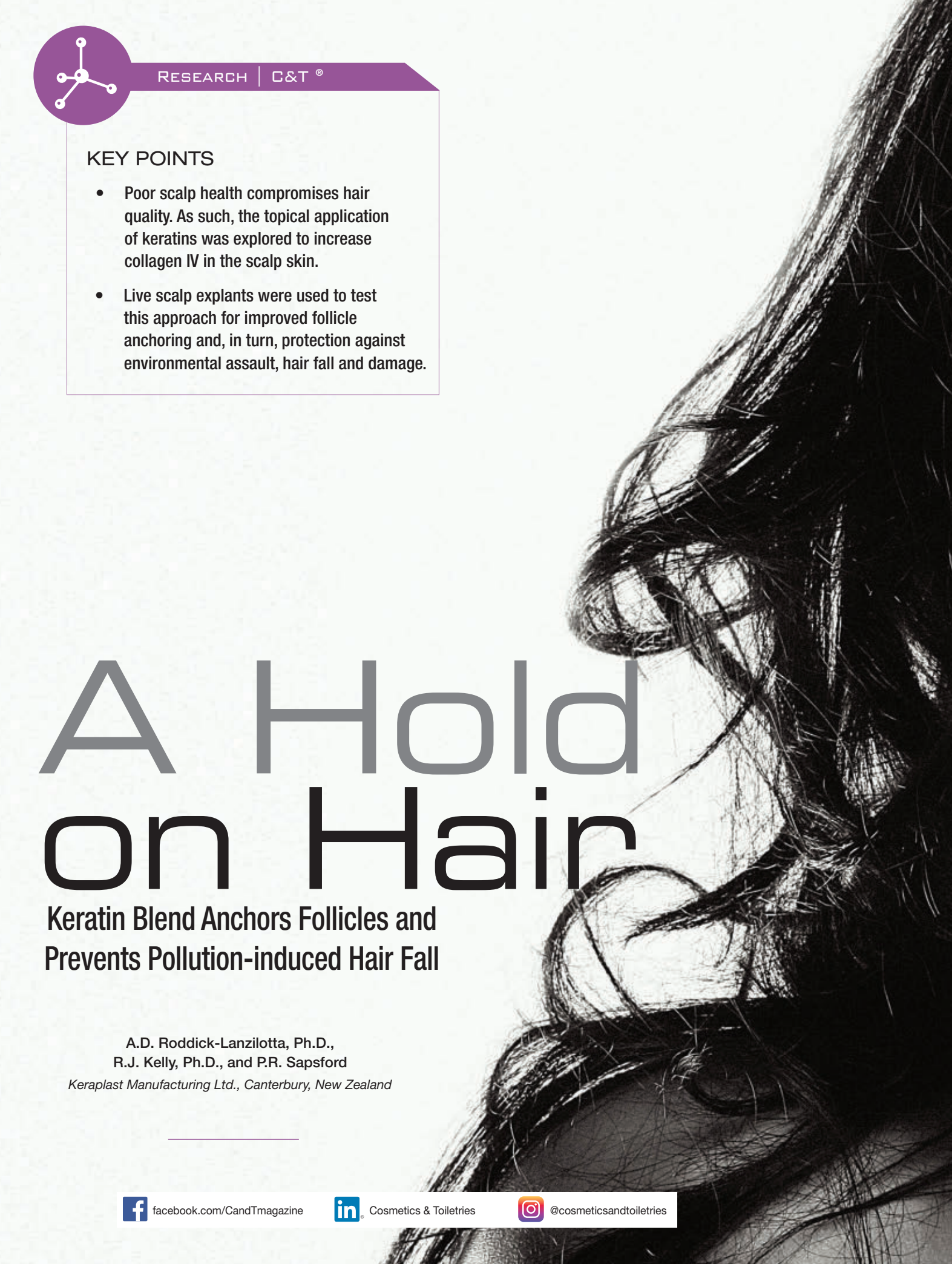
KEY POINTS

- Poor scalp health compromises hair quality. As such, the topical application of keratins was explored to increase collagen IV in the scalp skin.
- Live scalp explants were used to test this approach for improved follicle anchoring and, in turn, protection against environmental assault, hair fall and damage.



