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Letter to the Editor

4-(4-Hydroroxyphenyl)-2-butanol (rhododendrol) activates the autophagy-lysosome pathway in melanocytes: Insights into the mechanisms of rhododendrol-induced leukoderma

Keywords:
Autophagy; Rhododendrol; Leukoderma

Rhododendrol [4-(4-hydroxyphenyl)-2-butanol, Rhododenol®] was first developed 5 years ago as a skin-whitening cosmetic that contained a 2% (w/w) formulation. It is also a natural ingredient present in many plants, such as the Nikko maple tree [1]. It competes with tyrosine for hydroxylation by tyrosinase and effectively inhibits melanin biosynthesis [2,3]. However, these cosmetics were withdrawn from the market in 2013 after rhododendrol reportedly caused a depigmentation disorder. It was reported that partial depigmentation appeared on the neck, the hands, and the face at sites of repeated application of these cosmetics, and the symptoms in 79% of affected patients disappear or begin to improve within 6 months after ceasing product use [The Japanese Dermatological Association Special Committee on the Safety of Cosmetics Containing Rhododendrol, 2014 (in Japanese)]. A previous report suggested that the melanocyte toxicity of rhododendrol is caused by its tyrosinase-catalyzed oxidation and production of cytotoxic reactive oxygen species (ROS) [4]. Another previous study reported that no ROS were detected in rhododendrol-treated melanocytes, but a tyrosinase-dependent accumulation of endoplasmic reticulum (ER) stress and/or activation of the apoptotic pathway appeared to contribute to cytotoxicity [5]. However, contradictorily, it was also recently reported that one melanocyte line with higher tyrosinase activity is resistant to rhododendrol cytotoxicity [6]. Furthermore, depigmentation symptoms have only been confirmed in ≈16,000 (2%) of 800,000 estimated users of cosmetic products containing rhododendrol. Interestingly, not all consumers and not all treated skin areas developed white blotching. It is still unclear why only some people suffer from these skin problems and why a spectrum of symptoms occurs. It appears likely that rhododendrol causes irritation and depigmentation disorders only under certain conditions, suggesting that its mechanisms might be more complicated than previously reported.

In this report, normal human epidermal melanocytes from moderately pigmented neonatal foreskin (HEMn-MP) cells were incubated with rhododendrol (300–3000 μM). Rhododendrol treatment at 300–900 μM did not affect melanocyte viability. However, the number of viable melanocytes strikingly decreased in response to rhododendrol treatment at 1500 and 3000 μM (Fig. 1A). These results demonstrated concentration-dependent rhododendrol-induced cytotoxicity in melanocytes. After rhododendrol treatment, cultured melanocytes were washed with cold PBS buffer, adherent cells were lysed for western blotting analysis (Fig. S1A) or were fixed for immunofluorescence staining (Fig. S1B). Treatment with higher concentrations (1500–3000 μM) of rhododendrol induced caspase-3 and poly (ADP-ribose) polymerase (PARP) cleavages in melanocytes (Fig. S1A). The cleaved caspase-3 positive melanocytes were also observed after treatment with high concentration of rhododendrol (Fig. S1B). All of these results together suggest that rhododendrol induces apoptotic cell death in HEMn-MPs.

Cultured HEMn-MPs were treated with non-cytotoxic rhododendrol concentrations (300–900 μM) for 120 h. Rhododendrol-treated cells were markedly less pigmented than untreated control melanocytes (Fig. 1B(a–c)). By western blotting, identical treatment with rhododendrol markedly down-regulated expression of the melanogenic factors tyrosinase, Pmel17 and Melan-A, while slightly increasing MITF expression (Fig. 1B(d)). By western blotting, expression of the autophagosome marker LC3II and lysosome marker LAMP1 were both markedly up-regulated in melanocytes by rhododendrol treatment at non-toxic concentrations (Fig. 1C). Furthermore, by immunofluorescence staining, increased LAMP1 and LC3II expression and decreased Pmel17 expression was also observed in 900 μM rhododendrol-treated melanocytes (Fig. 1D). Together, these results demonstrate that exposure of melanocytes to rhododendrol inhibits melanin production and also enhances the autophagy-lysosome pathway.

