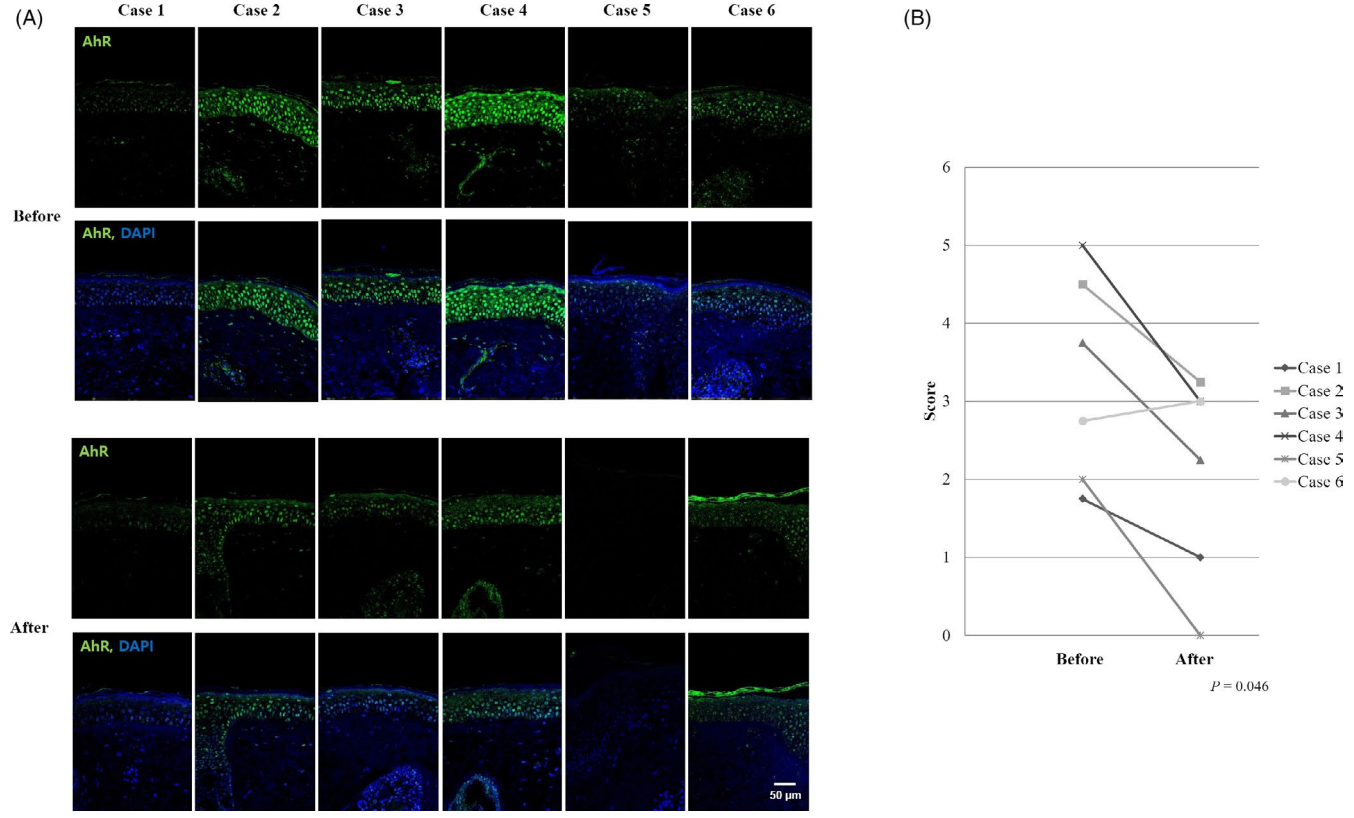

FIGURE 4 Changes of AhR expression after the treatment. A, Immunohistochemical staining before and after the treatment (green; AhR, x200). B, Changes of staining intensity of AhR

