An Experimental Study on Depigmenting Activity of 4-(p-Hydroxyphenyl)-2-Butanone in C57 Black Mice

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Abstract: An Experimental Study on Depigmenting Activity of 4-(p-Hydroxyphenyl)-2-Butanone in C57 Black Mice: Depigmenting Activity of HPB in Black Mice—Yoshiharu Fukuda, et al. Department of Public Health, Kumamoto University School of Medicine—The authors previously reported three cases of occupational leukoderma in workers engaged in a 4-(p-hydroxyphenyl)-2-butanone (HPB, Raspberry Ketone) manufacturing process. These cases suggested that HPB might be a chemical causing the leukoderma and it had depigmenting activity. The purpose of this study is to evaluate the depigmenting activity of HPB by using laboratory animals. HPB, its two crude products and monomethyl ether of hydroquinone as the positive control were topically applied to the dorsal surface of C57 black mice. Depigmentation was shown in black mice to which HPB and its crude products were topically applied but the effect produced by these chemicals was weaker than that seen with monomethyl ether of hydroquinone. Though evidence of a reduction in melanocytes and pigmentation was not documented in the microscopic analysis, spectrophotometric assay showed a decrease in melanin content in the hair of mice to which HPB and the crude product had been applied. The results indicated a depigmenting activity of HPB and supported the conclusion that the leukoderma which we reported in a companion paper was induced by exposure to HPB and/or its crude products. Nevertheless, the potential of this depigmenting activity is so weak that the development of leukoderma due to these chemicals may be limited to those who are occupationally exposed.

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Key words: Occupational leukoderma, Depigmentation, 4-(p-hydroxyphenyl)-2-butanone, Raspberry Ketone, C57 black mice

In our companion report¹, we discussed three cases of leukoderma in workers engaged in the workshop of one chemical factory from 1989 to 1992. The workshop produced 4-(p-hydroxyphenyl)-2-butanolone (HPB), which is called Raspberry Ketone and used commercially as a flavoring agent. In our epidemiological study, there were twenty-four workers who were engaged in the HPB manufacturing and packing process, and thirteen workers who could have been exposed to the chemicals. Two cases of leukoderma were accompanied with dermatitis, but another case was not. Moreover, seven other workers had complained of some symptoms of dermatitis and all of them may have been exposed to HPB and its two crude products (Distilled Raspberry Ketone, DRK and Recovered Raspberry Ketone, RRK) which were produced during the HPB manufacturing process and were detailed in our companion report¹.

The facts suggested that these cases of leukoderma and dermatitis were induced by exposure to HPB, RRK and/or DRK. The chemical structure of HPB is shown in Fig. 1, and it is a compound similar to several chemicals that are known to have a depigmenting activity²–¹⁵ but our report is the first one on depigmentation associated with HPB, and its depigmenting activity has not yet been evaluated. Gellin et al.¹⁵ established a method for screening for the depigmenting activity of chemicals by using experimental animals. In this study, we evaluated the depigmenting activity of HPB and its crude products in C57 black mice according to their method¹⁵.

![Fig. 1. Chemical structure of 4-(p-hydroxyphenyl)-2-butanone (HPB).](image-url)
Materials and Methods

Experimental Animals: Male C57 black mice, 8 weeks old, weighing an average of 22.7 g at the beginning of each experiment, were purchased from Shizuoka Laboratory Animal Center (Shizuoka, Japan). The animals were maintained in specific pathogen-free conditions in a temperature controlled room at 23°C, with a 12 h light/dark cycle (7:00–19:00) at the Laboratory Animal Research Center of our university. The mice were allowed free access to water and commercial laboratory chow. Each group of 5 or 6 animals was housed separately in a stainless-steel cage and identified by topical painting the skin. All these animal treatment were carried out in accordance with the principle of laboratory animal care as well as the guidelines on animal experiments in Kumamoto University, Japanese government animal protection and management law and Japanese government notification on feeding and safekeeping of animals.

Chemicals: HPB, RRK and DRK were supplied by the factory where the leukoderma were occurred. The purity of the HPB was 99.98% and that of the DRK was 90.99%, and the DRK included ten chemicals according to gas chromatography-mass spectrometry (GC-MS) assay. Five chemical impurities were identified by their chemical structures, one of them being phenol, and their maximum concentration was 1.1% and that of phenol was 0.3%. Except phenol, the characteristics and toxicity of the impurities were not established.

