A novel water-in-oil emulsion with a lecithin-modified bentonite prevents skin damage from urban dust and cedar pollen

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Abstract

OBJECTIVE: Particulate matter (PM), such as air pollutants and pol-lens, are known to cause skin ageing through skin inflammation. It is important to develop formulations which protect the skin from PM. We previously developed a conventional water-in-oil emulsion with a synthetic surfactant, distearyldimonium chloride, modified bentonite (C-W/O), which protects skin from allergens. In the present study, we developed a novel water-in-oil emulsion with a natural surfactant, lecithin, modified bentonite (N-W/O).

METHODS: The microrray analysis was performed using total RNA extracted from a reconstructed human epidermis (RHE) stimulated with urban aerosols or cedar pollen for 6 h in order to develop an epidermal inflammation model by PM for the evaluation of topical formulations. We then compared the efficacy of N-W/O and C-W/O to prevent epidermal degradation. Tissues and culture media were collected 24 h after the urban aerosol or cedar pollen stimulation for a histological assay, and the quantification of MMP1 and IL-8 secretion.

RESULTS: The expression levels of proinflammatory cytokines and chemokines, such as IL1A and CXCL8, and matrix metallopro-teinases, including MMP1, MMP3 and MMP9, were significantly up-regulated by the PM stimulation. As a result of ranking based on the pathway enrichment analysis, oxidative stress-related pathways, such as MAPK-mediated signalling, HIF-1 signalling, IL-1 signalling and ROS-induced cellular signalling, were ranked high in the urban dust- and cedar pollen-treated groups. A thickened stratum corneum, thinned vital layer and cleaved E-cadherin were observed by haematoxylin and eosin staining and immunohistochemical staining of E-cadherin in the PM treated groups. The secretion of MMP1 and IL-8 into the media was significantly increased by the PM stimulation. N-W/O prevented the degradation of epidermal integrity and secretion of inflammatory proteins more effectively than C-W/O.

CONCLUSION: The present results showed that N-W/O made using natural surfactant is useful at protecting skin from PM, such as urban aerosols and cedar pollen.

Résumé

OBJECTIF: Les particules en suspensions (PM), telles que les pol-luants atmosphériques et les pollens, sont connues comme des causes de vieillissement de la peau par inflammation cutanée. Il est essentiel de mettre au point des formules qui protègent la peau contre ces particules. Par le passé, nous avons mis au point une émulsion eau-dans-huile classique composée d’un tensioactif synthétique, de distéaryldimmonium chloride et de bentonites modifiées (E/H-C), qui protège la peau contre les allergènes. Dans la présente étude, nous avons conçu une nouvelle émulsion eau-dans-huile composée d’un tensioactif naturel, de lézithine et de bentoni-tes modifiées (N/E-H). Les résultats actuels ont révélé que la N-E/H protège les particules en suspensions telles que les aérosols urbains et le pollen de cèdre.

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Introduction
Particulate matter (PM) is a widespread air pollutant that comprises a mixture of particulate contaminants (including smog, tobacco smoke and soot), various types of dust, biological contaminants (such as pollen and house dust mite allergens) and gaseous contaminants (including exhaust gas from traffic). PM exerts adverse effects on human health by, for example, increasing the risk of cancer and pulmonary and cardiovascular diseases [1–3]. Recent studies reported that PM has a negative impact on skin and also induces extrinsic skin ageing, including wrinkling and pigmentation [4–6]. One of mechanisms by which PM induces skin ageing is by triggering the formation of intracellular reactive oxygen species (ROS) through mitochondrial damage and redox cycling and directly through particle surface reactivity [7]. Increases in ROS concentrations inhibit collagen synthesis as a result of the activation of matrix metalloproteases (MMPs) [8,9]. Furthermore, PM has been shown to enhance the production of proinflammatory cytokines and chemokines, such as IL-1α and IL-8, by human keratinocytes [10,11]. Since these PM-induced reactions lead to skin ageing, it is important to develop the formulations for the protection of skin from exposure to PM. These formulations should be made from natural ingredients including biosurfactants because they have low toxicity, biodegradability and higher stability under a wide range of physicochemical environments than synthetic surfactants [12].