A Hold on Hair

Keratin Blend Anchors Follicles and Prevents Pollution-induced Hair Fall

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The link between a healthy scalp and hair quality is now well-established.[1] Poor scalp health impacts hair follicle viability, and exposure to external factors such as UV radiation and pollutants can weaken scalp skin health and cause aging. The strong anchoring of hair in the follicle, however, relies on a strong basement membrane and the presence of collagen IV—the binding protein that makes up the primary structural component of the dermal-epidermal junction (DEJ), and the most important component in follicle anchoring.[2]

Soluble keratin proteins have been shown in vitro to increase the ability of skin cells to produce collagen IV and, as a result, improve the integrity of the DEJ.[3] Furthermore, they have been used in the development of dermatological treatments for the clinical management of wounds and the severe skin blistering disorder epidermolysis bullosa, in which the basement membrane is weakened due to a reduced expression of collagen IV.[4]

In addition, soluble keratin peptides are known to improve skin hydration and improve barrier function, particularly when the skin is challenged.[5] A combination of these materials was therefore deemed worth considering for

Cysteine-rich radical-scavenging plus improved sulfur metabolism may explain how keratin protects and neutralizes oxidizing pollution entities, improving follicle health.

applications to treat scalp skin characterized by poor hydration, inflammation and, ultimately, hair fall through the poor anchoring of hair follicles. This was the focus of the present study.

Previous literature has identified, via in vitro studies of balding dermal papilla cells, the contribution of pollution and cigarette smoke to hair fall.[6] Also, in France, in a study[7] of 1,011 individuals, a significant 44.2% declared they suffered from a sensitive scalp, which were associated with triggers including pollution, heat, emotions and shampoos. The subjects with sensitive scalp were also significantly more likely to suffer hair loss.

Symptoms of "sensitive scalp syndrome" (SSS) include itching and prickling in the scalp, dandruff, oily scalp and pain in the hair roots. In fact, SSS has been reported to arise from exposure to increasing levels of air pollution including particulate matter, smoke, heavy metals and gases, e.g., SO_2, NO_2 and ammonia, that settle on the hair and scalp.[8] Pollutants also can migrate into the dermis and through the hair follicle, leading to oxidative stress and hair fall.

The role that soluble keratin proteins can play in protecting hair from the oxidative effects of pollutants, specifically SO_2 and cigarette smoke, has been well-documented.[9] However, the question of how this protective effect impacts hair follicle health has not previously been addressed.

For one, studying follicle health requires overcoming the unique challenges of maintaining a living follicle while also allowing controlled exposure to actives and environments that represent real world conditions. Model systems in which skin structures and follicle systems are created artificially offer some promise.[10] However, recent developments in donor explant methodologies have progressed to a point to allow for the maintenance of live follicles in culture conditions, facilitating the controlled study of follicle development and health in a range of environments.[11]

The present article outlines a study performed by an independent testing laboratory[a] to investigate the impact of adding keratin actives to live explants of scalp skin containing follicles. Various tests, described next, were performed to determine the effects of a specific keratin active blend[b] in terms of anchoring follicles and protection against damaged associated with urban pollution and cigarette smoke.

The soluble keratin extracts used are isolated using proprietary processes to control protein molecular weight and cysteine availability. The blend of oxidized keratin, keratin and hydrolyzed keratin maintains key structural characteristics from the native keratin sources to ensure the bioactivity of the resulting ingredient.

The global anti-pollution skin care market was estimated at US $9.07 billion in 2018 and is likely to expand further at a CAGR of 4.2% from 2019 to 2025.

