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Protective Effects of Sacran, a Natural Polysaccharide, Against Adverse Effects on the Skin Induced by Tobacco Smoke

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Synopsis

Recent increases in air pollution have raised concerns about its adverse effects on human health. Sacran is a natural polysaccharide isolated from a cyanobacterium. We previously reported that sacran improves skin conditions because of its effects as an artificial barrier against external stimuli, which suggested that sacran might protect the skin against air pollutants. The goal of this study was to characterize the potential of sacran to protect human skin against damage from air pollutants and to compare sacran with hyaluronic acid (HA). Sacran that was topically applied on the skin stayed on the surface or in the stratum corneum. Sacran-treated filters had a shielding effect against benzo[a]pyrene (BaP) and aldehyde compounds contained in tobacco smoke. Sacran suppressed the upregulation of cytochrome P4501A1 messenger ribonucleic acid (mRNA), which is a xenobiotic-metabolizing enzyme induced by BaP, and other responses against tobacco smoke in HaCaT keratinocytes. Furthermore, topical application of a serum containing 0.04% sacran on the skin reduced levels of carbonylated proteins in corneocytes of tobacco smokers. Sacran showed superior effects in every characteristic measured, compared with HA. We conclude that sacran ameliorates the oxidative stress initiated by tobacco smoke by shielding the skin surface and protects human skin.

INTRODUCTION

Recently, the adverse influence of various air pollutants, such as polycyclic aromatic hydrocarbons (PAHs), volatile organic compounds, nitrogen oxides, and particulate matter, on human health has caused increased concern in the world, especially in India, China, Saudi Arabia, and African and Southeast Asian countries (1). In 2016, the World Health Organization (WHO) reported that more than 80% of people living in urban areas where air pollution is monitored are exposed to air pollutants beyond the limitations

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of the WHO. Several studies have shown that air pollutants interfere significantly with the normal functions of lipids, deoxyribonucleic acid, and proteins in human skin, which is the outermost line of defense of the human body, via oxidative damage (2–10). Those effects lead to skin aging; inflammatory or allergic conditions, such as atopic dermatitis, psoriasis, and acne; and skin cancer (11,12). It is easily imagined that the route of air pollutants that damage human health is through inhalation. However, recent epidemiological studies regarding the skin have reported that human beings living in places where they are exposed to much higher levels of air pollutants, such as China and India, suffer from skin problems such as pigmented spots and wrinkles at a high frequency (13). In addition, the number of people who suffer from sensitive skin in Japan increases every year, and about 30% of them feel that the causative factor of their sensitive skin is air pollution (14). In general, it has been demonstrated that PAHs induce inflammation by activating the aryl hydrocarbon receptor (AhR) signaling pathway (15). AhR is a chemical receptor that responds to exogenous and endogenous chemicals by inducing or repressing the expression of several genes that have protective or toxic effects (15). In that regard, it has been clarified that the activation of AhR induces the overexpression of cytochrome P4501A1 (CYP1A1) and generates excess reactive oxygen species (ROS) (15–17).

In daily life, we are frequently exposed to tobacco smoke and exhaust gas, which are types of air pollutants. This raises the possibility that we suffer from adverse effects of aldehyde compounds (ACs), benzo[a]pyrene (BaP), and other chemicals because they are major components in tobacco smoke and exhaust gas. Among various tissues of the body, it is of greatest concern that the skin is intensively influenced by air pollutants because of its localization at the outermost surface of the body. ACs cause protein carbonylation in the skin, and it has also been reported that ROS are generated from carbonylated proteins (CPs) when irradiated with blue light through a type-I photosensitization reaction (18). The sum of these facts suggests that ACs initiate an oxidation loop due to the accumulation of CPs in the skin. On the other hand, BaP, which is a representative PAH that is produced in industrial combustion processes, enhances the secretion of inflammatory cytokines from human epidermal keratinocytes because of the excess production of ROS through the activation of AhR signaling (19). Thus, it is considered a possibility that air pollutants directly cause these adverse actions because of their penetration through the skin. To avoid that risk, it is very important to develop an effective approach to prevent/reduce the penetration of air pollutants, PAHs, and ACs, into the skin. Thus, we designed an approach to shield the skin surface using a topically applied film to interfere with the penetration of air pollutants.

Sacran, a large molecular weight polysaccharide isolated from *Aphanothece sacrum* (Suizenji-nori) algae, has unique characteristics and is composed of 11 kinds of monosaccharides, including ionized groups containing sulfate groups and carboxylic acid groups (20–22). We have previously reported that sacran forms a hydrophobic gel-like film in combination with polyols and improves skin conditions in individuals who suffer from atopic dermatitis (23–25). From those results, we expected that sacran would protect the skin against environmental stimuli such as chemicals and air pollutants (e.g., tobacco smoke) because of its formation of an artificial skin barrier.

Thus, the purpose of this study was to clarify whether sacran reduces skin damage caused by air pollutants focusing on oxidative stress compared with hyaluronic acid (HA), which is commonly used in skin care products.

MATERIALS AND METHODS

MATERIALS

Sacran was extracted from the algae *Aphanothece sacrum* (Suizenji-nori) and was purified (19). Bio-sodium hyaluronate (MMW), as a general HA, was obtained from SK Bioland (Chungnam, Cheonan, South Korea), and 1,3-butylene glycol was obtained from Kokyu Alcohol Kogyo Co. (Tokyo, Japan). Dulbecco's modified Eagle medium (DMEM) and Hanks' Balanced Salt solution with Ca^{2+} and Mg^{2+} (HBSS) were obtained from Nissui Pharmacy (Tokyo, Japan), and fetal bovine serum (FBS) was obtained from Invitrogen (Carlsbad, CA). The BCA Protein Assay Reagent kit was purchased from Pierce Chemical Co. (Rockford, IL). LabCyte EPI-MODEL reconstructed human epidermal equivalents (RHEEs) at 12 d, and their assay medium were obtained from Japan Tissue Engineering (Aichi, Japan). 6-[6-(Biotinylamino)hexanoylamino]hexanoylhydrazine (biotin-AC5-hydrazide) and Hoechst 33342 solution were obtained from Dojindo Laboratories (Kumamoto, Japan). DyLight650-labeled streptavidin was obtained from Thermo Fisher Scientific Inc. (Waltham, MA). Acrolein monomer, NBD-hydrazine (4-hydrazino-7-nitrobenzofurazan-hydrazine), and 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride were purchased from Tokyo Chemical Industry Co. (Tokyo, Japan). Fluorescein-5-thiosemicarbazide (FTSC), 2′,7′-dichlorodihydrofluorescein diacetate (H_2DCFDA), and BaP were purchased from Sigma-Aldrich (St. Louis, MO). Dimethyl sulfoxide was purchased from Nacalai Tesque (Kyoto, Japan). 2-Mercapto-ethanol (2-ME) and SYBR® Green Real-Time polymerase chain reaction (PCR) Master Mix for real-time PCR analysis were obtained from Thermo Fisher Scientific Inc. (Kanagawa, Japan). RLT buffer and the RNeasy Mini Kit were purchased from Qiagen (Hilden, Germany).