In the present study, we used a reconstructed human epidermis (RHE) with PM-induced inflammation (urban aerosol and cedar pollen) to evaluate the protection efficacy of a novel water-in-oil emulsion with a lecithin-modified bentonite (C-W/O) with 700 μL TRизо® reagent and 140 μL chloroform was added. A total of 350 μL of the aqueous layer (containing RNA) was transferred to new RNase-free tubes after centrifugation at 12 000 g at 4 °C for 15 min. The aqueous layer was added to the same volume of 70% ethanol, and immediately mixed by pipetting. The mixture was transferred to an RNeasy spin column placed in a 2-mL collection tube and subjected to total RNA extraction according to the manufacturer’s instructions. The quality and concentration of total RNA were assessed using a Nanodrop ND-1000 spectrometer (Thermo Fisher Scientific, MA, USA). Total RNA obtained was used in a DNA microarray analysis with SurePrint G3 8x60K Microarrays (Agilent Technologies, Inc., CA, USA) as described previously [15]. The Agilent protocol ‘One-Color Microarray-Based Gene Expression Analysis (Low Input Quick Amp Labeling), Ver6.9, December 2015’ was used for sample preparation and array processing. Cy3-labelled cRNA was subjected to hybridization by an incubation in a hybridization oven (Agilent Technologies, Inc.) for 17 h. Hybridized slides were scanned with the G2505C scanner (Agilent Technologies, Inc.), and data were obtained using Agilent Feature Extraction software (version 10.7.1.1, Agilent Technologies, Inc.) with defaults for all parameters. Microarray data analyses were performed using GeneSpring GX (version 14.5) software (Agilent Technologies, Inc.).

The significance of differences in gene expression between the control and treated groups was assessed using Welch’s t-test. Differentially expressed genes (DEGs) were defined according to the following three criteria: (i) Significance level, \( P < 0.05 \). The gene expression ratio is >1.5 or <0.67, and all flags of each sample are ‘Detected’. (ii) Significance level, \( P < 0.01 \). The gene expression ratio is 1–1.5 or 0.67–1, and the flag of each sample is ‘Detected’. (iii) Significance level, \( P < 0.01 \). The gene expression ratio is >4, including the ‘Not Detected’ flag in the control group. (iiib) Significance level, \( P < 0.01 \). The gene expression ratio is <0.25, including the ‘Not Detected’ flag in the exposure group. DEGs were imported for a pathway enrichment analysis into MetaCore® software (Thomson Reuters, NY, USA).

Materials including emulsion samples (Table I)
N-W/O was formulated using a lecithin-modified bentonite made from hydrogenated lecithins (Lucas Meyer Cosmetics Inc., International Journal of Cosmetic Science published by John Wiley & Sons Ltd on behalf of Society of Cosmetic Scientists and the Société Française de Cosmétologie International Journal of Cosmetic Science, 42, 229–236

| Ingredients (w/w%) | Emulsion sample |
|--------------------|----------------|
|                    | Base formula | N-W/O† | C-W/O† |
| Modified clay      | Lecithin-modified bentonite | 2.0 | 2.0 |
| Distearylmonium-modified bentonite | | |
| Surfactant         | Sorbitan fatty acid esters | 2.5 | 0.5 |
|                    | Dimethicone copolyol | 4.0 | |
| Oil                | Hydrocarbon     | 32.0 | 32.0 |
| Water phase        | Glycerine       | 15.0 | 15.0 |
|                    | Butylene glycol | 6.0 | 6.0 |
| Water              |                | 41.0 | |
| Total              |                | 100.0 | 100.0 |

*Novel water-in-oil emulsion with a lecithin-modified bentonite.
†Conventional water-in-oil emulsion with a synthetic surfactant-modified bentonite.

Materials and methods
RHE model
A three-dimensional RHE model, LabCyte EPI-MODEL (Japan Tissue Engineering Co., Ltd., Aichi, Japan), which is composed of normal human epidermal keratinocytes that form a multilayered structure, was maintained according to the manufacturer’s instructions.