Source: Grand View Research

Materials and Methods

Scalp explants: Live scalp skin explants were harvested from a 58-year-old woman undergoing a facelift procedure. Sixteen shaved explants measuring 0.4 × 1.0 cm were stored in survival

[a] *Laboratoire Bio-EC Confidential Report: Study 18E4285 (2019)*

[b] *Functional Keratin (INCI: Oxidized Keratin (and) Keratin (and) Hydrolyzed Keratin)*

medium at 37°C in a humid, 5% CO_2 atmosphere. The experiment was conducted for seven days; Day 0 to Day 6. Day 0 involved sampling and product application. Keratin actives were re-applied on days 2 and 5, prior to pollution exposure. Pollution exposure occurred on Day 5 and sampling for analysis was conducted on Day 6.

Keratin actives were applied at a level of 1 µL per explant and spread using a small spatula. Half the volume (0.3 mL per well) was renewed on days 2 and 5. The explants were distributed into seven batches, as summarized in **Table 1**.

Pollutant exposure: Explants chosen for pollution exposure, i.e., samples P, PK1, PK2 in **Table 1**, were placed in wells with 900 µL of Hanks' balanced salt solution (HBSS) and exposed to a mixture of gases using the Air World Simulator Platform for 2 hr. Pollutants and levels included: SAE Dust 100 mg/m^3, NO_2 17 ppm, SO_2 8 ppm and CO 833 ppm. Following this, the explants were exposed to exhaust gases generated with a diesel generator[c] for 1 hr; the exhaust composition was as follows: PM1 500 µg/m^3, NO 3.96 ppm, NO_2 0.84 ppm and CO 5.8 ppm.

The explants P, PK1 and PK2 were then placed in wells with 900 µL HBSS and exposed to smoke from two cigarettes for 1 hr[d]. The control (nonpollution)

[c] *Honda EX4D*
[d] *PolluBox is a registered trademark of Laboratoire BIO-EC.*

Table 1. Summary of Explant Distribution

Sample name	Conditions	Number of explants	Sampling time
C0	Biopsy control	1	Day 0
C	Control	2	Day 6
K1	Keratin product K1	2	Day 6
K2	Keratin product K2	2	Day 6
P	Pollutants	3	Day 6
PK1	Keratin product 1 + Pollutants	3	Day 6
PK2	Keratin product 2 + Pollutants	3	Day 6

K1 INCI: Water (aqua) (and) Oxidized Keratin (and) Keratin (and) Hydrolyzed Keratin (and) Phenoxyethanol
K2 = 20% K1 in water

explants C, K1 and K2 were kept in 1 mL of HBSS during this time. At the end of pollutants exposure, all explants were placed back in 600 µL of survival medium.

Explant sampling: On Day 0, one explant from the batch C0 was collected and cut into two parts. One portion was fixed in buffered formalin solution and the other part was frozen at -80°C. On Day 6, 24 hr after pollutant exposure, two to three explants were collected from each of the C, K1, K2, P, PK1 and PK2 samples and treated using the same protocol. Seven different regions were observed: the epidermis, dermis, infundibulum, upper root sheath, bulge, lower root sheath and bulb. In this paper, the results for the hair bulb region are reported. From each explant, two to three follicles were observed and assessed by trained histologists. Results and conclusions were signed off by at least two histologists.

Histological processing: After fixation for 24 hr in buffered formalin, the samples were dehydrated and impregnated with paraffin using an automat[e]. The samples were then embedded in tissue blocks using an embedding station[f], from which 5 µm-thick serial sections were taken using a microtome[g] and mounted on histological glass slides[h]. Microscopic observations were made[j] and pictures were captured using a numeric camera[k] with storage software[m].

Collagen IV immunostaining: Collagen IV was stained on paraffin sections with a monoclonal anti-collagen IV antibody[n] diluted 1:200 in phosphate buffered saline (PBS), bovine serum albumin (BSA) (0.3%) and polysorbate 20[p] (0.05%), and incubated for 1 hr at room temperature. A biotin/streptavidin amplifying system was employed and visualization was accomplished using a violet substrate of peroxidase[q]. Immunostaining was performed using an automated slide-processing system[r] and assessed by microscopic observation.