By electron microscopy examinations, the number of stage IV melanosomes in the cells' cytoplasm markedly decreased, lysosome numbers also increased dramatically after rhododendrol treatment, and double-membrane enclosed autophagic vacuoles were observed to contain materials resembling stage II–III melanosomes (Fig. 2A). These results suggest that rhododendrol inhibits melanogenesis and induces subsequent autophagy-lysosome activation and autophagic melanosome degradation. To investigate the exact role of autophagy-lysosome activation in rhododendrol-treated melanocytes, we examined the effect of rhododendrol on autophagy-deficient melanocytes and autophagy-enhanced melanocytes. Cultured human melanocytes were pretreated with bafilomycin A1 or rapamycin for 3 h to inhibit or induce autophagy, respectively. Autophagy deficiency (bafilomycin A1 pretreated) significantly exacerbated rhododendrol cytotoxicity in cultured melanocytes, and this cytotoxicity was clearly abolished in melanocytes that were pretreated with rapamycin (Fig. 2B). These results suggest that autophagy induction significantly attenuates rhododendrol-induced melanocyte death. Protein expression levels of p62 and BiP were observed strikingly up-regulated by

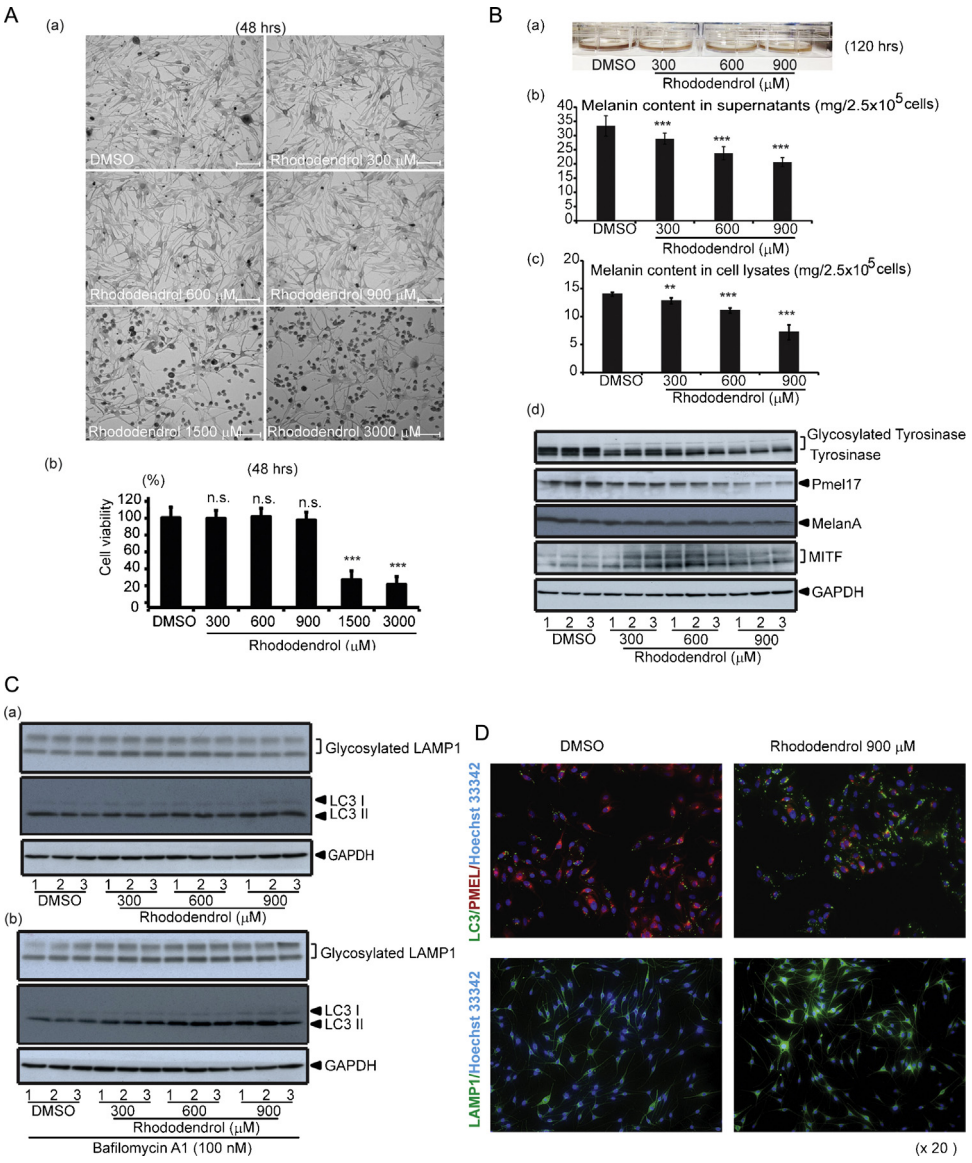

Fig. 1. Cultured human epidermal melanocytes were observed under phase contrast microscopy (A (a)), assessed by MTT assay (A (b)), cultured cells with medium were photographed (B (a)), melanin content both in culture medium (B (b)) and cell lysates (B (c)) were quantified with a Melanin Content Assay. Three days after rhododendrol treatment at the indicated concentrations, protein expressions in cell lysates were analyzed by western blotting ((B (d), C (a, b)). Melanocytes exposed to 900 μM rhododendrol were stained with the indicated antibodies and observed by confocal fluorescence microscopy (D).

rhododendrol treatment (Fig. S2). The p62 protein is known to mediate degradation of ubiquitinated proteins via the autophagosome-lysosome pathway [7], and BiP, a member of the HSP70 family found in the ER, is a marker of ER stress [8]. These results suggest that rhododendrol induced ubiquitin accumulation and ER stress in melanocytes. These findings also suggest that rhododendrol-induced ER stress might be one cause of reduced cell viability in rhododendrol-treated melanocytes.

