Preventing epigenetic changes fights urban pollution

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Abstract

Environmental pollution is one of the main contributing factors towards skin ageing and a dull complexion. For full protection, only a multi-level approach will succeed. Here an approach is described where a first line of defence is built up by a film-forming polysaccharide complex which physically prevents binding of pollutants. Activation of the cell's own detoxification system is used as the second line of defence. And because pollution also induces epigenetic changes with long-term effects, a third line of defence was established where these epigenetic changes are prevented or reversed.

Environmental pollution is one of the major areas of concern when it comes to skin ageing. In particular, air pollution is directly in contact with our skin and it contributes to skin ageing on a daily basis. The main sources of air pollution are industrial combustion (diesel exhaust fumes and coal), traffic and construction works. Air pollution consists of gases such as ozone and very fine particles that are known as particulate matter (PM). These particles, which are between 0.1 µm – 10 µm in size, can remain in the atmosphere for weeks and contain toxic compounds such as heavy metals and allergens. Particulate matter is especially dangerous for the skin as it not only sits on the surface but can also penetrate into pores and therefore transport toxic substances into deeper skin layers. In combination with exposure to UV light, these particles cause oxidisation reactions within the skin, which lead to the formation of reactive oxygen species (ROS), inflammation and the loss of collagen. ROS can cause protein carbonylation, and these damaged proteins contribute to skin ageing. Furthermore, lipid peroxidation caused by ROS leads to skin barrier dysfunction, which creates a vicious cycle as more PM can enter the skin. The result is irritated, uneven skin that will age more rapidly.

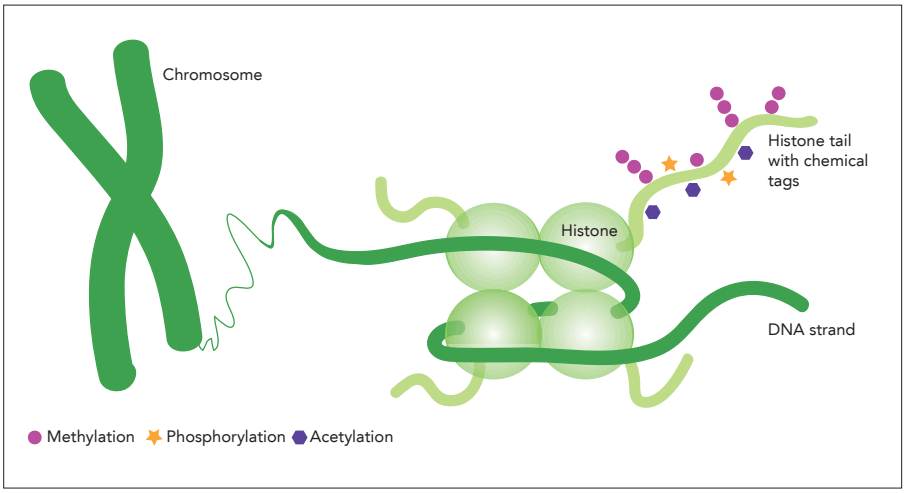

Figure 1: Epigenetic changes on histone proteins which are responsible for the packaging and organisation of the DNA strand.

Expression of detox and antioxidant enzymes in the skin

It is obvious that our cells are in constant danger by toxic influences from the environment and thus need an efficient defence or detoxification system. Fortunately, our cells are able to produce enzymes that assist in the detoxification and removal of toxic substances. The genes encoding for detoxification and antioxidant enzymes, which are also called phase II enzymes, are activated by a specific mechanism. The transcription of these genes is regulated by a special control sequence in the promoter region of the gene. To start the expression of detoxification and antioxidant enzymes, a specific protein interacts with this control sequence. This "switch-on-protein", which is called transcription factor Nrf2, is normally blocked by the repressor Keap1. A stimulus such as oxidative stress can disrupt the Nrf2-Keap1 complex. The released Nrf2 then binds to the control sequence and cells start to produce detoxification and antioxidant enzymes. In this way, cells can react to their environment by producing the correct response enzymes. Interestingly, a molecule found in plants of the *Brassicaceae* family, sulforaphane, has also been shown to disrupt the Nrf2-Keap1 complex and therefore activate the cellular detoxification system.

