Intradermal Concentration of Hydroquinone after Application of Hydroquinone Ointments Is Higher than Its Cytotoxic Concentration

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Ointments of the skin depigmentation agent hydroquinone (HQ) have been prepared by extemporaneous nonsterile compounding in our hospital. The HQ ointments were highly effective in the treatment of various types of skin pigmentation; however, various problems have emerged including chromatic aberration of the ointments, a relatively large variability of efficacy, and mild topical side effects including irritation. In this paper, the cytotoxicity of HQ was assessed in vitro using rat skin fibroblasts as the concentration with 50% survival after 24 h exposure to be 16.5 mm. The intradermal concentrations at 2 h after application of the HQ ointments was also estimated to be 358 mM and 51.7 mM in stratum corneum and viable tissue (viable epidermis+dermis), respectively, by an in vitro rat skin permeation study with rat full-thickness abdominal skin and Franz-type diffusion cells.13)

Key words hydroquinone; ointment; cytotoxicity; intradermal concentration

MATERIALS AND METHODS

Chemicals HQ and L(+)-ascorbic acid (AsA) were purchased from Nacalai Tesque, Inc., Kyoto, Japan. Sodium sulfite anhydrous (Na2SO3) was from Wako Pure Chemical Industries, Ltd., Osaka, Japan. Glycerin (GP grade) was from Nippon Shinyaku, Co., Ltd., Kyoto, Japan. Hydrophilic ointment (JP grade) was from Merck-Hoei, Ltd., Osaka, Japan. All other chemicals were of the highest purity available.

Preparations of HQ Ointments Table 1 shows the formulation of the 5% or 10% HQ ointments for clinical use (Rp. 5100 or 10100, respectively).9,10) HQ (5 or 10 g, respectively), AsA (1.6 g), and Na2SO3 (0.5 g) were mixed in a mortar and then 10 ml of glycerin was added to make a suspension. The suspension was mixed with hydrophilic ointment to give 100 g of HQ ointment. An extemporaneous nonsterile compound of HQ ointment was packed as 10 g per patient. A 1% HQ ointment (Rp. 1100) was also prepared for hypersensitive patients. The in vitro rat skin permeation study was conducted using 5% HQ ointment (Rp. 5100).

Cell Culture of Rat Skin Fibroblasts Rat skin fibroblasts, FR (ATCC CRL 1213), were obtained from the American Type Culture Collection and maintained in a culture medium consisting of Dulbecco’s modified Eagle’s medium (D-MEM with glucose (4.5 g/l), L-glutamine (4 mM) and sodium pyruvate (1 mM); Cat. No. 12800-017, Invitrogen Corp., Carlsbad, CA, U.S.A.) supplemented with 50 U/ml of penicillin and 50 µg/ml of streptomycin.11,12) FR cells were seeded into culture dishes (1.5×106 cells/100 mm dish), grown in a humidified atmosphere of 5% CO2–95% air at 37°C, and subcultured every 5 days with 0.25% trypsin (Invitrogen Corp.), when the cells were grown almost to confluence (3×105 cells/100 mm dish). FR cells can be propagated for at least 30 passages.

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**In Vitro Assessment of Cytotoxicity in Rat Skin Fibroblast FR Cells**

Cytotoxic effects of HQ, AsA, and Na₂SO₃ were assessed in FR cells by the WST-1 (tetrazolium salts) colorimetric assay using a Cell Counting Kit (Dojindo Laboratories, Kumamoto, Japan). Cells (1000 cells/well) were seeded on 96-well plates (Nunclon™ flasks, Nalge Nunc International, NY) in 100 µl of culture medium on Day 0, and 24 h later, the culture medium was changed to that containing a test substance at various concentrations on Day 1. After incubation for 24 h at 37 °C (on Day 2), the culture medium was exchanged for 110 µl of that containing WST-1 reagent solution (10 µl of WST-1 solution and 100 µl of the culture medium), and 3 h later, the absorbance was determined at 450 nm with a reference wavelength of 630 nm using a microplate reader (Sjeia Auto Reader II, Sanko Junyaku Co. Ltd., Tokyo, Japan) according to the manufacturer’s directions. The 50% cytotoxic concentration (EC₅₀) values in FR cells were calculated according to the sigmoid effect model as follows using the nonlinear least-squares fitting method (WinNonlin®, ver. 2.1, Pharsight Corp., CA, U.S.A.):

\[
E = E_{\text{max}} \times \left[ 1 - C^n \right] \times \left[ 1 + EC_{50}^m \right],
\]

where \( E \) and \( E_{\text{max}} \) represent the concentration in the medium and the sigmoidicity factor, respectively, and \( C \) and \( m \) represent the concentration in the medium and the sigmoidicity factor, respectively.

**In Vitro Rat Skin Permeation Study for HQ Ointment**

Full-thickness abdominal skin excised from male Wistar rats (Japan SLC, Shizuoka, Japan), 250—280 g, was used for the experiments. A Franz-type diffusion cell with an effective diffusional area (0.95 cm²) and the initial concentration (mg/ml) are the volume of reservoir solution (14.47 ml), the thickness of the stratum corneum and viable tissue, and reservoir solution at 2 h after application of HQ ointment. Thickness was measured using a thickness gauge (TH-102 Film Thickness Gauge, Tester Sangyo Co., Ltd., Tokyo, Japan). After weighing, HQ was extracted by using methanol and the content was measured.