SACRAN AQUEOUS SOLUTION

A 0.05% (w/v) sacran aqueous solution was used for experiments as noted in the text. As a representative anionic polysaccharide that is commonly formulated in skin care products, we used a 0.05% (w/v) HA aqueous solution.

BIOTIN CONJUGATION TO POLYSACCHARIDES

Polysaccharides were reacted with 2 mg/mL biotin-(AC5)2-hydrazide in the presence of 20 mg/mL 4-(4,6-dimethoxy-1,3,5-triazin-2-yl)-4-methylmorpholinium chloride and pyridine for 5 h. Biotin-conjugated polysaccharides were purified by dialysis against deionized water.

PENETRATION OF POLYSACCHARIDES INTO RHEEs

The penetration of biotin-conjugated polysaccharides through RHEEs was measured by quantification of biotin-conjugated polysaccharides in the culture medium using an Enzyme-linked immunosorbent assay (ELISA) and fluorescence histology. RHEEs were topically treated with biotin-conjugated polysaccharides, and then were cultured for 24 h at 37°C.

Each RHEE was separated into two pieces with a scalpel. Frozen thin sections of one piece of each RHEE embedded in optimal cutting temperature compound were prepared using a cryomicrotome, and the localization of biotin-conjugated polysaccharides in RHEEs was visualized by staining with DyLight650-labeled streptavidin. Nuclei were stained with Hoechst33342. Fluorescence images were taken with a Floid Cell Imaging Station (Thermo Fisher Scientific Inc.). The other piece of each RHEE was used to extract biotin-conjugated polysaccharides following homogenization in 500 µL phosphate-buffered saline without Ca^{2+} and Mg^{2+} [PBS(−)] at 2,700 rpm for 10 min with a µT-12 bead crusher (Taitec Corp., Saitama, Japan). Biotin-conjugated polysaccharides remaining in the RHEEs or penetrating into the culture medium through the RHEEs were quantified using an ELISA method. Briefly, the RHEE extract or culture medium was incubated with horseradish peroxidase-conjugated streptavidin (1:1,000) for 1 h at 37°C. The mixed solution was placed in wells of ELISA plates (MS-8596F, Sumitomo Bakelite, Tokyo, Japan) coated with biotin-conjugated bovine serum albmin (BSA), and then incubated for 1 h at 37°C. Each well was washed with PBS-T, then 150 µL 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS; Wako, Osaka, Japan) solution (0.3 mg/mL) in phosphate-citrate buffer (0.1 M, pH 4.0) containing a small amount of H_2O_2 was added to each well. After 30 min, the absorbance of each well was measured using a microplate reader (Spark 10M; TECAN, Männedorf, Switzerland) at 405 nm. Amounts of biotin-conjugated polysaccharides were determined using a calibration curve prepared with biotin as a standard substance.

TOBACCO SMOKE

Seven Stars® (tar: 14 mg, nicotine 1.2 mg, Japan Tobacco, Tokyo, Japan) was used a source of tobacco smoke.

TRAPPING EFFECTS OF POLYSACCHARIDES AGAINST TOBACCO SMOKE

The trapping effects of polysaccharides against tobacco smoke were examined by measuring ACs and BaP in PBS diffused with tobacco smoke using fluorescence methods (Figure 1). The smoke obtained from burning one piece of tobacco was introduced into PBS stirred with a magnetic stirrer passing through membrane filters (10 µm JH, Merck Millipore, Burlington, MA) that had been treated with or without polysaccharide by soaking in 1 mL polysaccharide

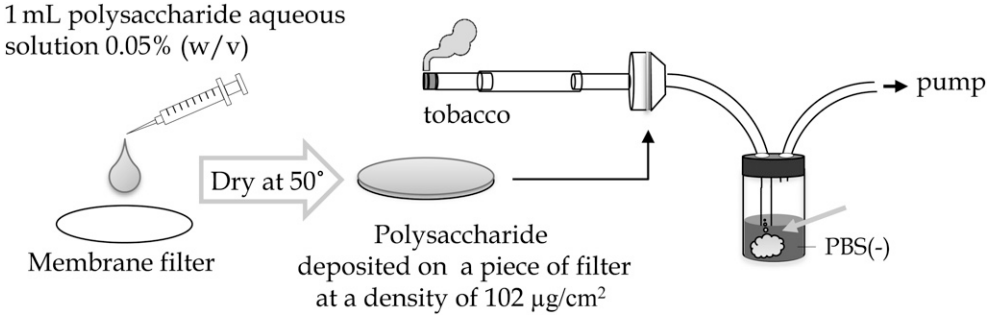


Figure 1. Method for treatment by tobacco smoke to PBS(−).

aqueous solution by aspiration with a vacuum pump, and then dried at 50°C (102 μg/cm^2 of polysaccharides were deposited on each filter). PBS(−) was reacted with 25 μM NBD-hydrazine in the presence of 0.05% trifluoroacetic acid for 30 min in the dark to determine the amount of ACs. ACs were quantified by measuring fluorescence intensity (FI) (Ex; 470 nm, Em; 550 nm) using a microplate reader with a calibration curve prepared with acrolein as a standard substance. BaP in the PBS(−) was quantified by measuring FI (Ex; 360 nm, Em; 450 nm) using a calibration curve prepared with BaP. Trapping efficiency is expressed as a percentage versus the value of the nontreated membrane filter.

