Factors Affecting Reduction in Preservative Efficacy in Nonwoven Fabrics

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Insufficient preservative efficacy leads to microbial contamination. Cosmetic-impregnated products composed of nonwoven fabrics, such as wipes and masks, can be contaminated with microbes owing to their special form. However, the reduction of preservative efficacy in cosmetic-impregnated products remains unverified. This study aimed to investigate whether preservative efficacy is reduced in nonwoven fabrics impregnated with a cosmetic liquid and the factors affecting this reduction. First, we evaluated the preservative efficacy of face wipes and confirmed that the preservative efficacy was reduced after impregnation of cosmetic liquids into nonwoven fabrics. We thus hypothesized that the adsorption of the antimicrobial components onto nonwoven fabrics decreases the preservative efficacy. Unexpectedly, the antimicrobial components were scarcely adsorbed onto the fabrics, while microbial growth activity was significantly increased on the fabrics, as determined through microbial calorimetry. Furthermore, the antibacterial effects were reduced in the nonwoven fabrics. These results indicate that the nonwoven fabrics enhanced microbial growth, thus decreasing the preservative efficacy. Our results provide novel insights into the microbial control of products composed of nonwoven fabrics.

Key words: Nonwoven Fabrics / Cosmetics / Preservative / Calorimeter.

INTRODUCTION

A proper preservative system is essential to prevent microbial growth in cosmetic products, thus potentially leading to a deterioration in the quality of the products or in disease pathogenesis. Similar to food products, cosmetic products are supplemented with antimicrobial agents to reduce the risk of microbial contamination upon repeated use. It is essential to determine the concentration of preservatives that is necessary to protect such products, without unnecessarily exposing the consumer to excessive preservative concentrations. There are concerns that cosmetic-impregnated products, such as face wipes and masks, increase the risk of microbial contamination owing to their special form, including nonwoven fabrics impregnated with a cosmetic liquid.

A preservative efficacy test is important to evaluate the preservative efficacy of cosmetic products. This test has been primarily designed for water-soluble or water-miscible cosmetic products, forcibly inoculating and mixing each test microorganism into the formulation, and then measuring the changes in the microbial count at predetermined intervals for a specific period and at a specific temperature. The protocol for the test is provided in the ISO 11930 (International Organization for Standardization, 2012), the Japanese Pharmacopoeia (2016), and so on. The preservative efficacy test for personal care products composed of nonwoven substrates should be conducted for products upon final packaging in accordance with the Cosmetic, Toiletry, and Fragrance Association technical guidelines (2007). However, packaged cosmetic-impregnated products include nonwoven fabrics and hence cannot be assessed in a manner similar to liquid products. Unfortunately, there is no detailed testing protocol for cosmetic-impregnated products.

It is well known that disinfectants including benzalkonium chloride or chlorhexidine gluconate cannot exert
sufficient bactericidal effects owing to their adsorption onto gauze or surgical cotton (Bloss et al., 2010; Kundsin and Walter, 1957; Yohkoh, 1986). Conversely, microbial growth reportedly increases upon attachment to materials with a broad surface area (Okada et al., 1998; Kobayashi et al., 1991). However, the mechanism underlying the reduction in the preservative efficacy in nonwoven fabrics remains unclear.

In this study, we investigated whether the preservative efficacy is reduced in nonwoven fabrics impregnated with a cosmetic liquid and the factors affecting this reduction.