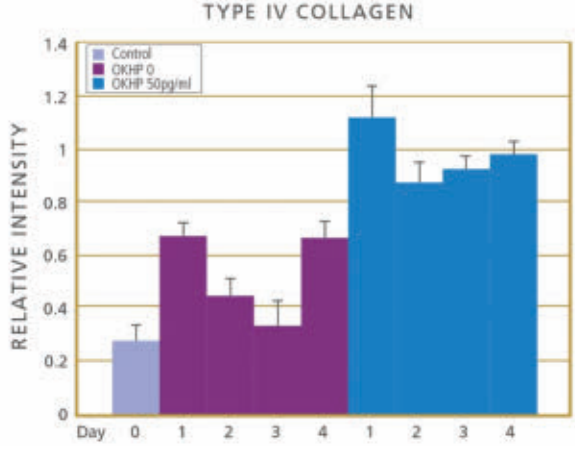

Figure 1. Relative intensities of protein synthesis normalized to β-actin

[e] *Leica PEARL*
[f] *Leica EG 1160*
[g] *Leica RM 2125 Minot-type*
[h] *Superfrost histological slides*
[j] *Leica DMLB or Olympus BX43 microscope*
[k] *DP72 Olympus*
[m] *CellD*
[n] *Novus biologicals, ref. NBP2-23608, clone COL-94*
[p] *Tween 20 (INCI: Polysorbate 20), Croda Inc.*
[q] *Vector, ref. PK-7200*
[r] *Dako, AutostainerPlus*

Nuclear factor erythroid 2-related factor 2 (Nrf2) immunostaining: Degradation of Nrf2 following pollutant exposure is indicative of oxidative stress. To visualize this degradation, paraffin sections were stained with a monoclonal anti-Nrf2 antibody[s] diluted to a ratio of 1:400 in PBS, BSA 0.3% and polysorbate 20[p] (0.05%) for 1 hr at room temperature with a biotin/streptavidin amplifying system, and visualized with a violet substrate of peroxidase[t]. Immunostaining, again, was performed using an automated slide-processing system[r] and assessed by microscopic observation.

Cell viability: The cell viability of epidermal, dermal and follicle structures exposed to pollution-induced oxidative stressed was observed on paraffin sections after Masson's trichrome staining, Goldner Variant. Cell viability was assessed by microscopic observation.

Results: Collagen IV

Collagen IV is the main structural component of basal membranes that in skin, localizes within the DEJ. The DEJ is important for the adhesion of the dermis to the epidermis, which therefore is integral in anchoring the bulb of the hair follicle. Previous in vitro studies on the oxidized keratin component (OKHP) of keratin products 1 and 2 showed a significant increase in collagen IV

[s] *Abcam, ref. ab76026, clone EP1809Y*
[t] *Vector, ref. SK-4600*

Pollutants can migrate into the dermis and through the hair follicle, leading to oxidative stress and hair fall.