In this work, an extract of garden cress sprouts (*Lepidium sativum*, belonging to the *Brassicaceae* family) was used as activator of the detoxification system in the skin. Cress sprouts have the highest concentration of sulforaphane.

Air pollution induces epigenetic changes in the skin

Epigenetics, which studies modulation of gene expression caused by mechanisms other than changes in the DNA sequence, can explain how environmental factors influence our body. Whereas oxidative stress acts at the moment, epigenetic modifications may lead to a long-term effect, even when the environmental stress is not present anymore. This is the reason why air pollution should be analysed for epigenetic effects. Epigenetics is based on chemical modification, mainly methylation or acetylation, of the nucleosome which comprises the DNA and histone proteins. The histone proteins are responsible for the packaging and organisation of the DNA (Fig 1). Modifications of histone proteins define the access of transcription factors to

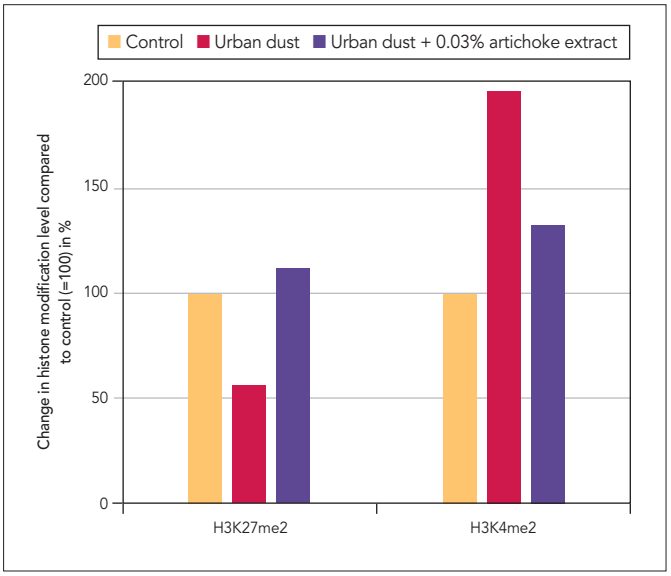

Figure 2: Artichoke extract protects against urban dust-induced epigenetic changes on histone 3.

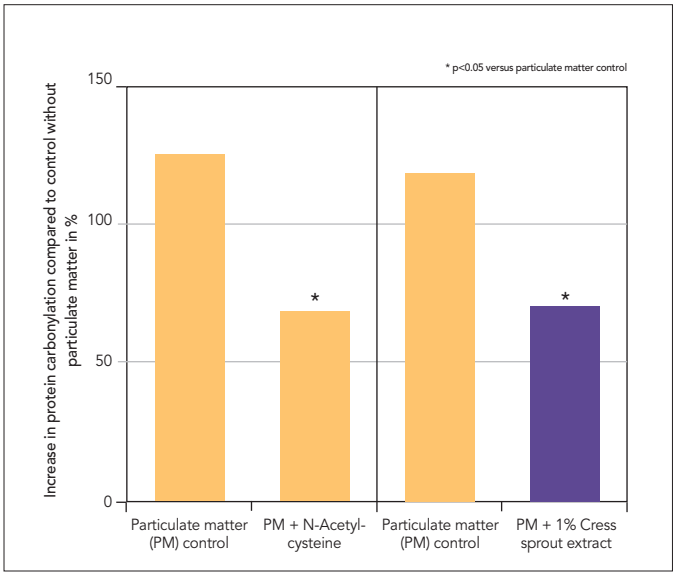

Figure 4: Cress sprout extract protects fibroblasts against pollution-induced protein carbonylation.

the DNA and thus whether a gene is active or not. Meanwhile, there are many publications about the effects of air pollution on the epigenetic pattern, indicating an epigenetic mechanism involved in the health problems induced by air pollution.[1] Recent studies showed air pollution-induced modifications of the histone H3 protein.[2] So far, the epigenetic effect of pollution on skin cells has not yet been elucidated.

