学 位 論 文 概 要

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学位申請者
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学位論文題目

In Vitro Methods for Predicting Leukoderma Caused by Quasi-Drug Cosmetics and Development of a New Skin Brightening Agent

学位論文の要旨

The field of cosmetology has recently attracted increasing attention across ages and genders. Therefore, many Japanese cosmetics companies constantly study new cosmetics materials. However, after five years on the market, brightening cosmetics containing 2% Rhododendrol (RD) were recalled for causing leukoderma. This suggests problems exist in the current materials evaluation method. Thus, this study attempted to (a) further clarify the mechanism of leukoderma caused by 4-substituted phenols, such as RD; to (b) develop a new *in vitro* evaluation method to replace the existing one; and to (c) find a new brightening material based on this method.

In Chapter II, the *in vitro* skin permeation rate and cytotoxic concentrations of brightening agents were studied using excised skin and cultured B16 melanoma cells. Pigment cell toxicity was observed by transmission electron microscopy (TEM). The levels of hydroxyl radicals (·OH) were measured, and the ·OH generation sites were determined in cultured B16 melanoma cells. Pigment cells cultured under conditions with high tyrosinase activity develop cytotoxicity when exposed to compounds known to cause leukoderma. It was found that only phenolic compounds, such as RD and raspberry ketone (RK), which cause leukoderma, generate high concentrations of ·OH.

In Chapter III, an *in vitro* skin permeation method was used to study the penetration of different formulations of RD-containing cosmetics, and an unknown substance was found after analyzing the skin metabolites of the RD-containing formulation by high-performance liquid chromatography (HPLC). Liquid chromatography-mass spectrometry (LC-MS) analysis revealed the compound to be RK, which has been reported to cause leukoderma, although its melanocyte cytotoxicity is weaker than that of RD (as shown in Chapter II). Further analysis suggests that alcohol dehydrogenase (ADH), an enzyme in the skin, may be involved in the oxidation of RD.

In Chapter IV, the new *in vitro* evaluation method developed in Chapter III was used to evaluate whether a new skin brightening agent induces leukoderma. Scutellarein inhibits melanogenesis without causing leukoderma. As a result, scutellarein may be a safe, active brightening agent.

In summary, the results of this study contribute to the elucidation of the mechanism of RD- or RK-induced leukoderma, and the proposed *in vitro* evaluation method can solve or reduce the safety issues associated with cosmetics.

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In Asian countries, “white” skin is considered attractive and has been sought after by women since ancient times. Therefore, many Japanese cosmetics companies continually study new brightening mechanisms and develop new materials. However, after being on the Japanese market for five years, brightening cosmetics containing 2% Rhododendrol (RD) were recalled in 2013 for causing leukoderma. This suggests that the existing evaluation method for cosmetics safety may be insufficient.

Therefore, this study focuses on the following three problems:
(1) whether the mechanism of RD-induced leukoderma has been clearly explained;
(2) what the issues of the existing quasi-drug cosmetics evaluation system are; and
(3) whether it is possible to design a new set of evaluation methods for quasi-drug cosmetics based on the principle of RD-induced leukoderma.

In previous studies, Ito et al. found that RD is easily oxidized to compounds, such as RD-quinone, by tyrosinase. The underlying mechanism of RD-induced leukoderma pathogenesis appears to be melanocyte tyrosinase catalysis to generate o-quinone from phenolic compounds, resulting in oxidative stress and cytotoxicity. In addition, some studies have attempted to employ *in vitro* skin permeation experiments to study situations in which consumers apply cosmetics daily, but they have not analyzed the metabolites from the perspective of safety. Some studies have attempted to explain the skin penetrability of RD using an infinite-volume test design, but this is a scientifically incorrect method.

Based on the above mechanistic assumption and previous studies, the objectives of the study were (a) to further clarify the mechanism of leukoderma caused by 4-substituted phenols, such as RD; (b) to develop a new *in vitro* evaluation method to replace the existing one; and (c) to find a new brightening agent based on this method.

In Chapter II, a new *in vitro* evaluation method is presented. First, skin permeation tests were carried out to determine the concentrations of each active ingredient to be added in the subsequent experiment. The concentrations of the test ingredients were determined from skin permeability coefficients calculated using Potts and Guy’s skin permeation coefficient prediction equation and Fick’s first law of diffusion.