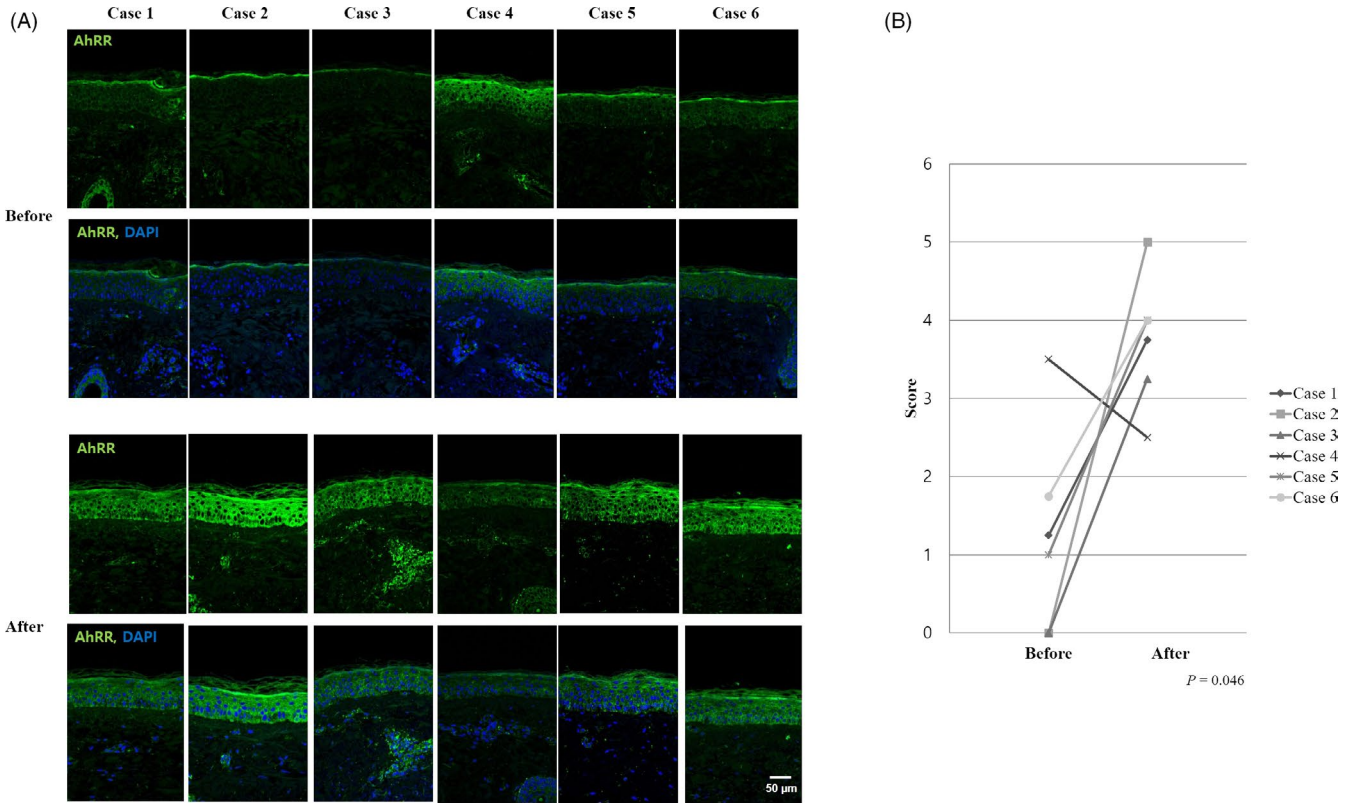

FIGURE 5 Changes of AhRR expression after treatment. A, Immunohistochemical staining before and after the treatment (green; AhRR, x200). B, Changes of staining intensity of AhRR

5 out of 6 volunteers (Figure 5B, mean score before: 1.250 ± 1.304, after 3.750 ± 0.837, $P = 0.046$). Negative control staining for AhRR revealed that there was no nonspecific binding (Figure S1).

4 | DISCUSSION

In this study, we demonstrated that hydroporation with anti-aging cocktail influenced the fate of epidermal stem cells and skin-environmental interaction. Anti-aging cocktail is a mixed solution containing copper-GHK, oligo hyaluronic acid, rhodiola extract, tranexamic acid, and β-glucan (GHR formulation). Copper-GHK plays roles of wound healing and tissue repair by boosting collagen synthesis.[10] Oligo hyaluronic acid was reported to induce thickening of the epidermis in a skin equivalent model.[11] *p*-Coumaric acid is one of the components of *rhodiolar* extract that completely inhibits melanogenesis through competitive inhibition activity between *p*-Coumaric acid and tyrosine.[12] Tranexamic acid can reduce epidermal pigmentation by decreasing vascularity and mast cell numbers in the melasma.[13] β-Glucan improves skin health due to its antioxidant, anti-wrinkling, anti-ultraviolet light, wound healing, and moisturizing effects.[14] Previously, we reported the anti-aging and skin lightening effect of hydroporation with GHR formulation.[1,2] After treatment, the proportion of p63+/HDAC1- basal cells was increased in most cases (Figure 3) although the mean ratio was not significantly different due to a small sample size. Thus far, p63 has been generally used as a stem cell marker based on p63 KO mouse study.[15] However, p63 is expressed in the upper layers of the epidermis, suggesting that it may not be a specific stem cell marker. To solve this drawback, we previously applied a combined staining method incorporating p63 and HDAC1 to localize the interfollicular epidermal stem cells in the skin. We found that a combined staining of both p63 and HDAC1 can be a new potential sensitive marker for identifying epidermal stem cells.[5] A characteristic feature of skin aging is a decrease in the capacity for wound repair and regeneration. Although the exact mechanism of aging has not yet been fully established, it is widely accepted that senescent changes in the stem cells are responsible.[16] Our results demonstrated that hydroporation with anti-aging cocktail can recover the stem cell potential and rejuvenate old skin. We think this effect is through the enhancement of dermal microenvironment since extracellular matrix protein, such as procollagen I, fibrillin, type IV collagen, and integrin α6 were increased after treatment. Copper-GHK and β-glucan, components of anti-aging cocktails, exert tissue-repairing effect by boosting collagen synthesis.[10,14] They are known to induce various growth factors, including PDGF, FGF, and TGF-β, which promote the production of collagen and other extracellular matrices in the skin. In particular, type IV collagen, which is known to be the main component of the basement membrane, was significantly increased after treatment. This means that hydroporation creates healthy basement membranes and determines the fate of epidermal stem cells.[17]

