INTRODUCTION

Depigmentation of the skin has been reported to be induced by damage to melanocytes in exposed sites, which then cannot produce melanin pigment following chemical exposure of the skin. For example, skin depigmentation due to exposure to 4-(4-hydroxyphenyl)-2-butanol (HPB) was reported in humans. However, the role of HPB as the causative material of this skin depigmentation was not clear. To evaluate whether HPB has the potential for skin depigmentation, we characterized its effects on the skin of pigmented guinea pigs. Following exposure to 30% HPB 3 times/day for about 20 days, we found that obvious skin depigmentation was induced in brown and black guinea pigs. In the depigmented skin, there was a marked reduction in melanin pigment, and decreased numbers of DOPA and S-100 positive epidermal melanocytes were observed histologically. In addition, the depigmentation gradually recovered spontaneously and the number of melanocytes in the skin also increased after terminating the application of HPB. Complete re-pigmentation needed 31 to 70 days to return to the original baseline level. These data indicate that skin depigmentation is induced by the toxicity of HPB to epidermal melanocytes, and that the induced skin depigmentation can recover by terminating the application of HPB.

Key words: Depigmentation, 4-(4-hydroxyphenyl)-2-butanol, Melanin, Melanocyte, Guinea pigs, Skin

Depigmentation of the skin induced by 4-(4-hydroxyphenyl)-2-butanol is spontaneously re-pigmented in brown and black guinea pigs

Yasutaka Kuroda1, Yutaka Takahashi1, Hitoshi Sakaguchi1, Kayoko Matsunaga2 and Tamio Suzuki3

1Safety Science Research Laboratories, Kao Corporation, 2606 Akabane, Ichikai, Haga, Tochigi 321-3497, Japan
2Department of Dermatology, Fujita Health University School of Medicine, 1-98 Kutsukakecho, Toyoake, Aichi 470-1192, Japan
3Department of Dermatology, Yamagata University Faculty of Medicine, Yamagata 990-9585, Japan

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ABSTRACT — Chemically induced depigmentation of the skin, which occurs following exposure (application or inhalation) to a depigmenting agent, is a disease with clinical findings similar to vitiligo. Recently, skin depigmentation possibly resulting from exposure to 4-(4-hydroxyphenyl)-2-butanol (HPB) was reported in humans. However, the role of HPB as the causative material of this skin depigmentation was not clear. To evaluate whether HPB has the potential for skin depigmentation, we characterized its effects on the skin of pigmented guinea pigs. Following exposure to 30% HPB 3 times/day for about 20 days, we found that obvious skin depigmentation was induced in brown and black guinea pigs. In the depigmented skin, there was a marked reduction in melanin pigment, and decreased numbers of DOPA and S-100 positive epidermal melanocytes were observed histologically. In addition, the depigmentation gradually recovered spontaneously and the number of melanocytes in the skin also increased after terminating the application of HPB. Complete re-pigmentation needed 31 to 70 days to return to the original baseline level. These data indicate that skin depigmentation is induced by the toxicity of HPB to epidermal melanocytes, and that the induced skin depigmentation can recover by terminating the application of HPB.

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Correspondence: Yasutaka Kuroda (E-mail: kuroda.yasutaka@kao.co.jp)
tion caused by RK or monobenzyl ether of hydroquinone (MBEH) was also reported (Fukuda et al., 1998a; Zhu et al., 2013). However, since melanocytes are not distributed in the epidermis of normal mice except for the ears and tail, the endpoints of depigmentation were bleaching of the ears and tail (Zhu et al., 2013) or the melanin content in the hair (Fukuda et al., 1998a).

4-(4-hydroxyphenyl)-2-butanol (HPB) has been formulated in topical products used by subjects concerned about pigmented spots on their skin (e.g., chloasma and ephelides). A recent report (Nishigori et al., 2014) suggested an association of HPB with skin depigmentation. In this study, we evaluated whether HPB has the potential to depigment skin. To assess the depigmentation potential of HPB, we chose the pigmented guinea pig model described above. However, it was difficult to obtain black guinea pigs and thus, we also examined brown guinea pigs, which have a brighter skin color and fewer epidermal melanocytes than black guinea pigs.