CELL CULTURE AND EXPOSURE TO TOBACCO SMOKE

HaCaT keratinocytes were cultured in DMEM with 5% FBS at 37°C in a humidified atmosphere containing 5% CO_2. HaCaT keratinocytes were inoculated at a density of 3.5×10^4 cells per well in 96-well plates. Cells were cultured in DMEM containing PBS diffused with tobacco smoke in the presence or in the absence of polysaccharides for 24 h.

mRNA EXPRESSION OF CYP1A1

The expression level of CYP1A1 mRNA in HaCaT keratinocytes, after culturing for 24 h in DMEM containing PBS diffused with smoke obtained from burning one piece of tobacco by aspiration with a vacuum pump, was evaluated using real-time quantitative PCR analysis. After removal of the medium from wells, washed adherent cells were processed for PCR analyses by the direct addition of 350 μL RLT buffer (Qiagen) containing 3.5 μL 2-ME to each well. Total RNA from the lysed cells was extracted using an RNeasy Mini Kit, according to the manufacturer's instructions. First-strand cDNA was then synthesized using a PrimeScript RT Master Mix and T100 Thermal cycler (Bio-Rad Laboratories, Hercules, CA). Real-time PCR analysis was performed using an Applied Biosystems StepOne Real-Time PCR System (Thermo Fisher Scientific Inc., Kanagawa, Japan) with 1 μL cDNA for each sample. SYBR Green Real-Time PCR Master Mix was used to detect products, and 10 μM concentrations of the primers were used: human CYP1A1 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) obtained from Takara Bio Inc. (Shiga, Japan) (i.e., NM_002046.5 and NR_045089.1). The relative amount of cDNA in each sample was normalized using GAPDH, and the melting curve was used to verify specificity.

ADVERSE EFFECTS OF TOBACCO SMOKE ON HaCaT KERATINOCYTES

The influence of smoke obtained from burning one piece of tobacco was measured for the following parameters: cell damage, cell viability, intracellular ROS, and intracellular CPs.

Cell damage was examined by measuring cell viability using the neutral red assay. Cells were cultured with DMEM containing 5% FBS and neutral red at a concentration of 33 μg/mL for 2 h. After washing with PBS, neutral red incorporated into the living cells was extracted with 30% MeOH aqueous solution with agitation. The absorbance at 550 nm of the resulting solution was measured using a microplate reader.

Intracellular ROS levels were measured using H_2DCFDA, a fluorescent probe for H_2O_2. Cells were treated with 20 μM H_2DCFDA in HBSS for 30 min. After lysing the cells with 0.1% Triton X-100 in PBS, the fluorescence (Ex; 485 nm, Em; 530 nm) was measured using a fluorescence microplate reader (Spectra Max Gemini; Molecular Devices, San Jose, CA). Intracellular ROS levels are calculated as FI per μg protein and are expressed as a fold change of the value of control cells. Protein concentrations were determined using a BCA Protein Assay Kit (Thermo Fisher).

Intracellular levels of CPs were determined by fluorescence labeling of the aldehyde group with FTSC. After fixation with cold MeOH for 10 min, cells were incubated with FTSC in 0.1 M MES-Na buffer (pH 5.5) and 2 μM Hoechst 33342 for 1 h. CPs and nuclei were quantified by image analysis using corneocytometry software (Ciel, Tokyo, Japan) after obtaining fluorescence images with a fluorescence microscope (Floid Cell Imaging Station; Thermo Fisher Scientific Inc).

BARRIER FUNCTION OF POLYSACCHARIDES AGAINST TOBACCO SMOKE

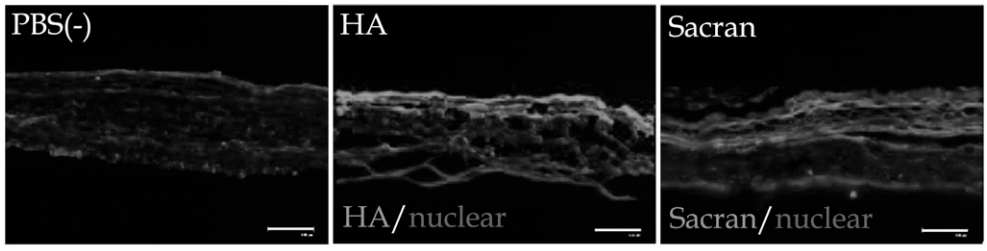
Ex vivo study. The barrier function of polysaccharides was characterized by measuring CP levels in corneocytes following exposure to tobacco smoke. Corneocytes were obtained from the upper inner arm of five human volunteers who were nonsmokers by the tape-stripping method using cellophane tape (Nichiban Co., Ltd., Tokyo, Japan) and were transferred to glass slides after dividing each piece of tape into four pieces. One piece of the corneocytes from each volunteer was used for the evaluation of one sample. The corneocytes were treated with 10 μL polysaccharide aqueous solution for 10 min at room temperature. As the control, H_2O, which was the solvent for the polysaccharide aqueous solution, was applied on corneocytes. After rinsing with running water and then drying, the glass slides with corneocytes were placed in a box filled with tobacco smoke for 2 h at room temperature. After a further incubation for 24 h at 37°C, CP levels of corneocytes were measured by fluorescence labeling as follows: Corneocytes on glass slides were immersed in 0.1 M MES-Na solution (pH 5.5) containing 20 μM FTSC for 1 h at 25°C in the dark. After rinsing, images were obtained using a fluorescence microscope (Floid Cell Imaging Station, Thermo Fisher). CP levels were quantified from the fluorescence images using corneocytometry software (Ciel). Informed consent was obtained from each volunteer after explaining the method of collecting corneocytes and the aim of the test. The *ex vivo* study was approved by the Ethical Committee of the Daito Kasei Kogyo Co., Ltd. and was performed three separate times using the same five volunteers.

Statistical analysis. All study data are expressed as means ± standard deviation (SD). Significant differences between experimental values were determined using the Wilcoxon rank-sum test, and p-values less than 0.05 are considered statistically significant.