Particulate matter
Urban dust collected on filters from the central ventilating system of a building in the centre of Beijing city between 1996 and 2005 (CRM No. 28, National Institute for Environmental Studies, Ibaraki, Japan) and Japanese cedar pollen (FUJIFILM Wako Pure Chemical Corporation, Osaka, Japan) were used in the present study.

Microarray analysis of the RHE inflammation model with PM exposure
RHE was exposed to 25 mg of urban dust or cedar pollen, while control samples were not. After six hours of PM exposure, total RNA was extracted from RHE using TRIzol® reagent (Thermo Fisher Scientific, MA, USA) and the RNeasy Mini Kit (Qiagen, Tokyo, Japan) modified from methods described in a previous study [14]. Briefly, all samples were homogenized using Biomasher® II (Nippi Inc., Tokyo, Japan) with 700 μL TRIzol® reagent, and 140 μL chloroform was added. A total of 350 μL of the aqueous layer (containing RNA) was transferred
Figure 1 Microarray analysis of the reconstructed human epidermis model after 6 h of urban dust or cedar pollen exposure. (A) A heat map shows fold changes in gene expression levels in the urban dust- and cedar pollen-treated groups from those in the control group. Many genes categorized as metabolism and antioxidant enzymes, cytokines and chemokines, proteases and growth factors were commonly up-regulated following the exposure to urban dust and cedar pollen. The experiments were performed in triplicate, and each data was shown in fold change/control column. (B) Comparison of the number of differentially expressed genes (DEGs) between the urban dust- and cedar pollen-treated groups was shown using a Venn diagram. Approximately 50% of up- and down-regulated DEGs were similar in the urban dust- and cedar pollen-treated groups.

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Preparation of lecithin-modified bentonite

A 1.75 (wt) % aqueous solution of hydrogenated lecithins was prepared, and citric acid (Iwata Chemical CO., LTD., Shizuoka, Japan) was added to pH 2.5–3.0. After pH-adjusted hydrogenated lecithin solution had been temperature-adjusted to 65 °C, 0.625 mol of bentonite was gradually added to 1 mol of hydrogenated lecithin. The mixture was stirred with a magnetic stirrer for 2 h. After stirring and cooling to room temperature, reagents were filtered by suction filtration and then washed once with an aqueous citric acid solution of pH 2.5 and three times with water to obtain a paste-like filtrate. The washed filtrate was dried under decompensation conditions (~1 Pa, 80 °C) for one day and then pulverized using a mixer to obtain a lecithin-modified bentonite powder.

Preparation of emulsion samples

The modified clay, surfactant and oil listed in Table I were mixed in a 100-ml test tube and homogenized by stirring for 5 min at 2000 rpm in a homogenizer (NS-360D, NS-7, MICROTEC CO., LTD., Chiba, Japan). Pre-mixed aqueous phase components were added and stirred with a homogenizer at 6000 rpm for 10 min to prepare creamy W/O emulsion samples. The base formula was prepared by hand stirring with a glass rod, and the internal water phase ratio was adjusted to achieve a similar creamy state to that of N-W/O and C-W/O.

Evaluation of the efficacy of N-W/O to protect against PM

RHE was treated with 100 μL of the base formula, N-W/O or C-W/O, before being exposed to 25 mg urban dust or cedar pollen, while control RHE was not exposed. Twenty-four hours after the exposure to PM, culture media were collected to quantify protein content based on ELISA as follows: quantification of MMP1 (ab100603, Abcam Inc., MA, USA) and IL-8 (D8000C, R&D Biosystems, MN, USA). MMP1 and IL-8 in the media were quantified according to the manufacturer’s protocol. A statistical analysis was performed with a one-way analysis of variance followed by the post-hoc Tukey–Kramer test.

RHE was harvested after 24 h of PM exposure for the MTT assay (403026, Japan Tissue Engineering Co., Ltd., Aichi, Japan) or histological assays. Formaldehyde-fixed RHE samples were embedded in paraffin blocks and stained with haematoxylin and eosin (H&E). And E-cadherin was stained immunohistochemically using Novocastra™ Liquid Mouse Monoclonal Antibody E-Cadherin (NCL-E-Cad, Leica Biosystems, Wetzlar, Germany). The primary antibody was detected using the biotinylated secondary antibody, horseradish peroxidase-labelled streptavidin and 3, 3′-diaminobenzidine (ab64259, Abcam Inc.).