In previous studies, depigmentation was reported to occur in about one month. In our study, we also evaluated the time for depigmentation when HPB was applied 3 times a day, which is an excessive experimental condition that results in a 15-fold higher exposure concentration than estimated use conditions.

**MATERIALS AND METHODS**

**Chemicals**

HPB was prepared by reducing RK with Raney Ni in EtOH (Carruthers, 1978). The purity was 100%, and the chemical structure is shown in Fig. 1. Ethanol (EtOH) as the vehicle was purchased from Wako Pure Chemical (Osaka, Japan).

**Animals**

Five female brown guinea pigs (kwl:A-1 strain, 7-weeks old), with brown hair and skin, were purchased from Tokyo Laboratory Animals Science (Tokyo, Japan). One black guinea pig (JY-4 strain, 1.5 years-old), with black hair and gray-black skin, was obtained from the Tokyo Metropolitan Institute of Public Health (Tokyo, Japan). All animals were housed with free access to standard food pellets and water. During the experiments, the animals were cared for in the experimental animal facility of the Kao Corporation. The Animal Care Committee of the Kao Corporation approved this study, and all experiments strictly followed the guidelines of that Committee.

**Experimental design**

The dorsal hairs of both strains of guinea pigs were cut with electric clippers and were shaved daily. Six dorsal areas (2 x 2 cm per area) on the back of each animal were used, as shown schematically in Fig. 2. All HPB solutions to be tested were prepared in 50% EtOH (ethanol:water = 1:1) daily. Twenty microliter aliquots of each test solution were applied 3 times per day to the appropriate area on the back of each animal.

In the brown guinea pigs, we created 6 treatment areas. Two treatment areas were exposed to 0.75 J/cm² UVB irradiation with an FL20SE lamp (Toshiba, Tokyo, Japan, wavelength spectrum 275-380 nm, peak 315 nm) 5 days before beginning the experiment, and then those 2 areas were treated with 30% HPB. Two other areas were only UV-treated. The final 2 areas remained intact with no treatment of any kind. One area from each pair of 2 areas was biopsied at the end of the study, and the other area was used to evaluate re-pigmentation (Fig. 2). To induce sufficient depigmentation, HPB was applied for 30, 40, 50, 60 and 97 days. To observe re-pigmentation, the HPB applications were terminated on days 31, 41, 51, 61 and 98. The re-pigmentation areas were then observed until the pigmentation returned to the original baseline level.

As for the study with the black guinea pig, a 30% HPB solution was applied continuously for 21 days to 2 areas. To observe re-pigmentation, the HPB application was stopped at day 22. One of the pair of 2 areas was biopsied at day 22 and the other was observed until the pigmentation returned to the baseline level.

**Skin color/visual grading**

Skin erythema and depigmentation were graded each day as negligible (−), slight (+), moderate (++) or marked (+++), according to a previous report (Tayama and Takahama, 2002). Briefly, skin color similar to the control areas was defined as negligible, otherwise it was defined as slight, moderate or marked in accordance with the difference in color relative to the control area.
Colorimetric measurements

A tristimulus colorimeter (Chromameter, CR-300, Minolta, Tokyo, Japan) was used to evaluate brightness changes of the skin. Color is expressed using the L*a*b* system (Robertson, 1977). In this study, the L* value (lightness) was used, and changes in this parameter are used as an indicator of skin depigmentation (Seitz and Whitmore, 1988). The L* value was measured in each application area (automatic averaging 3 times per point). The mean value of the application area in each animal was obtained from more than 3 animals.

Histological analysis

Skin samples were taken using a dermapunch (5 mm diameter, Maruho, Osaka, Japan) from isoflurane (Forene, Abbott Japan, Tokyo, Japan)-anesthetized guinea pigs. For split-dopa preparations, 3-(3,4-dihydroxyphenyl)-L-alanine (L-DOPA, Wako Pure Chemicals) was dissolved in phosphate buffered saline and split-tissue samples were prepared according to the method described in Staricco and Pinkus (1957). The number of whole dopa-positive melanocytes in each sample was counted using a light microscope (Biophoto or Optiphot-2, Nikon, Tokyo, Japan) and cell numbers were calculated per square mm.