RESULTS

PENETRATION OF POLYSACCHARIDES INTO RHEEs

In the histological study, it was observed that sacran as well as HA applied topically on RHEEs remained in the stratum corneum (Figure 2). In addition, the amount of sacran



	Penetration (%) into the culture medium	Retained in RHEEs (%)
HA	1.0±0.01	98.0±0.02
Sacran	1.0±0.01	99.5±0.03

Figure 2. Penetration of biotin-conjugated polysaccharides into RHEEs. RHEEs were treated topically with a biotin-conjugated polysaccharide aqueous solution (sacran or HA) as noted and then were cultured for 24 h at 37°C. The culture media were collected to quantity biotin-conjugated polysaccharides that penetrated through the RHEEs. The histology of biotin-conjugated polysaccharides is shown as representative images (scale bars, 100 μm). The penetration or retention of biotin-conjugated polysaccharides in RHEEs is expressed as a percentage of the amount of biotin-conjugated polysaccharides applied. PBS(−) was used as a control. Each value represents the mean ± SD of three experiments.

in the medium of RHEEs topically treated with sacran was approximately 1%, and more than 99% of the sacran was recovered from the RHEE homogenates (Figure 2). HA also showed similar results. These results indicate that polysaccharides topically applied on RHEEs stay in the stratum corneum and do not penetrate well to the living cell layers of the epidermis.

TRAPPING EFFECT OF POLYSACCHARIDES ON ACs AND BaP

To examine the trapping effects of polysaccharides, the amounts of ACs and BaP in PBS(−) diffused with tobacco smoke passed through membrane filters treated with or without polysaccharides were quantified. Sacran-treated membrane filters showed excellent trapping effects for both ACs and BaP (Figure 3). More than 90% of ACs and BaP were trapped in the sacran-treated membrane filter compared with the control (nontreated membrane filter). In the case of the HA-treated membrane filter, although it failed to significantly trap ACs, it showed a significantly high trapping effect for BaP compared with the control (Figure 3). Thus, although both sacran and HA have trapping effects on ACs and BaP, sacran showed a superior effect compared with HA.

SUPPRESSIVE EFFECTS OF POLYSACCHARIDES ON mRNA EXPRESSION OF CYP1A1 INDUCED BY TOBACCO SMOKE

HaCaT keratinocytes cultured in DMEM containing PBS diffused with tobacco smoke through a nontreated membrane filter showed a predominant upregulation of CYP1A1 mRNA levels. Cells cultured in DMEM containing PBS diffused with tobacco smoke

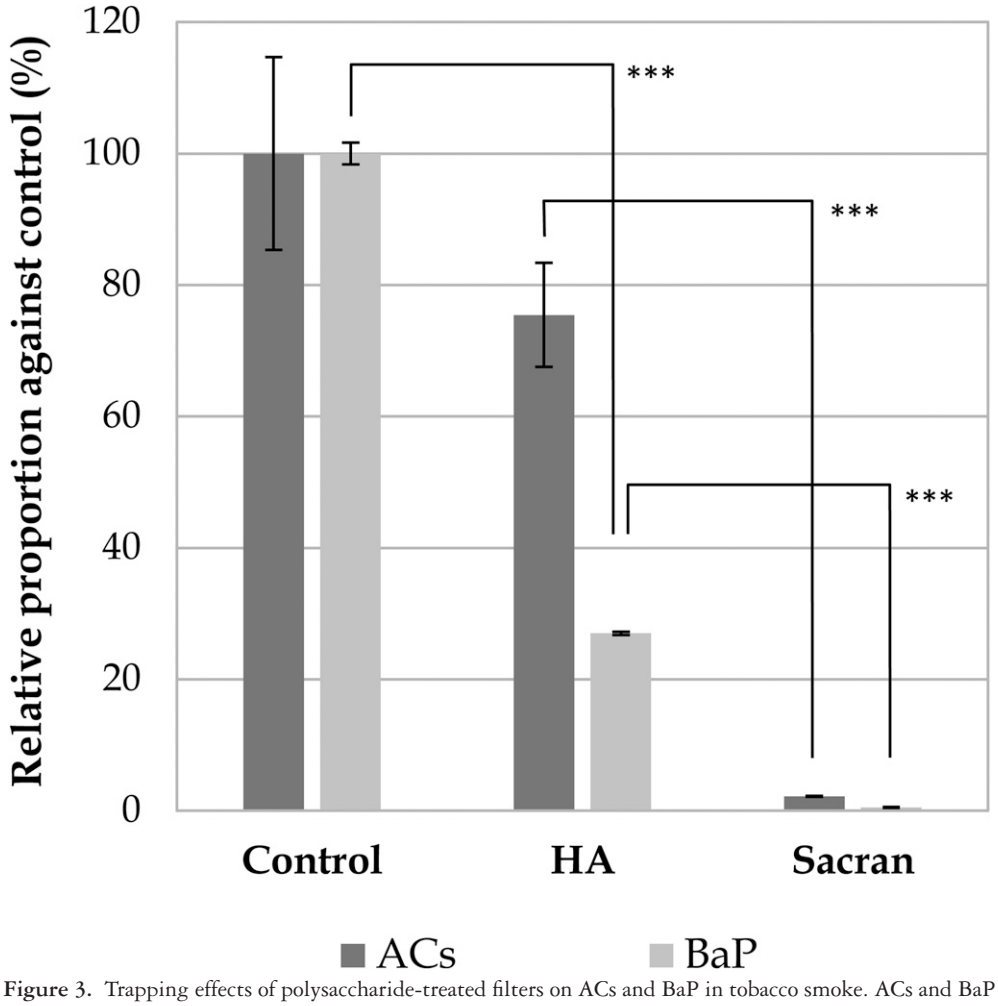


Figure 3. Trapping effects of polysaccharide-treated filters on ACs and BaP in tobacco smoke. ACs and BaP in PBS(−) diffused with tobacco smoke passed through filters treated with or without polysaccharides were quantified as follows: ACs were estimated by FI (Ex; 470 nm, Em; 550 nm) with NBD-hydrazine in the presence of trifluoroacetic acid. BaP was quantified by FI (Ex; 360 nm, Em; 450 nm). Calibration curves were prepared, and their concentrations were quantified. Each value represents the mean ± SD of three experiments. Wilcoxon rank-sum test, $***p < 0.001$. Trapping efficiency is expressed as a percentage versus the value of the H_2O-treated membrane filter diffused with tobacco smoke (Control).

through sacran or HA-treated membrane filters had significantly reduced CYP1A1 mRNA expression levels (Figure 4). On the other hand, when comparing sacran and HA, sacran showed a more significant downregulation of CYP1A1 mRNA expression levels than HA (Figure 4).