Results

Microarray analysis of the PM-induced inflammation in RHE

Fold changes in gene expression levels in PM-exposed samples from those in the control were shown using a heat map (Fig. 1a). Most genes categorized as metabolism and antioxidant enzymes, cytokines and chemokines, proteases and growth factors were commonly up-regulated following the exposure to urban dust and cedar pollen. On the other hand, most genes categorized as adhesion molecules in keratinocytes, cytoskeletal proteins and skin-hydrating factors maintained the same gene expression levels. The up-regulated expression levels of metabolism and antioxidant enzymes, cytokines and chemokines, proteases and growth factors were higher in the urban dust-treated group than in the cedar pollen-treated group. Changes in CYP1A1 and CYP1B1 mRNA levels were 544- and 253-fold higher, respectively, in the urban dust-treated group than in the control group. These up-regulated levels were markedly higher than those in the cedar pollen-treated group. The numbers of up-regulated DEGs in the urban dust- and cedar pollen-treated groups were 1793 and 1534, respectively, whereas those of down-regulated DEGs were 1480 and 1967, respectively. Approximately 50% of up- or down-regulated DEGs were the same in the
Figure 2. Evaluation of viability and preventative effects on MMP1 and IL-8 secretion by a novel water-in-oil emulsion with a lecithin-modified bentonite (N-W/O) and a conventional water-in-oil emulsion with a synthetic surfactant-modified bentonite (C-W/O) after 24-h exposure to urban dust or cedar pollen. (A) Viability was measured using the MTT assay. All samples maintained viability. (B-E) Culture media were collected to quantify (B, C) MMP1 and (D, E) IL-8. MMP1 and IL-8 secretion was significantly induced by the application of urban dust or cedar pollen, and significantly prevented by the N-W/O topical treatment. Data were presented as means ± SD. A statistical analysis was performed with a one-way analysis of variance followed by the post-hoc Tukey-Kramer test. The asterisks indicate statistical significance compared with the urban dust- or cedar pollen-treated groups. *P < 0.05, **P < 0.01.
(A) Histological assay following the application of urban dust

| Treatment                | H&E       | E-cadherin |
|--------------------------|-----------|------------|
| None / None              |           |            |
| Urban dust / None        |           |            |
| Urban dust / Base formula|           |            |
| Urban dust / N-W/O       |           |            |
| Urban dust / C-W/O       |           |            |

(B) Histological assay following the application of cedar pollen

| Treatment                | H&E       | E-cadherin |
|--------------------------|-----------|------------|
| None / None              |           |            |
| Urban dust / None        |           |            |
| Urban dust / Base formula|           |            |
| Urban dust / N-W/O       |           |            |
| Urban dust / C-W/O       |           |            |

Figure 3 Histological assay by haematoxylin and eosin (H&E) staining and immunohistochemical staining of E-cadherin at 24-h exposure to urban dust (A) or cedar pollen (B). A thickened stratum corneum, thinned vital layer and cleaved E-cadherin were observed with urban dust and cedar pollen exposure. N-W/O prevented the degradation of epidermal integrity and intercellular adhesion.

Regarding the results of histological observations of RHE, a thickened stratum corneum and thinned vital layer were observed in the 24-h PM-exposed groups by H&E staining, and cleaved E-cadherin in the epidermis was noted in these groups in the immunohistochemical analysis with the E-cadherin antibody. These epidermal disruptive effects by PM exposure were more prominent in the urban dust-treated group than in the cedar pollen-treated group (Fig. 3a, b). N-W/O and C-W/O prevented the degradation of epidermal integrity more than the base formula in RHE exposed to PM (Fig. 3a-e).