As the positive control, monomethy ether of hydroquinone (MMH), which is found to have strong depigmenting activity, was commercially obtained from Wako Chemical Ind. (Tokyo, Japan).

Hydrophilic ointment or acetone was used as the solvent. Concentrations of each chemical tested ranged from 5% to 20% in the hydrophilic ointment, and those in acetone ranged from 0.25 M to 1.0 M.

Methods of topical application: The chemicals were applied topically to the back skin of mice once a day. 5 days a week for 16 weeks. Hydrophilic ointment or acetone alone was chosen as a negative control. Applications of MMH were ceased at the time when depigmentation was significantly developed.

Each test area received 0.1 ml aliquots of the test compounds applied by a syringe, and this was rubbed in with a rubber-gloved finger.

Methods of oral application: Ten animals were used in an oral application experiment in which 0.2 ml of 0.2 M HPB in olive oil was inserted into the animal’s stomach with a stainless magensonde for mice once a day, 2 days a week for 16 weeks. Olive oil alone was given to 5 animals as the control.

Criteria for visual observation: The criteria used for assessing depigmentation and irritation as visually observed are according to Gellin et al. The scoring of depigmentation is as follows: no visual depigmentation (-), small spots or speckles of depigmentation (+), uniform hypopigmentation (+) and complete depigmentation (2+). An assessment was conducted before application each day.

Microscopic analysis: Under general anesthesia, biopsy of the skin of treated sites in animals to which, 20% HPB, 20% RRK and hydrophilic ointment alone were applied were taken at 16 weeks after the application. The tissues were prepared for light microscopic examination and stained with hematoxylin and eosin (H&E), dopa-reaction and silver (Fontana stain).

Spectrophotometric assay of melanin content: The hair on the treated sites of animals to which 20% HPB, 20% RRK and hydrophilic ointment alone were applied were sampled at 16 weeks after the application. A 30 mg hair sample from each animal was homogenized in distilled water at a concentration of 10 mg/ml. Aliquots of samples (100 µl) were transferred to screw-capped test tubes and mixed with 900 µl of Soluen 350. The tubes were heated twice in a boiling water bath. The solution was transferred to micro test tubes and centrifuged at 10,000 rpm for 10 min. The supernatants were analyzed for absorbances at 500 nm (A<sub>500</sub>), the A<sub>500</sub> values are referred to as total melanin. The method was detailed by Ito et al. and Ozeki et al.

Results

Visual observations: The results of topical application experiments are summarized in Tables 1 and 2.

Depigmentation was noted in animals treated with 20% HPB, 5% and 20% RRK, 5% and 20% DRK, and 5%, 10% and 20% MMH in hydrophilic ointment. Depigmentation in animals treated with 20% HPB, 5% DRK and 5% RRK was weak. That with 20% DRK and 20% RRK was moderate, and that with MMH was very strong. When acetone was used for the solvent, depigmentation was not noted in animals treated with HPB, RRK and DRK but was noted in those treated with MMH.

In animals treated with 20% MMH, depigmentation was noted at 3 to 4 weeks after application. In those with 20% HPB, 20% DRK, and 20% RRK, depigmentation was noted 4 to 5 weeks after application. In those with 5% DRK and 5% RRK, depigmentation was noted very late. In animals treated with 20% HPB, repigmentation occurred and depigmentation slightly recovered. Depigmentation observed in animals treated with 20% MMH was very severe, including loss of hair, and application was ceased at 7 weeks.

Macroscopic findings for the 20% HPB-, 20% RRK-, and 10% MMH- treated animals and the controls are shown in Fig. 2.

In animals which were given HPB orally, no visual sign was observed. In addition, no irritations such as...
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Erythema, edema or scaling were observed in any animals topically or orally treated with chemicals.

**Microscopic findings:** In animals treated topically with 20% HPB and 20% RRK, no reduction in melanocytes and melanin of the hair follicles were observed. On the other hand, the hair follicles of animals treated with HPB and RRK developed noticeably and the dermal hair papilla protruded into the hair bulb in H & E stain (Fig. 3 a, b). Melanocytes in the hair matrix of animals treated with chemicals were distinctly increased in dopa-reaction and