AMELIORATION OF ADVERSE EFFECTS INDUCED BY TOBACCO SMOKE ON HaCaT KERATINOCYTES

Treatment of HaCaT keratinocytes with tobacco smoke showed high cytotoxicity, but sacran or HA significantly reduced the cytotoxicity of tobacco smoke. Compared with the

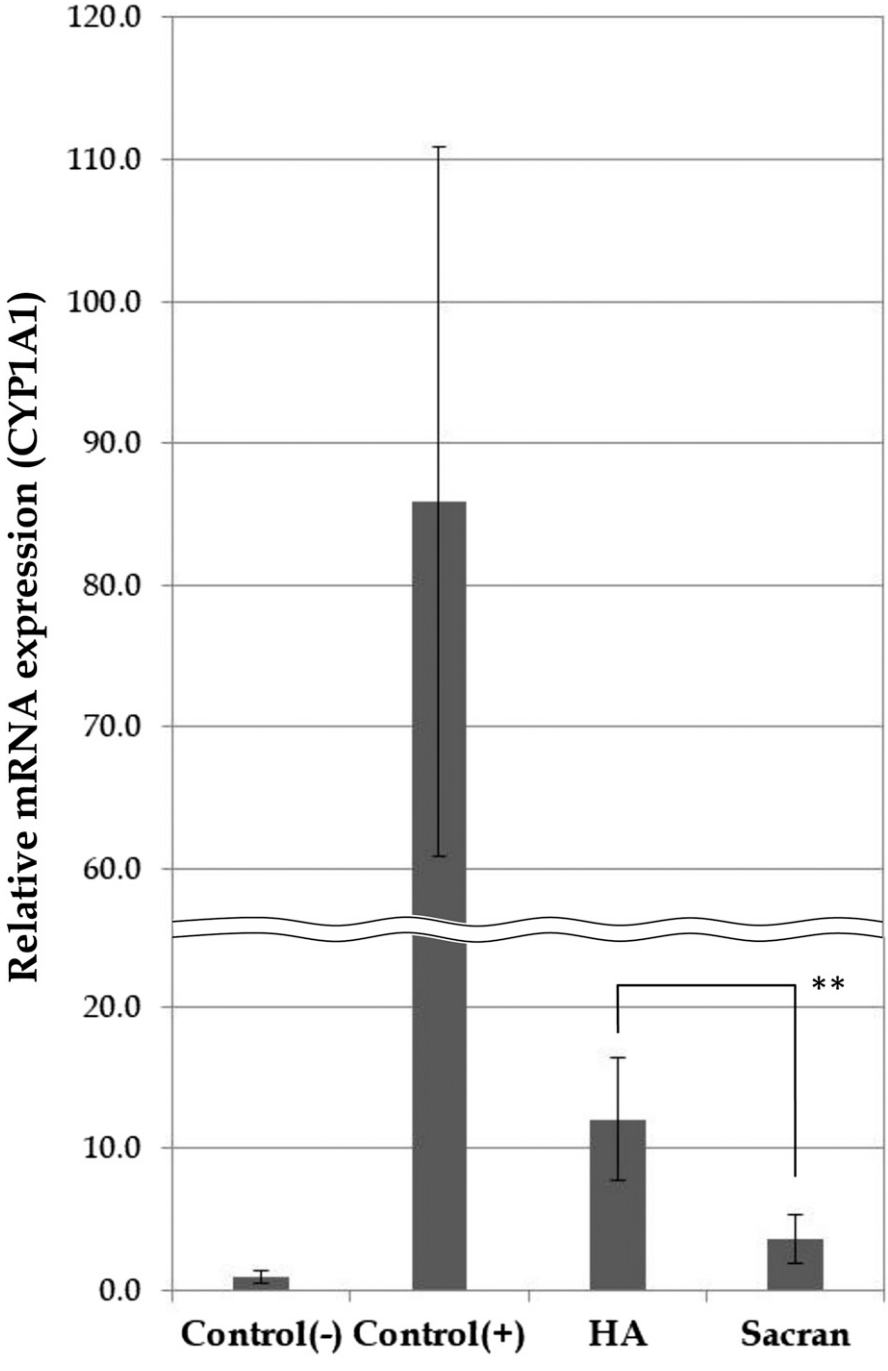


Figure 4. Suppressive effects of polysaccharides on CYP1A1 mRNA expression levels induced by tobacco. HaCaT keratinocytes were cultured in DMEM containing PBS diffused with tobacco smoke through filters treated with or without sacran or HA. After 24 h, total RNA was extracted and CYP1A1 mRNA levels were determined by real-time PCR. Each value represents the mean ± SD of four experiments. $^{**}p < 0.01$. Control(−) denotes sham-treated cells (PBS(−) nontreated with tobacco smoke) and Control(+) denotes cells treated with tobacco smoke diffused through a nontreated filter.

effects on the amelioration of cytotoxicity, sacran gave a significantly higher reduction in cytotoxicity than HA (Figure 5).

Regarding levels of intracellular oxidation, tobacco smoke strongly elevated intracellular ROS levels associated with intracellular CPs. Sacran significantly suppressed the elevation of intracellular ROS levels (Figure 6) and intracellular CPs (Figure 7). Although HA reduced levels of intracellular ROS, it failed to significantly suppress levels of intracellular CPs.

BARRIER FUNCTION OF POLYSACCHARIDES AGAINST TOBACCO SMOKE

In the *ex vivo* study, corneocytes exposed to tobacco smoke for 2 h had increased levels of CPs. Polysaccharide-treated corneocytes showed significantly lower levels of CPs after exposure to tobacco smoke than nontreated corneocytes. In addition, when exposed to tobacco smoke, sacran gave significantly lower CP levels in corneocytes than HA (Figure 8).