**MATERIALS AND METHODS**

**Preservative efficacy test**

We conducted our studies using two types of evaluation methods, including a liquid method and a method for impregnated fabrics. For the liquid method, we referred to the general method as a preservative efficacy test for cosmetics described in ISO 11930 (2012) and the Japanese Pharmacopoeia (2016). Three types of model solutions with different antimicrobial components were used as the liquid sample (TABLE 1). This formulation was commonly used for cleansing wipes. The test microbes used herein were *Escherichia coli* NBRC3972, *Staphylococcus aureus* NBRC13276, *Pseudomonas aeruginosa* NBRC13275, *Candida albicans* NBRC1594, and *Aspergillus brasiliensis* NBRC9455. Furthermore, a mixture of an equal amount of *E. coli* and *S. aureus* was conventionally used as mixed bacteria, i.e., each precultured microbial suspension was inoculated into 20 g of the liquid sample of the cosmetic liquid (TABLE 1). The inoculated preparations were stored at 35 °C for bacteria and 25 °C for fungi, and the viable cell count was determined on days 1 and 7 for bacteria and yeast and on days 7, 14, and 21 for molds. Bacteria were cultured on Soybean-Casein Digest agar with Lecithin & Polysorbate 80 (SCDLP, Nihon Pharmaceutical Co., Ltd., Tokyo, Japan) at 35 °C for 48 h and fungi were cultured on Glucose Peptone agar with Lecithin & Polysorbate 80 (GPLP, Nihon Pharmaceutical Co., Ltd., Tokyo, Japan) at 25 °C for 72 h.

In the impregnated fabric method, nonwoven fabrics comprising 50:50 pulp:rayon, a type of nonwoven fabric material generally used for cleansing wipes, were used. The liquid samples (TABLE 1) impregnated the fabric at 400% of the fabric’s weight. As numerous cleansing wipes were packaged together, when nonwoven fabrics were piled up, it was difficult to uniformly add the microbial suspension. Therefore, we used only one nonwoven fabric after impregnation for the test, i.e., a single nonwoven fabric of 1.25 g impregnated with 5 g of the solution was placed in a sterilized vial and sealed. Each test microbial suspension was prepared for determining the viable cell count after inoculation. Each precultured test microbial suspension was inoculated on a nonwoven fabric impregnated to the same final concentration as that used in the liquid method in a drop-by-drop manner with a pipette, for uniform spreading. The viable cell count was determined by immersing the single nonwoven impregnated fabric in physiological saline, suspending it using a stomacher, and then measuring the number of bacteria in the suspension. In addition, it was confirmed in advance whether the number of bacteria inoculated on the nonwoven fabric could be collected. Herein, the incubation conditions for microbial cultures and for viable counting were the same as those for the liquid method. It was confirmed that there were no microbes in the nonwoven fabric before use by a microbiological test.

**TABLE 1** The model formulation of cosmetic liquid used for the preservative efficacy test and the use test.

| Purpose          | Ingredient name          | A   | B   | C   |
|------------------|--------------------------|-----|-----|-----|
| Preservative     | EtOH                     | 5.0 | 7.0 |     |
|                  | Methylparaben            | 0.1 | 0.3 |     |
|                  | Phenoxyethanol           | 0.3 | 0.3 | 0.3 |
| Surfactant       | PEG-12 Laurate           | 5.0 | 5.0 | 5.0 |
|                  | PEG-60 Hydrogenated castor oil | 0.5 | 0.5 | 0.5 |
| Solubilizing agent | Dipropylene glycol        | 5.0 | 5.0 | 5.0 |
|                  | Water                    | 89.2| 84.1| 81.9|
Microbiological analysis after use

We used 20 nonwoven fabrics comprising 50:50 pulp:rayon impregnated with each model formulation of the cosmetic liquid (TABLE 1) at 400% of the fabric’s weight and packaged. The sample was used for 2 weeks, and the subjects included 32 healthy women. Residual samples after the use test were collected and the viable cell count was determined in the same way as that followed for the impregnated fabric method in the preservative efficacy test. Bacteria were cultured on SCDLP at 35 °C for 48 h and fungi were cultured on GPLP at 25 °C for 72 h.

Quantification of the adsorption of the antimicrobial components

Herein, 0.1% of methylparaben (MP) or 0.3% of phenoxyethanol (PhE) was dissolved as a 10% ethanol (EtOH) solution, which was previously confirmed to exhibit no effect on adsorption (Kajimoto and Umeda, 1994). To assess the characteristics of the nonwoven fabric in detail, three types of fabrics, including 100% pulp, rayon, and cotton were used herein. These nonwoven fabrics were impregnated with the solution containing antimicrobial components at 400% of the fabric’s weight. After storing them for 1 day or 1 month, the nonwoven fabric was placed in a syringe and compressed to squeeze out the solution used for impregnation. The antimicrobial components in the solution were detected by high performance liquid chromatography using an octa decyl silyl column at a wavelength of 254 nm for MP and 270 nm for PhE. Adsorptivity was determined from the quantified concentration of antimicrobial components before and after impregnation (Kajimoto and Umeda, 1994).