Our results indicate that the mechanisms of rhododendrol cytotoxicity in melanocytes is more complicated than its merely acting as a tyrosinase analog that competitively inhibits normal tyrosinase activity, the rate-limiting enzyme for melanogenesis. A possible tyrosinase-mediated mechanism is that rhododendrol reaction with tyrosinase generates reactive semi-quinone free radicals that induce cellular stress and cytotoxicity [9,10], and subsequently activates the autophagy-lysosomal pathway. Simultaneously, rhododendrol inhibits cellular melanogenesis by binding tyrosinase and by inducing autophagic melanosome degradation. However, cytotoxicity was only induced at higher rhododendrol concentrations or in autophagy-defective cells, as the autophagy-lysosome system becomes overloaded and cellular stress results from rhododendrol-originating quinones (Fig. 2C). This mechanism appeared to be specific as no cytotoxicity and no activation of autophagy-lysosome pathway were observed in cultured human primary keratinocytes or fibroblasts (data not shown). These data suggest that an impaired or dysfunctional autophagy-lysosome pathway in patients' melanocytes might be one explanation of why all consumers and all treated skin areas do not develop white blotching. Our findings suggest a possible role of autophagy-lysosome pathway in rhododendrol-induced depigmentation disorder, and individual variation in autophagic function may determine rhododendrol-application outcomes. These results expand our