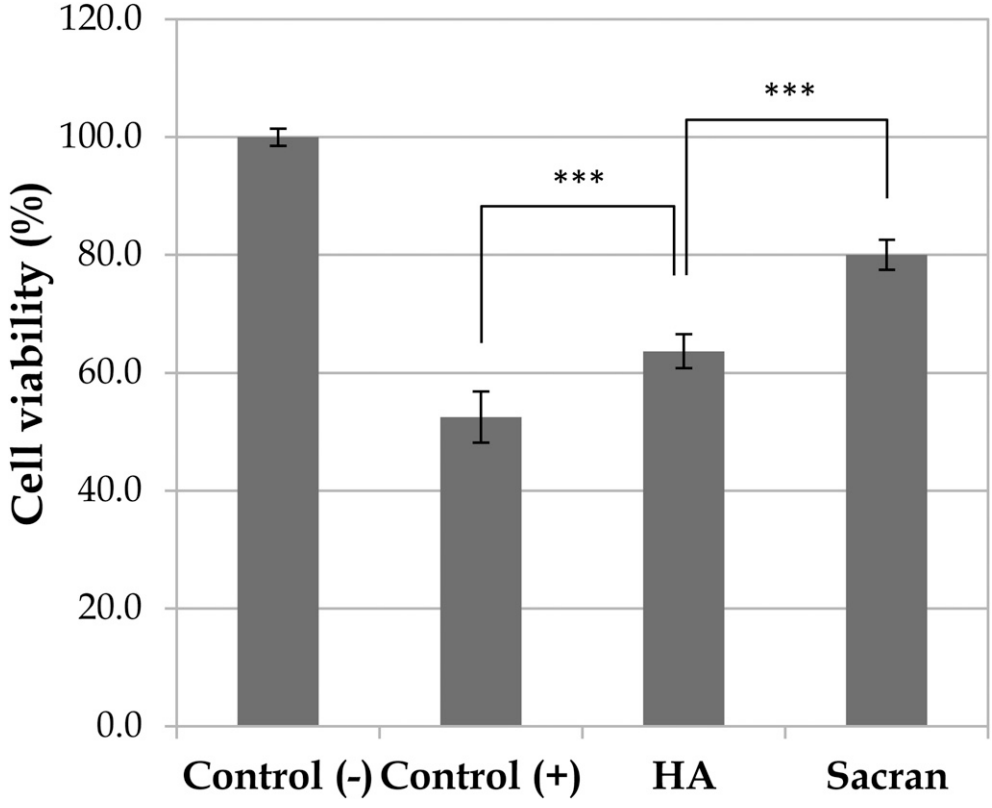


Figure 5. Amelioration of the cytotoxicity induced by tobacco smoke on HaCaT keratinocytes. HaCaT keratinocytes were cultured in DMEM containing PBS diffused with tobacco smoke through filters treated with or without sacran or HA for 24 h at 37°C. Cell viability was measured using the neutral red assay and is expressed as a percentage against sham-treated cells (Control(−)). Each value represents the mean ± SD of six experiments. Wilcoxon rank-sum test, ***$p < 0.001$.

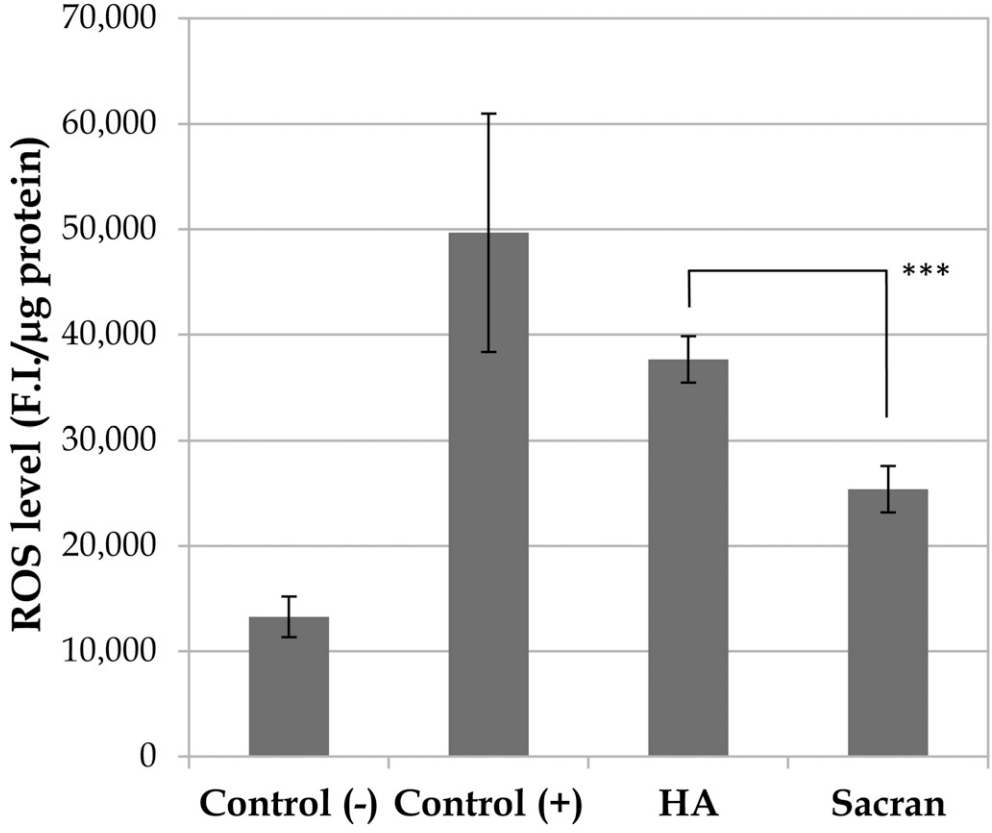


Figure 6. Amelioration of ROS generation induced by tobacco smoke in HaCaT keratinocytes. HaCaT keratinocytes were cultured in DMEM containing PBS diffused with tobacco smoke through filters treated with or without sacran or HA for 24 h at 37°C. Intracellular ROS levels were measured using H_2DCFDA. Each value represents the mean ± SD of six experiments. Wilcoxon rank-sum test, ***$p < 0.001$. Control(–) denotes sham-treated cells and Control(+) denotes cells treated with tobacco smoke diffused through a nontreated filter.