Measurement of microbial growth (calorimetric analysis)

Microbial growth on the nonwoven fabrics was measured using a multiplex batch calorimeter (ADVANCE RIKO, Inc., Yokohama, Japan), as previously reported (Takahashi, 1996). Microbial calorimetry is a method for monitoring microbial growth by determining the metabolic heat generated due to microbial growth and recording the evolution of heat in time under the form of a voltage signal (Antoce et al., 1996b; Okada et al., 1998).

The test microbes used herein were the same as those used for the preservative efficacy test. The used nonwoven fabric was 100% cotton, since it has the lowest adsorption capacity as compared with rayon and pulp. Thereafter, the nonwoven fabric was impregnated with 5 mL of brain heart infusion broth (BHIF, Nihon Pharmaceutical Co., Ltd., Tokyo, Japan) or glucose peptone broth (GP, FUJIFILM Wako Pure Chemical Industries, Osaka, Japan) at 400% of the fabric’s weight. Each of the precultured test microbes was inoculated to a final concentration of $10^3$ CFU/mL; thereafter, the lid of the vial containing the sample was sealed, and the thermograms associated with microbial metabolism were analyzed (Takahashi, 1996; Antoce et al., 1999). During the measurements, the temperature was maintained at 35 °C for bacteria and 30 °C for fungi. Moreover, microbial growth was measured with 5 mL of broth media without nonwoven fabrics. To confirm the effect of the material of nonwoven fabrics, we measured the microbial growth activity on 100% pulp or rayon fabric against P. aeruginosa, which had the highest growth activity on cotton among the test microbes. Furthermore, the growth of P. aeruginosa on cotton fabric impregnated with BHIF broth at 200, 400, 600, or 800% of the fabric’s weight was measured to determine the effect of the impregnation rate.

Determination of the minimum inhibitory concentration (MIC)

We examined the antimicrobial efficacy by measuring the MIC via calorimetric analysis and its operation techniques (Antoce et al., 1997; Okada 1998). Cotton fabric was impregnated with BHIF broth, including antimicrobial components (MP or PhE) at concentrations of 0% to 0.8%, at 400% of the fabric’s weight; this fabric showed the lowest level of preservative adsorption among the three nonwoven fabrics of cotton, pulp, and rayon. The microbes used herein were E. coli, S. aureus, and P. aeruginosa.

Each microbe was inoculated at $10^3$ CFU/mL, and the thermograms were analyzed at the same conditions as those for measuring microbial growth activity. The recorded signal can be analyzed to determine the MIC according to the method described in previous reports (Antoce et al., 1996a; Okada et al., 1999). Similarly, the MIC without nonwoven fabrics was determined.

RESULTS AND DISCUSSION

Effect of the impregnated nonwoven fabrics on the preservative efficacy

A preservative efficacy test with or without fabrics was conducted to investigate the effect of the nonwoven fabrics on the preservative efficacy (Fig. 1). All formulations of cosmetic liquid (TABLE 1) decreased the viable count of microbes over time during culture in both methods, excluding the results of formulation A against fungi. Furthermore, with an increase in the amount of preservatives from formulation A to C, the microbial decline rate increased. The results differed under the two test conditions, i.e., the microbial survival rate determined by impregnating fabrics was higher than...
Fig. 1-1. Effect of the impregnated nonwoven fabrics on the preservative efficacy. Fig. 1-1 indicates the preservative efficacy against mixed bacteria (E. coli and S. aureus) (a, b, and c) and P. aeruginosa (d, e, and f) and Fig. 1-2 indicates that of C. albicans (g, h, and i) and A. brasiliensis (j, k, and l) evaluated by a liquid method (○) and a method for impregnated fabrics (●). (a), (d), (g) and (j): results for formula A. (b), (e), (h) and (k): results for formula B. (c), (f), (i) and (l): results for formula C. These data are expressed as the mean ±SD of three experiments.
that obtained via the liquid method. These differences were remarkable among bacteria, although the samples with too high or too low preservative efficacy could not be compared between the two evaluation methods. In addition, the standard solution of 0.1% MP or 0.3% PhE excluding the surfactants from the formulation B as well as the model solution in TABLE 1 showed higher microbial survival rate with impregnating fabrics than that seen with the liquid method (data not shown).