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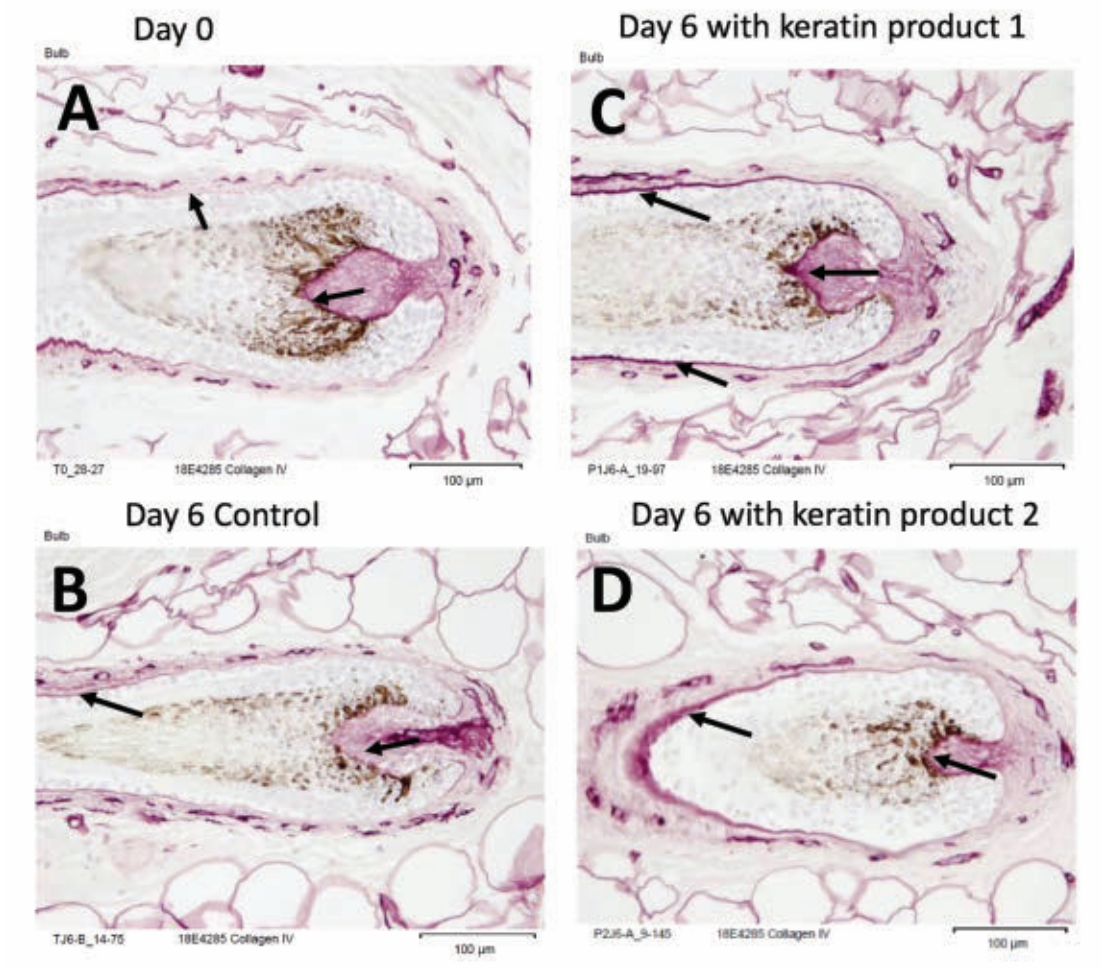
synthesis following injury to keratinocytes via a scratch assay model (see **Figure 1**).[3]

To investigate the effect of adding keratin products to scalp explants, samples were prepared and visualized as outlined in the Materials and Methods section. Samples included Day 0 control, Day 6 control, Day 6 with keratin product 1, and Day 6 with keratin product 2. Collagen IV was stained violet; representative images are shown in **Figure 2**.

On Day 0 (**Figure 2a**) collagen IV was present to some extent around the bulb and more clearly in the dermal papillae (see arrows). After six days, the application of keratin 1 and keratin 2 resulted in increased collagen IV expression in the dermal papillae and an increase in the follicle bulb, particularly following treatment with keratin 1. This is demonstrated by a thickening of the violet stain associated with the increased presence of collagen IV relative to the control.

Results: Nrf2

Nrf2 is a regulator of cellular resistance to oxidants.[12] It is ubiquitously expressed in a wide range of tissue and cell types. When subjected to oxidative stress, Nrf2 is activated and translocates from the cytoplasm of the cell to the nucleus. Once in the nucleus, Nrf2 binds to a common DNA sequence referred to as the *antioxidant response element* (ARE), which is the master regulator of the total antioxidant system. Thus, as noted, the degradation of Nrf2 following pollutant exposure is a useful indicator of how much oxidative stress cells have experienced.