For paraffin-embedded sections, the skin samples were fixed overnight in neutral-buffered 10% formalin (Kokusan Chemical, Tokyo, Japan) and were then embedded in paraffin (Fisher Scientific, Pittsburgh, PA, USA). Paraffin-embedded sections of vertical skin samples were prepared in two ways: one involved histopathological examination using Hematoxylin and Eosin (HE, Muto Pure Chemical, Tokyo, Japan) and Fontana–Masson (FM) staining as a marker for melanin granules; and the other involved the immunohistochemical examination of S-100 protein (polyclonal antibody, Code No. z0311; Dako Co., Glostrup, Denmark) as a marker for melanocytes in the epidermis. The primary antibody for S-100 was diluted at a ratio of 1:2,400 and was reacted for 50 min at room temperature after the specimen was treated with 3% H2O2 for 90 min at 55°C. Sections from both groups were stained with FM and S-100, and were counterstained using Kernechtrot solution (Merck, Darmstadt, Germany) and hematoxylin, respectively.

Statistics

Significance of differences was calculated by Student’s t-test (Microsoft Excel). A p-value of ≤ 0.01 is considered statistically significant.

RESULTS

Induction and recovery of depigmentation caused by HPB

To ascertain whether HPB has a depigmenting activity on melanocytes, a 30% solution of HPB was applied topically to brown and black guinea pigs continuously for up to a maximum of 97 days. A slight depigmentation (+) at the HPB-treated sites appeared on day 9 in 4 guinea pigs and was found in all 5 guinea pigs by day 10 (Table 1). With further treatment, the depigmentation gradually increased. The appearance of marked depigmentation (++) was observed between 17 to 21 days of treatment (Table 1). After the treatment of HPB was discontinued, the depigmentation disappeared over time. The re-pigmentation took 31 to 52 days from the day of HPB withdrawal to reach the same level as untreated skin (Table 1). Representative examples of baseline, depigmentation and re-pigmentation are shown in Fig. 3C through E. As for colorimetric measurements, the L* value (skin brightness) significantly increased at sites pre-treated with UV and then treated with HPB compared with the only UV-treated areas on days 14 and 21 (Fig. 3A). In addition, the L* values in HPB + UV-treated areas also increased compared to untreated skin on days 28 and 35. Continuous treatment with HPB sustained this augmentation (Fig. 3A). No skin erythema was induced in any of the animals (data not shown).
In the black guinea pig model, a slight depigmentation (±) of the sites treated with 30% HPB was first observed on day 5 and a marked depigmentation (++) was observed on day 19 (Table 1). It took 70 days for repigmentation to return to the same level as untreated skin after the treatment of HPB was discontinued (Table 1, Fig. 3F-H). As for the colorimetric measurements, the L* value increased up to day 21 post-withdrawal of HPB, after which this value decreased gradually to the baseline level (Table 1, Fig. 3B). Visual grading followed a pattern similar to the

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Table 1. Depigmentation by HPB application and repigmentation in guinea pigs

<table>
<thead>
<tr>
<th>Guinea pig skin color</th>
<th>No.</th>
<th>HPB Concentration</th>
<th>Application time / total application number (3 times per day)</th>
<th>Day of first appearance of slight depigmentation (±)</th>
<th>Day of first appearance of marked depigmentation (++)</th>
<th>Day to complete repigmentation post PHB withdrawal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Brown (N = 5)</td>
<td>1</td>
<td>30%</td>
<td>30 days/90</td>
<td>9</td>
<td>20</td>
<td>52</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>30%</td>
<td>40 days/120</td>
<td>9</td>
<td>17</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>3</td>
<td>30%</td>
<td>50 days/150</td>
<td>9</td>
<td>20</td>
<td>41</td>
</tr>
<tr>
<td></td>
<td>4</td>
<td>30%</td>
<td>60 days/180</td>
<td>10</td>
<td>21</td>
<td>31</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>30%</td>
<td>97 days/291</td>
<td>9</td>
<td>17</td>
<td>44</td>
</tr>
<tr>
<td>Black (N = 1)</td>
<td>1</td>
<td>30%</td>
<td>21 days/63</td>
<td>5</td>
<td>19</td>
<td>70</td>
</tr>
</tbody>
</table>