In this work, human epidermal keratinocytes were used to study the effects of urban dust and benzo[a]pyrene on the epigenetic pattern of the histone H3 protein. The assay was then used to screen a series of plant extracts in order to find cosmetic actives that protect the skin against air pollution.

Materials and methods

Pollution-induced epigenetic changes in keratinocytes

● Cultures and treatments

Normal human epidermal keratinocytes (2nd passage) were seeded in 175 cm^2 flasks (125 000 cells) and cultured in Keratinocyte-SFM supplemented with EGF (0.25 ng/ml), pituitary extract (25 µg/ml) and Gentamycin (25 µg/ml) for 24 hours. The medium was then replaced with culture medium containing or not (control) the pollutants and the test compounds. The urban dust (Ref. NIST, Standard Reference Material (SRM) 1649b) was used as stock solution (100 mg/ml in ethanol/water, 2:1) at 0.01 mg/ml in the culture medium. Cells were then subcultured from 2nd to 6th passage every week with treatment after each subculture. At the 7th passage, the cells were seeded in 12-well plates (350 000 cells/well) and the treatment was renewed. Cells were then incubated for 24 hours. At the end of the incubation, the medium was discarded and the cells were washed in phosphate buffered saline (PBS) solution.

● Quantification of histone modifications

The quantification of total histone H3 and 21 histone H3 modifications was performed using the EpiQuick Histone H3 Modification Multiplex Assay Kit (Colorimetric) from Epigentek (Ref. P-3100-96) following the supplier's instructions. For each experimental condition, 100 ng of total histone extract was deposited per well.

Pollution-induced protein carbonylation in fibroblasts

Normal human dermal fibroblasts were seeded in 6 well plates and cultured for 24 hours in culture medium. The medium was removed and replaced by the assay medium containing or not (control) the reference N-Acetylcysteine (NAC), or cress sprout extract. After 7 hours, the medium was removed and the particulate matter (PM_{10}-like) added to the assay medium. 16 hours after treatment, cells were washed with PBS and proteins were extracted from HDF for further analysis. All experimental conditions were performed by triplicate (n=3). Extracted proteins were quantified by the Bradford method and split into equal amounts for analysis. Oxidatively damaged (carbonylated) proteins were labelled with specific functionalised fluorescent probes and samples were resolved by high-resolution electrophoresis separation via 4-20% gradient SDS-PAGE. Total proteins were post-stained with SyproRubyTM protein gel stain. Image acquisition for carbonylated and total proteins was performed using the Ettan® DIGE imager. Image processing and analysis was performed using ImageJ.

	Enzyme expression relative to control (%)	
Concentration of cress sprout extract	0.05%	0.2%
Detox enzymes		
NADPH: quinone reductase 1	75	214
Heme oxygenase 1	212	4182
Thioredoxin reductase 1	184	2316

Figure 3: Cress sprout extracts activates detoxification enzymes in keratinocytes.

Results

Preventing epigenetic changes caused by pollution

To test whether air pollution influences the epigenome of skin cells, a novel assay was designed that combined the long-term treatment of keratinocytes with pollution with the subsequent analysis of histone modifications. The EpiQuick Histone H3 modification assay is designed for measuring 21 histone H3 modifications simultaneously. Each histone H3 modified at specific sites is captured by an antibody that is coated on the strip wells and specifically targets the respective histone modification pattern. The captured histone sites are detected with a detection antibody, followed by a colour development reagent.

Exposure of the keratinocytes to urban dust or benzo[a]pyrene was found to strongly modulate methylation, acetylation

Figure 5: Depolluphane facilitates to wash off particulate matter from the skin.