A = explant at time of biopsy; B = control after 6 days in survival medium; C = treated with keratin 1, Day 6; D = treated with keratin 2, Day 6

Figure 2. Collagen expression around hair follicle (violet)

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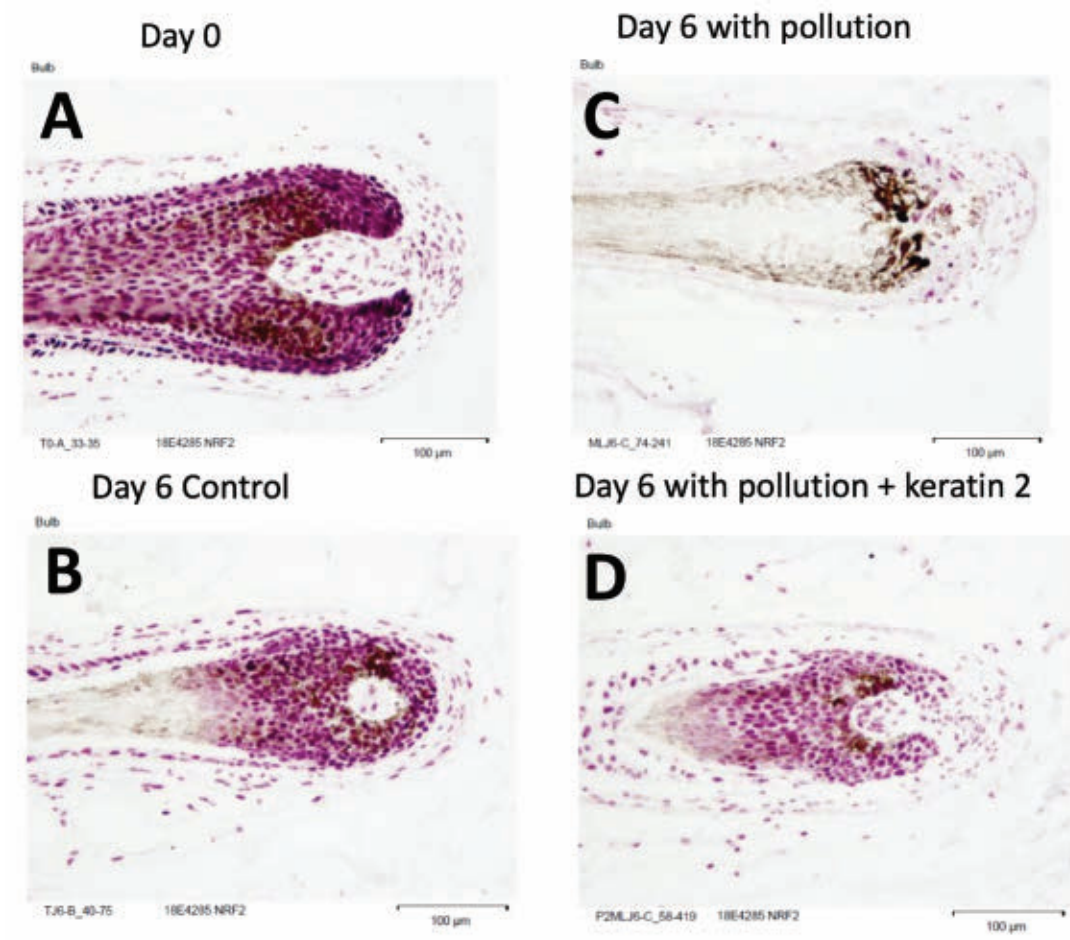
Samples including Day 0 control, Day 6 control, Day 6 without pollutants and Day 6 keratin product 2 + pollutants were prepared and visualized as outlined in the Materials and Methods section. Representative images of Nrf2-stained hair follicles are shown in **Figure 3**.

Nrf2 is clearly stained in the initial follicle sample (see **Figure 3a**). After 6 days in survival medium, the amount of Nrf2 visualized decreased but is still clearly visible (see **Figure 3b**). Exposure to pollution, as detailed in the Materials and Methods section, caused oxidative stress, which substantially decreased the amount of Nrf2 observed (see **Figure 3c**). Conversely, the application of product 2 prior to exposure to pollutants (see **Figure 3d**) showed Nrf2 levels similar to the Day 6 control without pollution (**Figure 3b**), indicating the follicle was protected from oxidative stress.