Fig. 3. Changes of skin brightness caused by HPB. (A) L* values of 3 dorsal areas in brown guinea pigs. Values are shown as means ± S.D. (Day 0-28, n = 5; Day 35, n = 4; Day 42-49, n = 3). (B) Time course of L* values for skin treated with 30% HPB and untreated skin of the black guinea pig. HPB was withdrawn on day 21. Representative photographs of dorsal skin from a brown (C-E) and a black (F-H) guinea pig. (C) UV-treated skin; (F) Untreated skin; (D, G) Depigmented skin; (E, H) Repigmented skin. ** p < 0.01 (HPB+UV-treated vs only UV-treated), † p < 0.01 (HPB+UV-treated vs untreated).
L* value, which indicated that the application of HPB induced the skin depigmentation and that withdrawal of HPB resulted in re-pigmentation.

Quantification of dopa-positive melanocytes in the epidermis

Table 2 shows the representative number of dopa-positive melanocytes. In brown guinea pigs, the number of dopa-positive melanocytes per square mm was 99 in the only UV-treated site on day 0. The numbers of dopa-positive melanocytes decreased to 14 and 0.66 in the only UV-treated, untreated and UV + HPB-treated depigmented skin on day 41, respectively. The number of dopa-positive melanocytes (0.66) in the UV + HPB-treated area was largely eliminated on day 41, although the number of dopa-positive melanocytes in the only UV-treated area was reduced by about 50%. Moreover, dopa-positive cells in the UV + HPB-treated area were clearly fewer than in the untreated site. The number of dopa-positive melanocytes per square mm was 31 in the UV + HPB-treated area on day 78 post-withdrawal of HPB, which was more than the untreated site. Taken together, these results suggest that the number of dopa-positive melanocytes was markedly decreased and then increased along with the depigmentation caused by HPB and the subsequent re-pigmentation.

In the black guinea pig, the number of dopa-positive melanocytes per square mm was 90 on the day before the beginning of the experiment and decreased to 2.2 after treatment with HPB for 22 days (Table 2). However, the number of dopa-positive melanocytes increased to 24 after 69 days post-withdrawal of HPB. Thus, dopa-positive melanocytes were markedly decreased and then increased with the application or the removal of HPB, respectively, just as occurred in the brown guinea pigs.

Localization of melanocytes and melanin

The immunohistochemical localization of melanocytes and melanin content in brown guinea pigs and the black guinea pig (vertical sections) are shown in Figs. 4 and 5, respectively. The number of S-100 positive melanocytes in the basal layer and the quantity of melanin granules in the epidermis decreased in the HPB-treated depigmented skin in comparison with the only UV-treated and untreated skin (Figs. 4A, B, D, E and Figs. 5A, B, D, E). Melanocytes and melanin granules were almost undetectable in brown guinea pig skin (Figs. 4B, E). On the other hand, when re-pigmentation was achieved, melanocytes in the basal layer and melanin granules in the epidermis were recovered (Figs. 4C, F and Figs. 5C, F).

Effect on keratinocytes

Topical application of HPB marginally induced epidermal thickening in brown and black guinea pigs (Figs. 4G-I and Figs. 5G-I). However, marked inflammatory mononuclear cell infiltration was not observed in the HPB-treated skin. HPB-treated skin and untreated skin had almost the same number of keratinocyte layers, but different sizes of keratinocytes. Epidermal thickening returned to normal
on day 78, when re-pigmentation was observed.

**DISCUSSION**

The present study shows that the frequent continuous topical application (3 times per day) of a high concentration (30%) of HPB to the backs of brown and black guinea pigs induces significant and patchy skin depigmentation. This was caused by the reduction of dopa-positive and S-100-positive epidermal melanocytes as well as a decrease in the amount of melanin granules. However, these epidermal changes spontaneously recover after withdrawing the application of HPB. The results in brown guinea pigs were similar to those in the black guinea pig.