or phosphorylation at almost all of the analysed 21 histone H3 sites. It was also possible to reproduce the results of Ding *et al.* 2016 which found increased acetylation at lysine 9 in rats after exposure to traffic-related air pollution. The same epigenetic change was also found in steel workers who inhaled higher amounts of particulate matter and heavy metals.[3] These investigations also showed an increase in lysine 4 dimethylation which also could be reproduced in our experiment with urban dust. The assay was then used to screen a series of plant extracts for protective effects against pollution-induced epigenetic changes. Surprisingly, an artichoke extract turned out as a very efficient protector. Keratinocytes treated with 0.03 % artichoke extract exhibited a histone modification profile similar to control cells that did not come into contact with urban dust. Shown in Figure 2 are two modification examples, of Histone 3 lysine 27 dimethylation (H3K27me2) and Histone 3 Lysine 4 dimethylation (H3K4me2). However, there were more than 10 other such histone modification changes caused by urban dust that all together led to an average change of histone modification levels of 50%. When cells were treated with 0.03 % artichoke extract, the histone modification levels were also closer to that of control cells with an average modification level change of only 19%. The experiment was also conducted with Benzo[a]pyrene where, although the histone modification pattern was different, a profile closer to unpolluted keratinocytes was as well observed for more than 10 different histone modifications in cells treated with 0.03 % artichoke extract (on average 45 % change with Benzo[a]pyrene treatment vs on average only 11 % change with additional artichoke extract treatment compared to control cells, data not shown).

Activation of detoxification enzymes by a cress sprout extract

The ability of a cress sprout extract, rich in sulforaphane, to activate enzymes that help in the skin detoxification process was investigated in an assay with normal human epidermal keratinocytes. Three antioxidant enzymes were chosen as representatives of 'Detox' enzymes: NADPH:quinone reductase 1 (NQO1) is a major anticarcinogenic enzyme that transforms quinones into stable hydroquinones. Heme oxygenase 1 (HO-1) is induced following exposure to oxidative stress such as UV, which indicates its role in cellular defence. Thioredoxin reductase 1 (TrxR1) works together with NADPH in order to control the redox balance of the cell.

The keratinocytes were incubated for 24 hours with 0.05 % or 0.2 % cress sprout extract. Following incubation, the cells were harvested and total RNA was extracted. Gene expression of the detox enzymes was

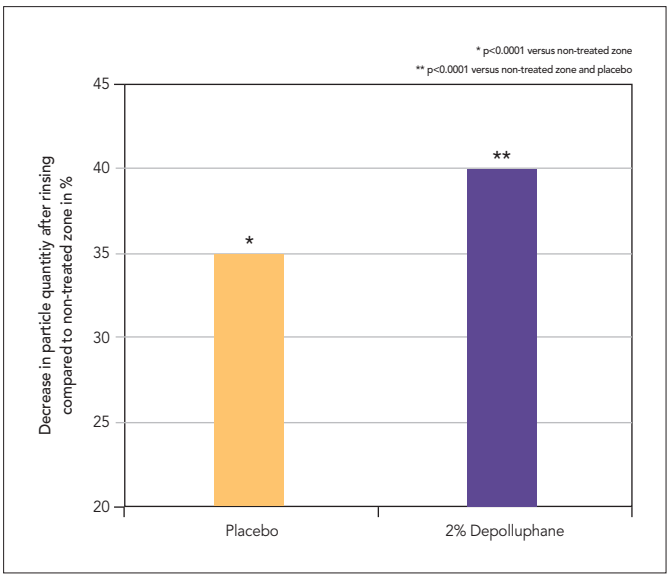

Figure 6: Decrease in quantity of particulate matter which still adheres to the skin after rinsing.

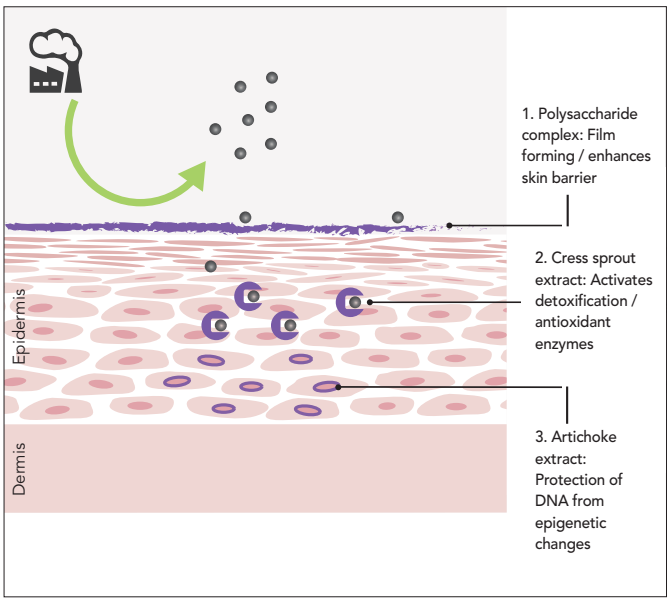

Figure 7: Triple action against pollution.