Results: Cell Viability

Cell viability was assessed to determine the overall health of scalp cells in response to oxidative stress caused by pollution exposure. The samples Day 0 control, Day 6 control, Day 6 with pollutants and Day 6 keratin product 2 + pollutants were prepared and visualized as outlined in the Materials and Methods section.

Keratin expression was stained in red. On Day 0 and Day 6, without pollution exposure (see **Figure 4a** and **4b**, respectively) keratin expression was clearly observed (i). Following



A = explant at time of biopsy; B = control after 6 days in survival medium; C = following pollution exposure, Day 6; D = treated with keratin product 2 and exposed to pollution, Day 6

Figure 3. Nrf2 expression in hair follicle (violet)

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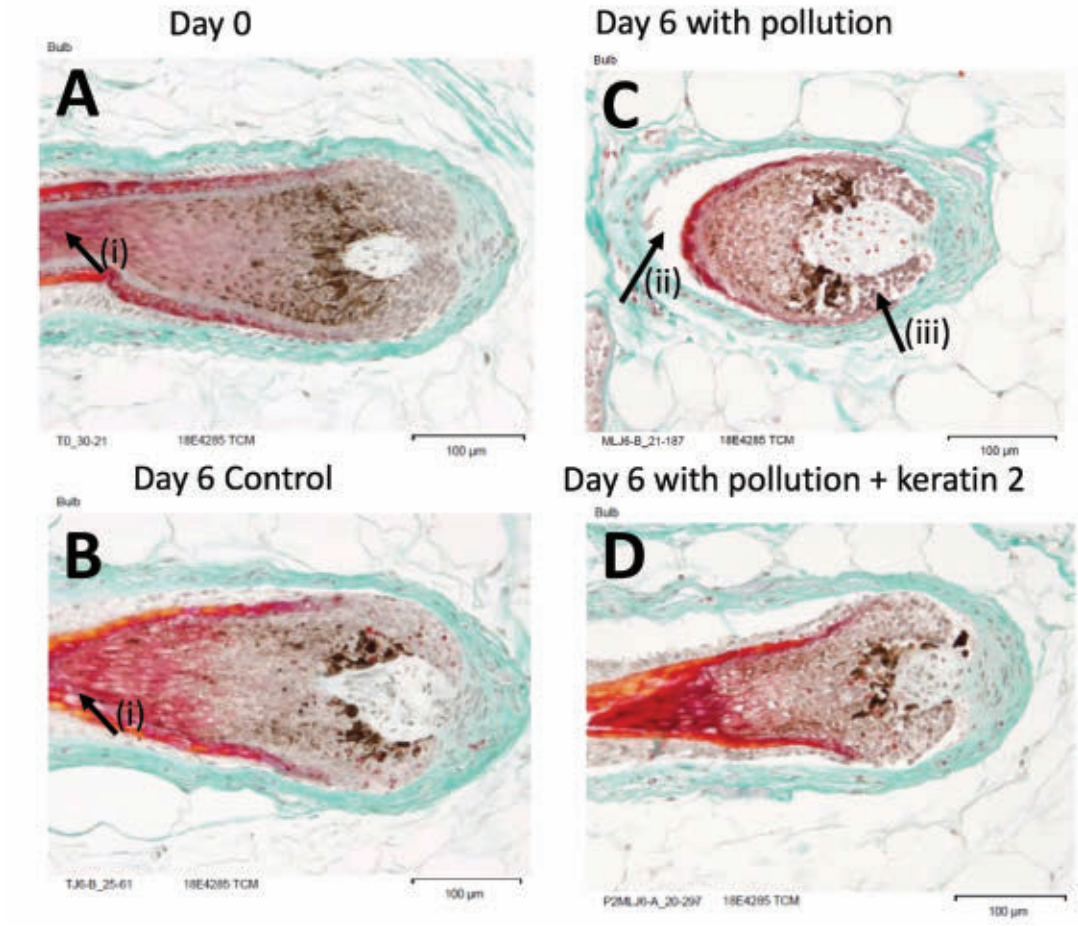
exposure to pollution, keratin expression was decreased, separation between the bulb and surrounding tissue was increased (ii) and poor cell morphology, characterized by the increased occurrence of pyknotic nuclei, was observed.