DISCUSSION

This study aimed to investigate the antipollution effects of sacran using tobacco smoke as a representative air pollutant. In a previous study, we identified the unique characteristics of sacran that result in the formation of a gel-like sheet. That gel-like sheet suppresses water evaporation and penetration by chemicals (23). The application of a sacran solution improves itching and facial rashes in patients with atopic dermatitis based on a questionnaire survey (24). In addition, sacran showed improvement of corneocyte maturation in healthy volunteers who had a history of atopic dermatitis (24). These effects have been considered to be based on the shielding effects of the skin against external stimuli due to the physicochemically unique properties of sacran. Thus, sacran is also expected to have protective effects against air pollutants. To identify the potential effects of sacran against air pollutants, we conducted various examinations focusing on oxidative stress using tobacco smoke as a representative air pollutant. Furthermore, to demonstrate whether sacran exhibits specific effects, we also examined the effects of HA, which is commonly formulated as an anionic polysaccharide in cosmetic products. In this study, to evaluate the effects of sacran, the effects of HA were compared as a representative polysaccharide.

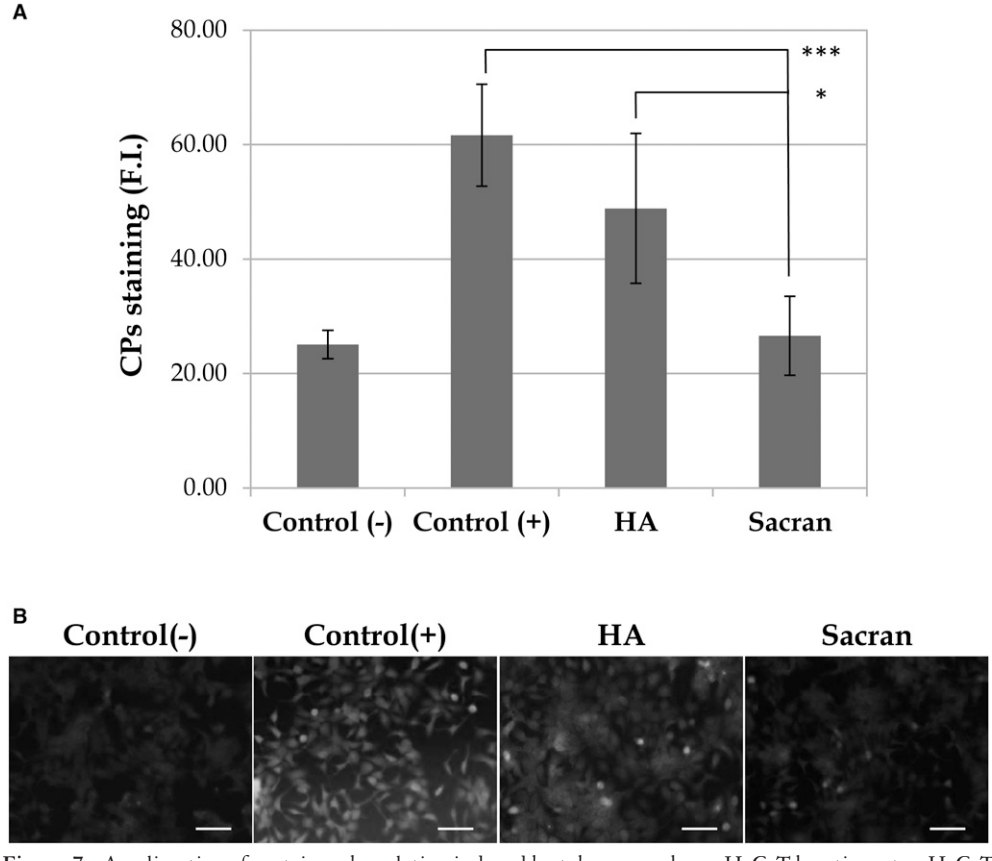


Figure 7. Amelioration of protein carbonylation induced by tobacco smoke on HaCaT keratinocytes. HaCaT keratinocytes were cultured in DMEM containing PBS diffused with tobacco smoke through filters treated with or without sacran or HA. After 24 h, intracellular CP levels were estimated by FI of FTSC labeling. (A) CP levels were quantified by image analysis, and each value represents the mean ± SD of six experiments. Wilcoxon rank-sum test, $*p < 0.05$, $***p < 0.001$. (B) Representative images of CPs in HaCaT keratinocytes after each treatment (scale bar, 100 μm). Control(−) denotes sham-treated cells and Control(+) denotes cells treated with tobacco smoke diffused through a nontreated filter.

First, to examine whether sacran functions as a shield, we monitored the localization of sacran topically applied on the surface of RHEEs from a histochemical viewpoint. More than 99% of sacran stayed in or on the stratum corneum of RHEEs, and HA also exhibited a similar behavior (Figure 2). Based on those results, it seemed likely that sacran and HA would function as an artificial barrier because they remained in or on the stratum corneum.

In the next examination, to investigate whether sacran remaining on the skin surface functions as a barrier against penetration from the chemical aspect, we determined the amount of ACs or BaP in PBS diffused with tobacco smoke through membrane filters treated with sacran or HA. Sacran or HA reduced levels of ACs or BaP in PBS, and the amounts of ACs and BaP in PBS were significantly lower after passing through sacran-treated membrane filters (Figure 3). Furthermore, although tape-stripped corneocytes treated with tobacco smoke had increased levels of CPs, topical treatment with sacran or HA reduced the level of CPs in corneocytes treated with tobacco smoke (Figure 8). Regarding that ability to reduce levels of CPs, sacran was significantly superior to HA. The sum of these results