assessed via RT-qPCR. Results showed that the antioxidant enzyme NQO1 was moderately stimulated at 0.05% and strongly stimulated at 0.2 % cress sprout extract (Fig 3). HO-1 and TrxR1 were both stimulated strongly, even at the lower cress sprout extract concentration.

A cress sprout extract protects against protein damage caused by pollution

Particulate matter from air pollution can enter the skin and cause oxidisation reactions within the skin through the formation of free radicals. These oxidisation reactions damage important cell components such as proteins and lipids, and thus contribute to inflammation and skin ageing. The cress sprout extract which was shown as an efficient inducer of antioxidant enzymes could protect against pollution-induced protein damage.

In order to test the protective effect of the cress sprout extract against particulate matter, normal human dermal fibroblasts were either pretreated or not (PM control) with 1% cress sprout extract for a period of 7 hours. Subsequently, the medium containing the cress sprout extract was removed and the assay medium was exchanged to a medium that contained particulate matter (PM_{10}-like) to mimic pollution stress. Protein carbonylation was measured as a marker for oxidative protein damage caused by pollution (Fig 4). N-Acetylcysteine served as a positive control. A significant reduction in carbonylated proteins in cells treated with 1% cress sprout extract was observed which showed that the extract protects cells from free radicals caused by particulate matter.

A smart polysaccharide complex to shield the skin from pollution particles

The cress spout extract was sprayed on a carrier that is based on a mixture of different polysaccharides [Depolluphane (INCI: Lepidium sativum sprout extract and Pullulan and Sodium Carboxymethyl Betaglucan and Caesalpinia Spinosa Gum and Maltodextrin and Aqua)]. This smart polysaccharide complex performs various functions on the skin:

- The film-forming capability of the complex shields the skin from unwanted exposure to pollutants
- The biochemical activity of the complex enhances the skin's immune function and helps to strengthen the skin barrier.

The shielding effect of the polysaccharide complex with the encapsulated cress sprout extract was tested in a clinical study. Twenty-one women aged between 20 and 44 years (average age: 31 years) received a standardised single application of a cream containing 2% complex and the corresponding placebo cream in distinct 4x4 cm zones on their forearms. 20 minutes after product application, microparticles that modelled atmospheric pollution (1 µm on average) were applied to the forearm in the 2% complex and placebo-treated zones as well as to a non-treated zone as a negative control. The rinsing of the forearm was performed by spreading a standardised amount of water on the forearm and wiping each zone three times with a dry cotton pad. Pictures of the defined zones were taken with a Hirox video microscope before and after the rinsing of the microparticles.

Representative images of the average effect showed that the microparticles were more efficiently rinsed off in the zones that were pretreated with 2% complex (Fig 5). The pictures were also quantified by image analysis. The surface (in pixels) covered by the microparticles was measured before and after rinsing, and compared to the non-treated zone. Results showed that a single treatment with 2 % complex resulted in a significantly more efficient removal of microparticles compared to the non-treated zone as well as the placebo treated zone (Fig 6).

Conclusion

For complete protection of the skin against pollution, a cosmetic ingredient was developed based on two plant extracts, cress sprouts and artichoke which were encapsulated into a smart polysaccharide complex [Depolluphane EpiPlus (INCI: Lepidium Sativum Sprout Extract and Cynara Scolymus Leaf Extract and Pullulan and Sodium Carboxymethyl Betaglucan and Caesalpinia Spinosa Gum and Maltodextrin and Aqua)]. This new cosmetic active protects the skin from urban pollution in a threefold manner over the course of three distinct timelines (Fig 7):

- Immediately shields the skin against particulate matter
- In the short-term fortifies the skin's own defense mechanism by activating detoxification enzymes
- In the long-term protects the skin by preventing epigenetic changes that are caused by pollution.

References

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