The combination of reduced keratin expression and poor cell morphology indicates that hair viability decreased following exposure to pollution, which can result in hair fall. Treatment with keratin product 2 prior to pollution exposure, however, appeared to protect the follicle against pollutant-induced alterations (see **Figure 4d**), with keratin expression and cell morphology more like the control sample (see **Figure 4b**). This was particularly evident in the upper part of the follicle and epidermis.

Discussion

The impact of soluble keratin proteins on collagen IV expression, previously identified in a keratinocyte model,[3] appears to increase collagen IV in the basement membrane of the hair follicle. As the role of this binding protein is to anchor the hair follicle, this increase in occurrence would be expected to lead to improved anchoring of the hair in the scalp.

Keratin's mechanistic role in wound healing has been associated with the healing response,[13] encouraging cell proliferation and basement membrane protein synthesis. Keratins are typically intracellular proteins, so the occurrence of intact keratin protein in the extracellular matrix outside of the cell only occurs as a result of cell



A = explant at time of biopsy; B = control after 6 days in survival medium; C = following pollution exposure, Day 6; D = treated with keratin product 2 and exposed to pollution, Day 6

Figure 4. Cell viability

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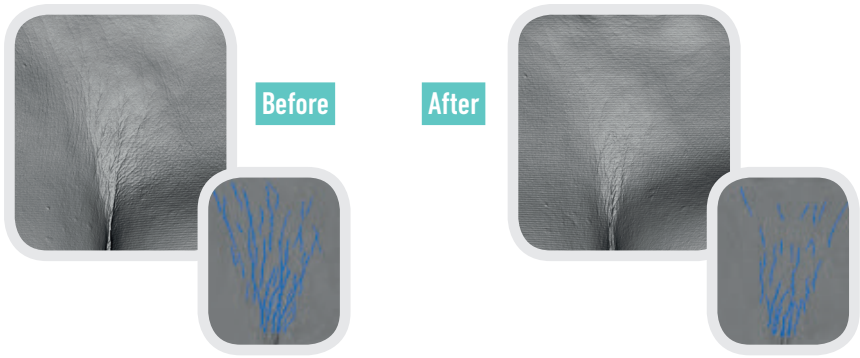

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injury, as would occur in a wound. In the hair follicle model studied, the occurrence of soluble keratin proteins in the extracellular environment may be leading to a similar wound response, encouraging an increase in basement membrane proteins and resulting in the strengthening of the follicle anchor observed.

The radical scavenging ability of intact keratin proteins on hair is well-established and associated with the antioxidant characteristics of the amino acid cysteine.[9] In skin cells, the cysteine-rich protein may also encourage anti-inflammatory and antioxidant response by providing cysteine for the sulfur-dependent pathways of glutathione and taurine—important in cellular oxidative and inflammatory response. An improved anti-inflammatory response in the presence of keratin has been reported in vitro[14] and is consistent with observations in wound-healing studies of keratin-based wound treatments.[15]

Similar protection appears to be functioning in hair follicles. The combination of cysteine-rich radical-scavenging and improved sulfur metabolism may explain the mechanism by which the applied keratin is providing protection and neutralizing the oxidizing impact of pollution components, resulting in improved follicle health in the presence of pollution. This is manifested in the follicle model studied as a strong antioxidant response, visualized through Nrf2 expression, and improved cell morphology since the cells experience less oxidative stress and therefore better maintain their ability to express keratins in the hair fiber and retain a healthy cell structure.

Conclusions

Soluble keratin proteins and peptides have previously shown efficacy for wounded and injured skin. Here, the biological response to these keratins has now been demonstrated in explants of hair follicles, to improve follicle anchoring by increasing collagen IV in the hair bulb and further prevent the oxidizing, inflammatory effects of pollutants. This functionality is of particular use in the development of topical products targeting scalp health.

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