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Concentration</th>
<th>Depigmentation</th>
<th>Case</th>
<th>Minimal</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPB</td>
<td>5%</td>
<td>-</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10%</td>
<td>-</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20% ±</td>
<td>5/5</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>DRK</td>
<td>5% ±</td>
<td>1/5</td>
<td>86</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20% +</td>
<td>5/5</td>
<td>26</td>
<td></td>
</tr>
<tr>
<td>RRK</td>
<td>5% ±</td>
<td>3/6</td>
<td>56</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20% +</td>
<td>5/5</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td>MMH</td>
<td>5% 2+</td>
<td>5/5</td>
<td>25</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10% 2+</td>
<td>5/5</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td></td>
<td>20% 2+</td>
<td>5/5</td>
<td>20</td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>-</td>
<td>0/5</td>
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**Table 1.** Results of topical application of test compounds with hydrophilic ointment

- HPB=4-(p-hydroxyphenyl)-2-butanone (Raspberry Ketone; RK),
- DRK=Distilled RK, RRK=Recovered RK, MMH=monomethyl ether of hydroquinone.
- Depigmentation: score of depigmentation; no visible depigmentation (-), small spots or speckles of depigmentation (±), uniform hypopigmentation (+) and complete depigmentation (2+).
- Case in which visual depigmentation was noted in/N.
- Minimal: minimal interval in days for depigmentation to be observed.

<table>
<thead>
<tr>
<th>Chemical</th>
<th>Concentration</th>
<th>Depigmentation</th>
<th>Case</th>
<th>Minimal</th>
</tr>
</thead>
<tbody>
<tr>
<td>HPB</td>
<td>0.25 M</td>
<td>-</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>0.5 M</td>
<td>-</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0 M</td>
<td>-</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td>DRK</td>
<td>0.25 M</td>
<td>-</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0 M</td>
<td>-</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td>RRK</td>
<td>0.25 M</td>
<td>-</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.0 M</td>
<td>-</td>
<td>0/5</td>
<td></td>
</tr>
<tr>
<td>MMH</td>
<td>0.25 M 2+</td>
<td>5/5</td>
<td>20</td>
<td></td>
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<tr>
<td></td>
<td>0.5 M 2+</td>
<td>5/5</td>
<td>32</td>
<td></td>
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<tr>
<td></td>
<td>1.0 M 2+</td>
<td>5/5</td>
<td>18</td>
<td></td>
</tr>
<tr>
<td>control</td>
<td>-</td>
<td>0/6</td>
<td></td>
<td></td>
</tr>
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</table>

**Table 2.** Results of topical application study of test compounds with acetone

- HPB=4-(p-hydroxyphenyl)-2-butanone (Raspberry Ketone; RK),
- DRK=Distilled RK, RRK=Recovered RK, MMH=monomethyl ether of hydroquinone.
- Depigmentation: score of depigmentation; no visible depigmentation (-), complete depigmentation (2+).
- Case in which visual depigmentation was noted in/N.
- Minimal: minimal interval in days for depigmentation to be observed.
silver stain (Fig. 3 c, d). This indicates that the follicular cycle was in the anagenetic stage.

Inflammatory findings such as infiltration of inflammatory cells, hyperkeratosis, acanthosis and extension of capillary vessels, were not found in any of the mice.

Spectrophotometric assay of melanin content: The results of the spectrophotometric assay for melanin content are shown in Table 3. The $A_{500}$ values for hair samples from mice treated topically with 20% HPB and 20% RRK were significantly (p<0.05) decreased as compared with the control.
Discussion

In 1939, Oliver reported occupational leukoderma in workers engaged in a leather manufacturing company. These cases of leukoderma were induced by mono-benzyl ether of hydroquinone (MBEH) contained in rubber gloves. Several hydroquinone compounds were shown to have depigmenting activity. In addition, several phenol and catechol compounds, para-tertiary butyl phenol (pTP), ortho phenyl phenol (oPP), para-octyl phenol (pOP), 4-tertiary butyl catechol (4BC) and others, were reported to have depigmenting activity. The chemical structure of 4-(p-hydroxyphenyl)-2-butanone (HPB) is similar to alkylphenols having depigmenting activity, though it has keto group in its molecule (Fig. 1).

In this study, we evaluated the depigmenting activity of HPB and its crude products (DRK and RRK). These results supported our suggestion that the occupational leukoderma we reported was induced by exposure to HPB and its crude products. But the potency of depigmentation of these chemicals is very weak as compared with that of MMH, which is known to have strong depigmenting activity. The depigmentation observed in animals treated with 20% HPB, 20% DRK or 20% RRK is weaker than that in animals treated with 5% MMH. This suggests that leukoderma due to HPB and its crude products might occur only after high dose exposure such as occupational exposure as well as other depigmentation agents.