Next, the viable cell count after use was measured with using samples of the same formulations as those used in the preservative efficacy test. We calculated the percentage of samples in which each number of bacteria (the order of $10^0$, $10^1$, $10^2$, $10^3$, or $10^4$ CFU/mL) was detected among all samples collected after use (Fig. 2). The extent of microbial contamination tended to be high in the formulation with a small amount of preservative. Particularly in formulation A, 43.8% of the samples (14/32 samples) had a contamination of $10^4$ CFU/mL. However, in formulation C, the proportion of samples with viable cell count of $<10$ CFU/mL was 95% or more. In addition, the number of viable fungal cells was inconsequential compared to the number of viable bacterial cells in all the samples. Most of the detected microbes were identified to be *Staphylococcus epidermidis* (data not shown). Others, such as *Klebsiella oxytoca*, *Staphylococcus warneri*, *Pseudomonas fluorescens*, and *Bacillus subtilis* were also detected, but considerable individual differences were observed between subjects. These general bacterial species are widely distributed in human skin and the surrounding environment. Therefore, we propose that preservative efficacy in nonwoven fabrics can be sufficiently evaluated using microbial species that are defined as standard microbes for preservative efficacy testing of cosmetics.

Compared to the results of the preservative test (Fig. 1), microbial contamination after use occurred even in samples with sufficient antibacterial preservative potential in the liquid method, such as formulation A. These data indicate that fabric impregnation could help assess the risk of cross-contamination with high accuracy compared to that by the liquid method. Therefore, in the preservative efficacy test of the cosmetic-impregnated products composed of nonwoven fabrics, it is necessary to evaluate not only the solution, but also the condition in which the solution is used to impregnate the nonwoven fabric. Moreover, we confirmed that the nonwoven fabric, including 100% cotton or rayon also had higher viable cell counts detected by impregnating fabrics rather than the liquid method (data not shown).

These results suggest that preservative efficacy was reduced upon impregnation into nonwoven fabrics. To clarify the reason underlying this reduction, we assessed the adsorption of the antimicrobial components onto the nonwoven fabrics.

**Adsorption of the antimicrobial components onto the nonwoven fabrics**

We determined the adsorptivity of each of the antimicrobial components on the different types of nonwoven fabrics (100% pulp, rayon, and cotton). The adsorption of MP onto the nonwoven fabrics peaked at approximately 20% after storage for 1 day or 1 month. Moreover, the adsorption of MP on cotton was significantly lower than that on other nonwoven fabrics (One-way ANOVA and Tukey-Kramer test, $p<0.05$).
Furthermore, the adsorption of PhE on the three types of nonwoven fabrics after storage for 1 day or 1 month was less than 10% (Fig. 3(b)), which was significantly lower than that of MP (Student’s t-test, p<0.05).

Adsorptivity was markedly lower than that for benzalkonium chloride, where more than 80% was adsorbed against a cotton product as previously reported (Kajimoto and Umeda, 1994). Cationic bactericides, such as benzalkonium chloride, are considered to have high adsorptivity because an electrostatic attraction force occurs between the component and the fiber (Bloss et al., 2010; Kajimoto and Umeda, 1994). However, it is considered that MP and PhE, which are nonionic components and widely used in cosmetics, are less affected by the adsorption onto the nonwoven fabrics than ionic components. These results suggest that the adsorption of the antimicrobial agents in cosmetic-impregnated products is relatively lower than that in ionic substances. Furthermore, EtOH and polyhydric alcohol were confirmed to have no effect on MP adsorption to nonwoven fabrics at a concentration of 10% or less (data not shown). Thus, although we evaluated the preservative efficacy test using the model solutions (TABLE 1), we consider that these formulations will not be a factor in the promotion of the adsorption of antimicrobial agents to nonwoven fabrics. Thus, to determine the other contributing factors, we investigated the possibility of the activation of microbial growth on the nonwoven fabrics.