These results are similar to previous reports where 1-5% 4-isopropylcatechol induced a potent skin depigmentation of the ear and dorsal skin of black guinea pigs (Bleehen *et al.*, 1968), or where topical application of 2% or 5% HQ to the skin induced a more potent depigmentation in black guinea pigs (Jimbow *et al.*, 1974) or where 5% PHQ induced more skin depigmentation on the backs of JY-4 black guinea pigs (Tayama and Takahama, 2002). For the chemicals reported in the literature, a concentration of 1-5% was reported to induce potent skin depigmentation by application once per day. Yet, for HPB, obvious skin depigmentation could not be achieved unless a 30% concentration was topically applied 3 times per day. On the other hand, a 10% concentration of HPB topically applied 1 time per day did not induce skin depigmentation compared to baseline skin levels (data not shown). Skin depigmentation caused by the toxicity of epidermal melanocytes is a common phenomenon among these
reports and our findings. Alkyl phenols, such as monobenzyl ether of hydroquinone (MBEH), monomethyl ether of hydroquinone, p-tertiary amyl phenol and p-tertiary-butyl catechol, have potent depigmenting capacities (Gellin et al., 1979). Common chemical features of those structures are the hydroxyl group that could bind at the 4 (or para) position and the non-polar side chains at position 1 of the aromatic ring (Bleehen et al., 1968). HPB (Fig. 1) has a similar feature among the alkyl phenols mentioned, however, HPB differs by not having non-polar side chains.

HPB is similar in chemical structure to RK, in which 3 cases of occupational leukoderma have been reported in chemical factory workers (Fukuda et al., 1998b). Two mechanisms have been suggested for the RK-induced depigmentation: toxicity to melanocytes and inhibition of melanogenesis (Fukuda et al., 1998c; Lin et al., 2011). Fukuda et al. (1998c) reported that the 50% growth inhibition concentration of B16 melanoma cells by RK was 0.13 mM, but that a 1 mM RK solution enhanced the tyrosine hydroxylase activity of B16 cells. On the other hand, Lin et al. (2011) reported that 0.6 mM RK did not show any cytotoxicity although it strongly inhibited melanogenesis in B16 cells. The mechanisms involved remain controversial.

Besides the changes elicited in melanocytes, an effect of HPB on keratinocytes was also observed. An epidermal thickening was observed in our study and was similarly reported with other depigmentation reagents (Gellin et al., 1979; Jimbow et al., 1974; Tayama and Takahama, 2002). Taken together, we suggest that the HPB-induced depigmentation occurs via selective melanocyte toxicity.

These chemicals also have a structural similarity to
tyrosine and may have a competitive inhibition effect with tyrosinase (Denton et al., 1952). Riley (1969a, 1969b, 1970, 1971 and 1975) suggested that these chemicals are incorporated into melanogenic cells and form semiquinone free radicals, which lead to the destruction of the lipoprotein membrane, and thus cause melanocyte death. In addition, HPB may be metabolized by tyrosinase. In fact MBEH can be metabolized to a quinone form and can generate cytotoxic reactive oxygen species (van den Boorn et al., 2011). Hariharan et al. (2010) showed that 4-tertiary butyl phenol induces apoptosis. In contrast, MBEH induces not the apoptotic but the necrotic pathway leading to melanocyte death. If HPB was to induce melanocyte necrosis, an inflammatory reaction would have been observed. In the present study, we could not detect a marked increase in inflammatory mononuclear cells even when a high concentration of 30% HPB was continuously applied. Thus, we hypothesize that HPB induces melanocyte apoptosis. However, further studies will be required to reveal the detailed mechanism(s) involved.

Our results demonstrate that epidermal melanocytes in the basal layer are selectively disrupted. We also found that the depigmented skin gradually re-pigments because dermal melanocytes in the basal layer re-emerge without any treatment. Speculation regarding the mechanism(s) underlying the re-emergence of epidermal melanocytes leads to 2 possible explanations. First, this phenomenon might be attributed to the migration and differentiation of melanocyte stem cells (McSCs). McSCs in the bulge or secondary hair germ can be a reservoir, not only for follicular melanocyte but also for cyclic hair pigmentation but also for epidermal re-pigmentation (Nishimura, 2011). The second possible explanation is that surrounding epidermal melanocytes might migrate to depigmented sites. The former mechanism is likely since the migration of McSCs from the bulge or secondary hair germ to the epidermis is enhanced by UV-B irradiation (Chou et al., 2013).

In conclusion, we demonstrate that HPB has a depigmenting activity via its selective toxicity to epidermal melanocytes not only in black guinea pigs but also in brown guinea pigs. We further show that this depigmentation is reversible.

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Re-pigmentation of depigmented skin caused by HPB in guinea pigs