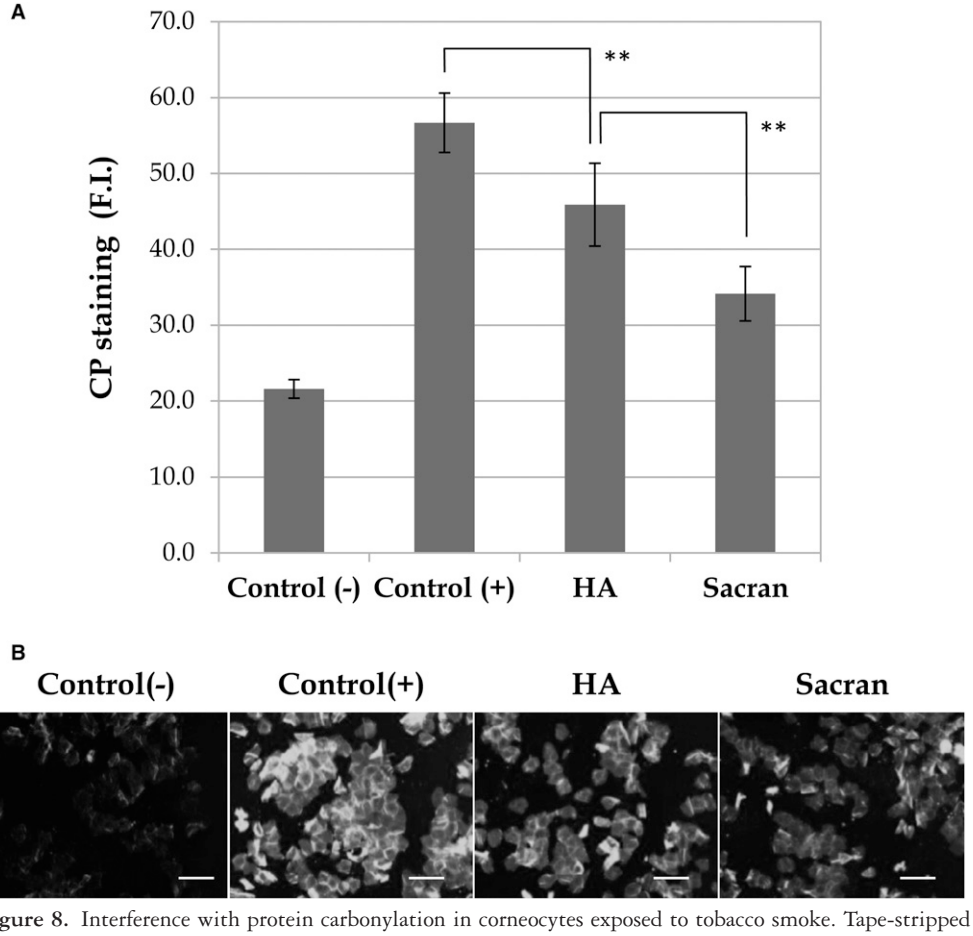


Figure 8. Interference with protein carbonylation in corneocytes exposed to tobacco smoke. Tape-stripped corneocytes were treated with sacran or HA, placed in a box filled with tobacco smoke for 2 h, and then were incubated for 24 h at 37°C. The levels of CPs in corneocytes was quantified by image analysis. (A) Changes in protein carbonylation in corneocytes. Each value represents the mean ± SD of three independent experiments using the same five volunteers. Wilcoxon rank-sum test, $^{**}p < 0.01$. (B) Representative images of CPs in corneocytes after each treatment (scale bar, 100 μm). Control(−) denotes sham-treated corneocytes and Control(+) denotes corneocytes treated with tobacco smoke without polysaccharide treatment.

demonstrates that sacran reduced the penetration of ACs and BaP in tobacco smoke and that the effect was higher than that of HA.

To investigate the effects of sacran from the biological viewpoint, we examined the responses of HaCaT keratinocytes to tobacco smoke. In general, it is known that BaP upregulates CYP1A1 through the activation of AhR signaling. CYP1A1 metabolizes PAHs by introducing a hydroxyl group to detoxify BaP. It has been reported that superoxide anion radicals are synthesized as a side product in the metabolic process (15–17). Thus, the effects of sacran as a barrier against tobacco smoke were evaluated regarding CYP1A1 mRNA expression levels in HaCaT keratinocytes. Sacran and HA suppressed mRNA expression levels of CYP1A1 stimulated by tobacco smoke, and, furthermore, the suppression by sacran was significantly higher than that by HA (Figure 4). In addition, sacran ameliorated the cytotoxicity and elevations of intracellular ROS and intracellular CPs

initiated by tobacco smoke (Figures 5–7). The effects of sacran also were higher than those of HA. The reduction in CP level was understood to originate from the improving effects of sacran on skin conditions from the aspect of oxidation because the long-term application of sacran reduced CP levels in corneocytes obtained from healthy volunteers (25).

Sacran is a unique cyanobacteria-derived sulfated polysaccharide that is composed of 11 kinds of monosaccharides, which contain a sulfate group in 11% of them and a carboxyl group in 22% of them. However, the detailed structure of sacran regarding its amino acid sequence and amounts of lesser components has not been identified. Considering the manner of the shielding effects indicates the possibility that polysaccharides trap BaP and/or ACs in their matrix. However, sacran and HA showed different intensities in their shielding effects, despite the difference in the presence or absence of sulfate groups in their chemical structures. Because carrageenan (a sulfated polysaccharide) did not show a shielding effect in a preliminary study (data not shown), it is hard to explain the difference in the shielding effects between sacran and HA against BaP or ACs according to the presence or absence of sulfate groups. In our previous study, we found that sacran and HA showed different emulsification abilities against squalene (data not shown). Sacran was able to emulsify squalene itself, but HA failed to emulsify squalene. Those results suggested the possibility of the presence of the hydrophobic domain to hold nonpolar chemicals in the matrix formed by sacran. It might be considered that the hydrophobic domain in the matrix of polysaccharides depends on the difference of molecular weights. However, the manner of the shielding effects of sacran is not beyond a region of hypothesis. Clarifying the mechanism involved in the shielding will require further study.

CONCLUSION

We conclude that sacran protects against adverse effects initiated by exposure to tobacco smoke, which is a representative air pollutant, because of shielding effects. These findings strongly support the possibility that skin care products formulated on sacran will protect the skin against air pollutants.

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