Skin is the most outer surface of the body. Therefore, it serves as a barrier against the environment and provides protection from physical and chemical harm, as well as infections. We analyzed the changes of AhR to determine the interaction between the skin and the environment. The aryl hydrocarbon receptor is a ligand-activated transcription factor involved in the regulation of biological responses to aromatic (aryl) hydrocarbons.[18] This receptor has been shown to regulate xenobiotic-metabolizing enzymes, such as cytochrome p450.[19] AhR binds to several exogenous ligands, such as natural plant flavonoids, polyphenolics, and indoles, as well as synthetic polycyclic aromatic hydrocarbons and dioxin-like compounds.[20] AhR is ubiquitously expressed and regulates cell growth, differentiation, and apoptosis. It is also reported that AhR seems to have a negative impact on aging.[21] Moreover, AhR expression levels in humans could serve as a predictor for vascular and organ aging.[22] Furthermore, AhR is closely related to skin pigmentation, photocarcinogenesis, and skin inflammation.[8] Particulate matter (PM) pollutants can induce skin aging via AhR. Thus, the role of AhR in the prevention of skin aging, in midst of heightened concerns for severe air pollution, has been receiving more attention recently. In this study, interestingly, there was a significant decrease in the expression levels of AhR after hydroporation (Figure 3B,C). Since environmental organic pollutants, like dioxins, furans, and polychlorinated biphenyls (PCB), act as ligands for AhR,[19] our results suggest that hydroporation with anti-aging cocktail may protect the skin against these environmental pollutants. One of the possible cause of AhR reduction is transforming growth factor- β (TGF-β). TGF- β regulates the synthesis of collagen and other extracellular matrix proteins in the human skin.[23] As mentioned above, copper-GHK and β-glucan have been demonstrated to induce TGF-β. In a previous study, TGF-β suppressed expressions of AhR and AhR-related genes. However, further investigation is needed to determine the exact mechanisms underlying AhR reduction following hydroporation with the anti-aging cocktail. In this present study, we observed a dramatic increase in the levels of AhRR in 5 out of 6 cases. AhRR is known to negatively regulate AhR signaling via its inhibitory transactivation.[24] Moreover, AhRR have anti-inflammatory activity associated with tumor suppressive.[9]Our results clearly showed that the levels of AhRR can be increased after anti-aging treatment of the skin. In other words, regulating the levels of AhR and AhRR can be a new approach to regulate skin aging, which is extensively exposed to environmental hazards, such as air pollutants and UV.

One limitation of this study is the small sample size. Nevertheless, our data showed consistent and statistically significant results in most cases. Further investigation with larger sample sizes is necessary to confirm our results. In addition, our study did not show the effect of hydroporation alone, without anti-aging cocktail, and did not specifically analyze the effect of each ingredient included in the cocktail. In our previous report, expressions of extracellular matrix proteins, including procollagen I and fibrillin, were more prominent in the area treated with hydroporation using an anti-aging cocktail compared to the area treated with hydroporation using normal saline.[1] We suspect that the superficial mechanical peeling effect of hydroporation alone is not enough to exert a noticeable anti-aging effect. Further study is needed to investigate the detailed anti-aging effects of hydroporation using anti-aging cocktails.

In conclusion, hydroporation with anti-aging cocktail may be effective in the recovery of stem cell potential of basal cells by enhancing the dermal microenvironment. In addition, this treatment may regulate the level of AhR/AhRR and protect the skin from the harmful environment.

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SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

How to cite this article: Shin J-W, Choi H-R, Yang S-H, et al. The increase of interfollicular epidermal stem cells and regulation of aryl hydrocarbon receptor and its repressors in the skin through hydroporation with anti-aging cocktail. *J Cosmet Dermatol*. 2019;18:1133–1139. https://doi.org/10.1111/jocd.12798