**Statistical Analysis**

All data presented are the mean±S.D. or S.E. The equivalence of variance was tested with the F-test. Statistical comparisons were made using the unpaired t-test. p-Values of less than 0.05 were considered significant.

**RESULTS AND DISCUSSION**

Cytotoxic effects of HQ, AsA, and Na₂SO₃ were assessed in FR cells by the WST-1 colorimetric assay to give EC₅₀ values of 16.5±3.2 µm, 660±198 µm and 2.60±0.60 mm, respectively (n=4; ±S.D.). The EC₅₀ value was 1.40±0.40 mm (n=4; ±S.D.), when sodium salt of AsA was used in the assay instead of AsA, and the cytotoxic effect of AsA was, in part, due to its acidifying effect. The EC₅₀ value for HQ was 56.0±37.2 µm (n=4; ±S.D.), when AsA and Na₂SO₃ were added at a weight ratio of 5:1.6:0.5, suggesting AsA and Na₂SO₃ had no or very little protective effect. Thus, it was speculated that the topical side effects were mainly due to HQ rather than AsA and Na₂SO₃.

**Table 1. HQ Ointments for Clinical Use**

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>HQ</td>
<td>5 g (10 g)</td>
</tr>
<tr>
<td>L(+)-Ascorbic acid</td>
<td>1.6 g</td>
</tr>
<tr>
<td>Sodium sulfite anhydrous</td>
<td>0.5 g</td>
</tr>
<tr>
<td>Glycerin</td>
<td>10 ml</td>
</tr>
<tr>
<td>Hydrophilic ointment</td>
<td>ad 100 g</td>
</tr>
</tbody>
</table>

1% HQ ointments were also prepared (Rp. 1100) for hypertensive patients.
products containing higher HQ concentrations. Herein, by been reported with inappropriate use of unregulated OTC formulations of HQ in FDA-regu-
lated products have been limited to a small number of cases.

<table>
<thead>
<tr>
<th></th>
<th>Amount (mg)</th>
<th>Volume (ml)</th>
<th>Concentration (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Donor</td>
<td>13.2±0.24</td>
<td>0.300</td>
<td>400</td>
</tr>
<tr>
<td>Stratum corneum</td>
<td>0.13±0.01</td>
<td>0.0033</td>
<td>358</td>
</tr>
<tr>
<td>Viable tissue b</td>
<td>0.17±0.01</td>
<td>0.0299</td>
<td>51.7</td>
</tr>
<tr>
<td>Reservoir solution</td>
<td>0.40±0.01</td>
<td>14.47</td>
<td>0.251</td>
</tr>
<tr>
<td>Total</td>
<td>13.9±0.27</td>
<td></td>
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</tr>
</tbody>
</table>

a) Each value represents the mean±S.E. of 3 experiments. b) Viable epidermis + dermis.

3.5×10⁻³ cm and 31.5×10⁻³ cm, respectively. Thus, their volume was estimated to be 3.3×10⁻³ cm³ and 29.9×10⁻³ cm³, respectively, and the HQ concentration in stratum corneum and viable tissue was estimated to be about 358 mm and 51.7 mm, respectively (Table 2). The concentration in the stratum corneum was similar to that in the ointment at 2 h after application, and the concentrations in the stratum corneum and viable tissue were more than 1000 times higher than the EC₅₀ value obtained in the rat skin fibroblast, FR cells, that is, 16.5 µm.

HQ is a high-volume commodity chemical used as a reducing agent, antioxidant, polymerization inhibitor and chemical intermediate. HQ is a natural ingredient in many plant-derived products, including vegetables, fruits, grains, coffee, tea, beer and wine, and believed to be safe for hu-
mans. In fact, there are few reports of adverse health effects associated with the production and use of HQ, and in our hospital, the major side effect was limited to mild irritation after topical application of HQ ointments. However, recent investigations have suggested that HQ might show the nephrotoxicity, mutagenicity or carcinogenicity under a cer-
tain condition. Adverse events associated with external exposure to HQ and permeation efficiency across the skin contrib-
ute the overestimation of cytotoxicity. Various types of cells are alive in the skin and the cross-talks among the cells might contribute the in vivo integrity of the skin and the flux of HQ across rat skin was almost constant under steady-state condition (Fig. 1), suggesting the evaluation only with fibroblasts was not enough. In addition, the HQ distribution would be heterogeneous in vivo. Finally, species differences in the cytotoxicity of HQ and permeation efficiency across the skin should be considered. Collectively, further experiments with various types of cells and under various experimental condi-
tions should be addressed to obtain the conclusions concerning the relationship between HQ and the topical side effects after application of HQ ointments.

HQ is susceptible to acid-base and oxidation–reduction matrixes forming various chemical structures including p-BQ, and p-BQ is thought to be potentially important for the action of HQ in the biological system. In previous reports, it has been shown that AsA and Na₂SO₃ were effective in suppressing the production of p-BQ from HQ. Here, it was also demonstrated that the cytotoxicity of HQ was not altered by antioxidants, AsA and Na₂SO₃.

In this study, the cytotoxicity of HQ was assessed in vitro using rat skin fibroblasts. The intradermal concentrations after application of the HQ ointments was also estimated by an in vitro rat skin permeation study with rat full-thickness abdominal skin and Franz-type diffusion cells. It was demon-
strated that the intradermal concentration of HQ was much higher than that eliciting cytotoxicity, suggesting that the topical side effects after application of HQ ointment were due to the cytotoxicity of HQ.

REFERENCES


September 2003