A study with laboratory animals gave false positive results but these false positive results caused by the chemicals induced marked irritation. In this study, because HPB did not induce any irritation detected in visual observations or microscopic findings, we may reject the possibility of false positive results.

But the photopach test in one of two cases of leukoderma showed a positive reaction. In addition, nine of 13 workers who were exposed to the chemicals had complained of dermatitis. The facts suggested that HPB and its crude products have irritant potency or photosensitivity. Although this study did not show the irritant potency of the chemicals, exact studies may clarify these activities.

The reason why depigmentation was not noted in animals topically treated with HPB and its crude products in acetone whereas depigmentation did occur in animals topically treated with the same chemicals in hydrophilic ointment may be attributed to the difference in solvents. Gellin et al. reported there is a difference between solvents in the development of depigmentation, and that acetone was easy to apply but that it had given false negative results.

On the other hand, the depigmentation of HPB was weaker than that of DRK and RRK. The purity of DRK is 90.99% and it includes several impurities such as phenol (0.3%) and other unknown chemicals detected by GC-MS assay. Although RRK was not assayed, RRK was thought to include some chemicals according to the HPB manufacturing process. As phenol does not have a depigmenting activity but an irritant potency, it might cause the dermatitis or worsen the depigmentation. It is possible that there are other chemicals which have depigmenting activity in crude products and we must investigate the toxicity of these impurities.

An interesting observation is that the depigmentation induced by HPB gradually decreased while the application was continued. Microscopic analysis of biopsied skin of animals treated with HPB did not show a clear reduction in melanocytes and melanin but did show a hair cycle activation. This finding indicates that the hair cycle was activated in compensation for the depigmentation hazard. The peak of depigmentation was 4 to 5 weeks after treatment and depigmentation was reduced thereafter. Melanin production in the hairs was influenced by the hair cycle, so that if we want to exclude the influence of the hair cycle, we should shave or pull out the hairs on the treated site before application, or should use newborn mice.

This study does not explain the mechanism of the depigmenting effect of HPB. In earlier reports, two main causes of depigmentation by chemicals have been discussed. First, depigmenting agents have a cytotoxic effect on melanocytes. Some studies showed that most of these chemicals have a selective cytotoxic effect on melanocytes. Second, they may inhibit of enzymatic action on melanogenesis. Because most depigmenting agents are similar to tyrosine in chemical structure, it has

<table>
<thead>
<tr>
<th>Chemical</th>
<th>N</th>
<th>A_{500}</th>
<th>(mean±SD)</th>
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<tbody>
<tr>
<td>20% HPB with hydrophilic ointment</td>
<td>5</td>
<td>0.600±0.05*</td>
<td>(87.6%)</td>
</tr>
<tr>
<td>20% RRK with hydrophilic ointment</td>
<td>5</td>
<td>0.592±0.12*</td>
<td>(86.4%)</td>
</tr>
<tr>
<td>control (hydrophilic ointment)</td>
<td>5</td>
<td>0.965±0.03</td>
<td>(100.0%)</td>
</tr>
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*significantly (P<0.05) reduced compared with the control.
been suggested that they inhibit tyrosinase and/or other enzymes involved in melanogenesis. In fact, a few compounds were found to inhibit the synthesis of melanin without having melanocyte cytotoxicity\textsuperscript{19, 21, 22}. The mechanism by which depigmentation of HPB develops may be clarified by \textit{in vitro} studies such as a biochemical assay and cell culture studies of melanoma or melanocytes. To determine whether HPB and its impurities have specific cytotoxicity for melanocytes or an inhibitory action on melanogenesis, further investigations are required.

**Conclusion**

In this experimental study with C57 black mice, the depigmenting activity of 4-(\textit{p}-hydroxyphenyl)-2-butane (HPB) and its crude products was evaluated. It supported the conclusion that the leukoderma we observed in the workers who engaged in an RK manufacturing process was due to exposure to these chemicals but the potency of the depigmenting activity is weak compared with the established depigmenting agent; monomethyl ether of hydroquinone. We could not find irritation in mice to which those chemicals were simply applied. Neither was depigmentation noted in animals given HPB orally. These result suggests that leukoderma induced by these chemicals may be limited to occupational exposure, and that it will be possible to protect workers from their toxicity by the standard method of chemical use management.

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**References**