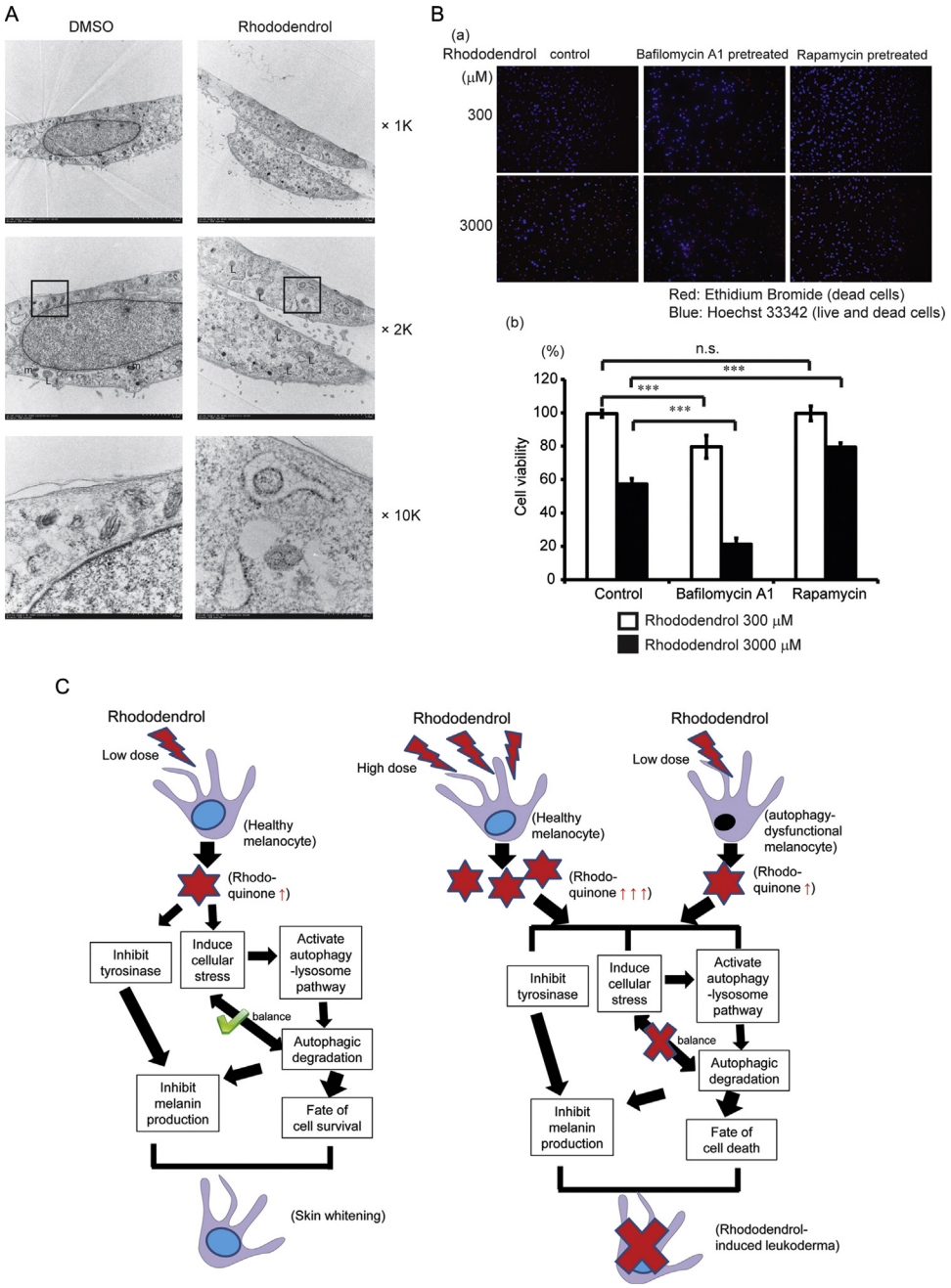

Fig. 2. Cultured human epidermal melanocytes exposed to 900 μM rhododendrol or DMSO for 72 h followed by bafilomycin A1 treatment for an additional 3 h, and then observed by electron microscopy (A). m: melanosome; L: lysosome. Cultured human epidermal melanocytes were pretreated with 100 nM bafilomycin A1 or 100 nM rapamycin for 3 h, followed by rhododendrol treatment for 72 h at the indicated concentrations. Cell staining with ethidium bromide (red) and Hoechst 33342 (blue) was observed using confocal fluorescence microscope (B (a)). After these treatments, cell viability was also evaluated by MTT assay (B). Schematic illustration of the possible mechanisms of rhododendrol-induced leukoderma (C).

understanding of the mechanisms underlying the rhododendrol-induced leukoderma.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jdermsci.2015.01.006.

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Letter to the Editor

An immune pathological and ultrastructural skin analysis for rhododenol-induced leukoderma patients

Keywords:
Rhododenol-induced leukoderma; Histopathological and ultrastructural analyses of the skin; Immune-competent cells

A B S T R A C T

As reported in the mass media on July 2013, numerous consumers who had used the cosmetic ingredient containing rhododendrol (4-(4-hydroxyphenyl)-2-butanol, Trade name; rhododenol), which is a melanin inhibitor isolated from Acer nikoense Maxim, released from Kanebo Cosmetics Inc. (Tokyo, Japan) noticed leukoderma patches on their face, neck and hands. We have experienced 32 cases that developed leukoderma after using such cosmetics so far and skin biopsy samples in some cases were obtained from both leukoderma and pigmented lesions. A histopathological analysis for skin lesions obtained from such patients notably showed basal hypo-pigmentation, melanin incontinence, and remaining melanocytes in most patients which is not relevant in vitiligo vulgaris. Subsequently, we comprehensively carried out immunohistochemical analyses of immune-competent cells infiltration to assess the effect of the cellular immune response to inducible hypopigmentation. Furthermore, detailed morphological observations performed by electron-microscopy notably showed the presence of melanocytes with only a small number of melanosomes, dermal fibroblasts containing melanosome globules and melanophages whereas no damage associated with melanosome transfer and the basal layer apparatus. These findings provide a cue to diagnose as rhododenol-induced leukoderma differentiate from vitiligo vulgaris and for rhododendrol to induce local immunity in addition to melanocyte damage.

Rhododenol-induced leukoderma was found to have occurred in approximately 2% of the consumers who had used the cosmetics containing rhododendrol and the total number of such patients is estimated to be more than 9000 individuals. Since the products in question produced by the Kanebo corporation had been sold on the Asian market in not only Japan, but also in Korea and Taiwan, the associated health hazard is thus considered to be a serious and widespread problem. Recent research has unveiled the biochemical and physiological mechanism of melanocyte cell damage induced by rhododenol-metabolites after tyrosinase reaction [1,2]. Soon after rhododendrol was oxidated by endogeneous tyrosinase in cytoplasm, the metabolite form could thus become toxic to melanocytes due to endoplasmic reticulum and oxidative stress production [2] in addition to NO production [3], however, it is not yet investigated whether lesional immune reaction could affect the pathogeny of rhdodenol-induced leukoderma. Previously, we analyzed histopathological alteration and the infiltration of immune-competent cells in vitiligo skin followed by an investigation of the local immune milieu on melanocyte dysfunction and disappearance in the occurrence and maintenance of vitiligo vulgaris [4–7]. In this communication, we obtained hypopigmented skin specimens from patients and carried out an immune-pathological analysis of 32 lesions and an ultrastructural analysis of 6 lesions to assess morphological change of skin component cells and local cellular immune reaction. Comprehensive infiltrating cells number was summarized in Table 1. A small number of melanosomes were found remaining in the basal layer while many were present in the dermis, along with melanin incontinentia