The cytotoxicity of the active ingredients was then studied in cell-based experiments. Cytotoxicity was observed with RD, raspberry ketone (RK), and magnolignan (ML). High concentrations of RD, RK, or ML showed high cytotoxicity in B16 melanoma cells with high tyrosinase activity or with added tyrosinase. This means that leukoderma caused by RD is not due to inhibition of melanin biosynthesis, but it is instead due to the destruction of melanocytes by a toxic substance produced by tyrosinase activity on RD.

After studying the hydroxyl radical (·OH)- and hydrogen peroxide (H_2O_2)-generating activity of each active ingredient, it was found that 4-substituted phenol shows the strongest

·OH-generating activity, and RD and RK have stronger H_2O_2-generating activity than the other active ingredients. The mechanism of ·OH production appears to be a Fenton reaction occurring between the liberated H_2O_2 and Cu(I)−Cu(I) of tyrosinase, which has been confirmed by testing the effects of chelating agents on the generation of ·OH. Furthermore, RD and its oxidized form, RK, have stronger ·OH-generating activity, and RD is more potent than RK in the generation of ·OH, in the presence of tyrosinase.

Chapter II shows that skin depigmentation agents that produce high amounts of ·OH at the estimated skin concentrations are RD and ML, both of which cause leukoderma. Therefore, the new *in vitro* evaluation method developed here may be used to determine whether a given ingredient will cause leukoderma.

In Chapter III, the mechanism of RD-induced leukoderma was further investigated. First, RD-containing cosmetics were repeatedly applied on the skin of 7-week-old mice, mimicking the human usage of the product. Cosmetics, especially those aimed at brightening skin, are often applied at night, before sleeping; thus, the maximum duration for which they remain on the skin tends to exceed 8 hours. Therefore, a 24-hour skin permeation test using finite-volume conditions was implemented to simulate the metabolism of RD in the skin, and an unknown substance was found after analyzing the metabolites of the RD-containing formulation by high-performance liquid chromatography (HPLC). Characterization of the substance by liquid chromatography-mass spectrometry (LC-MS) showed that it was RK.

After analyzing various factors, it was found that the conversion of RD to RK in the skin permeation test may be caused by (a) ambient temperature, (b) cosmetic formulations, (c) skin, and (d) enzymes in the skin. Therefore, the skin permeation test was redesigned to determine the factors that cause RD oxidation. The results show that the enzymes on the skin and the formulation of emulsion cosmetics may be involved in the oxidation of RD to RK.

Alcohol dehydrogenase (ADH), an enzyme commonly found in organisms, has been reported to oxidize RD to RK. In this chapter, ADH was found to be able to oxidize RD to RK at a level of 0.98 units/mL or higher. In addition, the metabolism of RD to RK in human skin homogenate was demonstrated. Addition of NAD^+, the coenzyme of ADH, causes more RD to be metabolized to RK in the skin homogenate. Although RK has been reported to induce leukoderma, the experiments in Chapter II suggest that the melanocyte cytotoxicity of RK is less than that of RD. Thus, the metabolism of RD may reduce its risk of inducing leukoderma.

In Chapter IV, the new *in vitro* evaluation method developed in Chapter III was used to evaluate whether new skin brightening agents would induce leukoderma. Scutellarein and baicalein, which are classified as drugs for clinical treatment because of their high anticancer activity, were tested for brightening activity and cytotoxicity. In this chapter, the skin brightening ability of the active substance was first confirmed based on melanin content, cellular viability, tyrosinase activity, and mushroom tyrosinase activity assays. The results show that scutellarein has skin-brightening activity, while baicalein does not. Western blot and reverse transcription-quantitative polymerase chain reaction (RT-qPCR) assays were used to study the mechanism of melanin inhibition by scutellarein. These experiments show that scutellarein affects melanin synthesis by inhibiting the expression of tyrosinase. Finally, the risk of leukoderma induced by scutellarein was studied by measuring the amount of ·OH generated in the presence of tyrosinase. Scutellarein showed no ·OH-generating activity compared to RD. These results indicate that scutellarein may be a safe brightening agent.

In summary, the results of this study contribute to the elucidation of the mechanism of leukoderma induced by RD and other 4-substituted phenols, and the *in vitro* evaluation method reduces the issues associated with cosmetics safety testing.

Summary

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