Discussion
Particulate matter increased the mRNA expression levels of inflammation-related genes and disrupted epidermal integrity in the RHE model. Gene expression and histological observations demonstrated that urban dust is a stronger inflammatory inducer than cedar pollen. Urban dust exerts markedly strong effects on skin because it contains a wide range of toxic contaminants, such as heavy metals, polycyclic aromatic hydrocarbons (PAHs), including benzo[a]pyrene (BaP), and endocrine-disrupting chemicals. Furthermore, urban dust may penetrate skin more easily than cedar pollen because the diameters of 99% of urban dust were less than 10 µm and 40%...
were 2 µm [16], which are markedly smaller than that of cedar pollen (30 µm) [17].

Urban dust and cedar pollen significantly activated oxidative stress-related pathways in the ranking based on the pathway enrichment analysis by MetaCore®. Approximately 50 % of up- and down-regulated DEGs in urban dust and cedar pollen, such as MMP1 and CXCCL8, were the same. According to previous findings, PM induces oxidative stress through the formation of ROS [18–21]. PAHs localize in mitochondria in which they induce major structural damage [18], and they produce superoxide (O$_2^−$) and the hydroxyl radical (•OH) by the redox cycling of NADPH-cytochrome P450 reductase [19,20]. In addition, pollen grain extracts exhibit NADPH oxidase activity and NADPH oxidases increase O$_2^−$ levels [21]. These previous reports suggest that the similar effects on the gene expression and histological damage by urban dust and cedar pollen in this study were induced by ROS.

On the other hand, approximately 50% of up- and down-regulated DEGs in urban dust and cedar pollen were different. Cytochrome P4501A1 (CYP1A1) and CYP1B1 mRNA were more strongly induced in the urban dust-treated group than in the cedar pollen-treated group. BaP, which is composed of urban dust, is a ligand of the aryl hydrocarbon receptor (AhR), and AhR signalling has been shown to induce CYP1A1 and CYP1B1, which produce ROS [22], in skin and keratinocytes [23–25]. Furthermore, has been shown to induce CYP1A1 and CYP1B1, which produce ROS [22], in skin and keratinocytes [23–25]. Furthermore, SLC7A11, SRXN1 and PIR, which were induced by urban dust [26], were detected as specific DEGs in urban dust samples. Cedar pollen exhibits serine protease activity [27], and Cry 1a, a peptide allergen of cedar pollen, activates protease-activated receptor 2 (PAR2) [28]. KRA5, a gene that is up-regulated by a PAR2 agonist, was only listed in the DEGs of the cedar pollen-treated group [29]. These findings suggested that ROS production is a common effect of urban dust and cedar pollen, and AhR and PAR2 signalling were specifically activated by urban dust and cedar pollen, respectively.

Matrix metalloproteinases and IL-8 were chosen representative targets as proinflammatory factors that cause skin ageing because these targets are commonly induced by AhR and PAR2 agonists [11,30,31]. In addition, MMP1 was selected as a typical matrix metalloproteinase related to skin ageing because it is elevated in the aged human skin and is responsible for the initiation of collagen fragmentation [32]. The significant up-regulation of these proinflammatory factors was significantly induced by PM exposure and prevented by N-W/O treatment. Since these targets are associated with the early stages of skin inflammation and inflammatory factors were activated by the skin penetration of PM, other inflammatory factors induced by PM may also be prevented by N-W/O treatment.

However, E-cadherin mRNA was unchanged by PM exposure in this study, and the destruction of the epidermis was clearly observed by H&E staining. We demonstrated the immunohistochemical staining for E-cadherin because it was known to be cleaved by MMP3 [33] strongly induced by PM exposure. As a result, cleaved E-cadherin was observed in PM-treated groups. It was suggested that MMP3 induced by PM exposure in the epidermis cleaves E-cadherin and causes skin destruction.

We previously reported that C-W/O decreased the skin severity score, the frequency of scratching behaviour, and the grade of TEWL more effectively than petrolatum, which was used as a control, after 8 weeks of treatment for atopic dermatitis-like skin symptoms in NC/Nga mice [13]. The efficacy by which the induction of skin inflammation was prevented by N-W/O tended to be higher than that by C-W/O based on MMP1 and IL-8 secretion and epidermal degradation.

In conclusion, N-W/O made using natural surfactant is useful at protecting skin from PM, such as urban aerosols and cedar pollen.

Conflict of Interest

None.

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