Growth activity on nonwoven fabrics
Microbial growth was investigated via calorimetric analysis with or without the cotton fabric. Microbial growth activity determined through culturing indicates that the levels of heat change per unit time reflect the specific growth rate. It was confirmed that there was no change in the growth activity on the nonwoven fabric before inoculation with microbes (data not shown). Microbial growth activity on cotton markedly increased with all test microbes in comparison with that without cotton (Fig. 4). Bacterial growth activity was greater than fungal growth activity, and in particular, the gram-negative bacteria P. aeruginosa and E. coli had higher growth activity on nonwoven fabrics than other test microbes. The flagella and cilia on the cell surface, which are structural characteristics of gram-negative bacteria, are involved in the adhesion of microbial cells to the support (O’Toole G, and Ghannoum M, 2004). In addition, it has been reported that flagella increase the contact area with the surface of the support and promote the binding between bacteria and the support (Ronn S. Friedlander et al., 2013). Therefore, the structural characteristics of these bacteria may influence the increase in the growth activity on the nonwoven fabric.

Moreover, as a result of testing of the different types of nonwoven fabrics, the growth activity of P. aeruginosa increased on all nonwoven fabric types, regardless of the fabric material (Fig. 5). However, microbial growth activity depended on the impregnation rate, which peaked at 400% of the fabric’s weight compared with lower (200%...
FIG. 4. Growth activity of the microbes measured with or without the nonwoven fabrics via calorimetric analysis. The gray line indicates the growth activity without fabric, and the black line indicates the growth activity with cotton fabrics. The impregnation rate was 400% of the fabric’s weight. The growth activity was tested using *E. coli* (a), *S. aureus* (b), *P. aeruginosa* (c), *C. albicans* (d), and *A. brasiliensis* (e). These data are expressed as the mean of three experiments.

FIG. 5. Growth activity of *P. aeruginosa* on the different types of nonwoven fabrics via calorimetric analysis. The growth activity was indicated with pulp (---), with cotton (--), with rayon (---) and without the nonwoven fabric (-----). The impregnation rate was 400% of the fabric’s weight. These data are expressed as the mean of three experiments.

FIG. 6. Growth activity of *P. aeruginosa* on cotton fabric impregnated with different impregnation rates via calorimetric analysis. The impregnation rate was 200(---), 400(--), 600(---), or 800(-----) of the fabric’s weight and without nonwoven fabric (-----). These data are expressed as the mean of three experiments.
of the fabric’s weight) and higher (600% of the fabric’s weight or more) impregnation rates (Fig. 6).

These findings raise the possibility that nonwoven fabrics with a larger surface area could allow for an increased microbial growth activity, owing to the increased contact area between microbes and air. Moreover, either a small amount of moisture or a large amount of moisture and a reduced amount of air affect the growth of microbes; therefore, there may be suitable ratio of the amount of moisture to air for microbial growth on the nonwoven fabric.

In particular, *P. aeruginosa* is an obligate aerobe, so its growth may be greatly affected by the amount of air available. Herein, microbial growth activity on agar, which also offers a broad surface, was equivalent to that in broth (data not shown), suggesting that an increase in microbial growth was activity specific for nonwoven fabrics.

Furthermore, we investigated the effect of nonwoven fabrics on the MICs of the preservative, through calorimetric measurements (Fig. 7). Nonwoven fabrics increased the MICs of MP and PhE against *P. aeruginosa*, especially among the test bacteria. Microbial species, such as *P. aeruginosa*, which potentially form biofilms (Matsuyama, 1999; Klausen et al., 2003), are considered to acquire resistance to antimicrobial material by adhering to and growing on a solid surface (Beveridge et al., 1997; Hoyle and Costerton, 1991; Mah and O’toole, 2001). Therefore, the MIC against *P. aeruginosa* may be increased in nonwoven fabrics. Precautionary measures should be taken against contamination by *P. aeruginosa*, a typical infectious bacterium causing keratitis (Willcox et al., 2008), especially when developing cosmetic-impregnated products intended for use around the eyes.

This study showed that the preservative efficacy is reduced when nonwoven fabrics are impregnated with cosmetic products, and this reduction largely stems from an increase in microbial growth on nonwoven fabrics. The present results provide novel insights into the microbial regulation of products used in nonwoven fabrics.

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