A unique system that can sensitively assess the risk of chemical leukoderma by using murine tail skin

Machiko Iida[a], Akira Tazaki[a], Yuqi Deng[a], Wei Chen[a], Ichiro Yajima[a], Lisa Kondo-Ida[a], Kazunori Hashimoto[a], Nobutaka Ohgami[a], Masashi Kato[a*]

[a]Department of Occupational and Environmental Health, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi, 466-8550, Japan.

[*]**Corresponding author:** Masashi Kato M.D., Ph.D. Department of Occupational and Environmental Health, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan. **E-mail:** katomasa@med.nagoya-u.ac.jp

Conflict of interest: The authors have no conflict of interest to declare.

Abstract

Chemical leukoderma is a patchy hypopigmentation in the skin. Phenol derivatives such as raspberry ketone have been reported to cause the development of occupationally induced leukoderma. Recently, 2% (w/w) rhododenol, a reduced form of raspberry ketone used in a skin-lightening agent, also caused the development of leukoderma in >16,000 users, about 2% of all users, in Asian countries including Japan. However, a method for assessing the risk of leukoderma caused by 2% rhododenol has not been established despite the fact that the development of leukoderma caused by 30% rhododenol was previously shown in animal experiments. Establishment of a novel technique for risk assessment of leukoderma in humans caused by external treatment with chemicals is needed to prevent a possible future chemical disaster. This study demonstrated that external treatment with 2% rhododenol and the same concentration of raspberry ketone caused the development of leukoderma in murine tail skin without exception with significant decreases in the amount of melanin and number of melanocytes in the epidermis. Thus, a novel *in vivo* technique that can assess the risk of leukoderma caused by 2% rhododenol was developed. The unique technique using tail skin has the potential to prevent chemical leukoderma in the future.

Keywords

Chemical leukoderma, Raspberry ketone, Rhododenol, Risk assessment, Tail skin

1. Introduction

Chemical leukoderma, a patchy hypopigmentation in the skin, is caused by various chemicals such as phenol derivatives and catechol derivatives (Hariharan et al., 2010; Harris, 2017; Lengagne et al., 2004). Leukoderma that is occupationally induced by raspberry ketone, 4-(4-hydroxyphenyl)-2-butanone (Fig. 1a), which is a phenol derivative, has been reported (Fukuda et al., 1998). In 2013, more than 16,000 patients developed leukoderma caused by using skin-lightening agents that contained 2% (w/w) rhododenol (4-(4-hydroxyphenyl)-2-butanol) (Fig. 1a), a reduced form of raspberry ketone (Nishigori et al., 2015; Sasaki et al., 2014). The rhododenol-induced leukoderma was improved or completely resolved in 72% of the patients (Nishigori et al., 2015), but the remaining patients were resistant to conventional therapy (Watabe et al., 2018). External treatment with 2% rhododenol caused leukoderma in about 2% of the skin-lightning agent users (Nishigori et al., 2015), indicating that these patients with leukoderma must be hypersensitive to rhododenol. A previous study showed that external treatment with 30% but not 10% rhododenol caused the development of leukoderma in the dorsal skin of haired guinea pigs, in which skin pigmentation was induced by ultraviolet light irradiation before treatment with rhododenol (Kuroda et al., 2014). It was also shown that external treatment with 30% but not 2% rhododenol caused the development of leukoderma in the dorsal skin of hairless transgenic mice carrying murine stem cell factor under control of the human keratin 14

promoter (Abe et al., 2016). These previous studies are important for risk assessment of chemical leukoderma. However, cases of chemical leukoderma have been occurring for more than 75 years (Harris, 2017), indicating that there is no system that is sensitive enough to assess the risk for chemical leukoderma at present. To prevent a possible chemical disaster in the future, the development of a novel technique that can sensitively assess the risk for chemical leukoderma, even with a low rhododenol concentration of 2%, is needed.

2. Materials and Methods

2.1. Mice and chemicals

After *hairless* homozygous (*Hr/Hr*) mice (HOS:HR-1) purchased from Hoshino Laboratory Animals, Inc. had been backcrossed to C57BL/6-mice for 6 generations, an inbred line was developed by brother-sister mating for >10 generations. The hairless mice at 10 weeks of age were used in this study. This study was approved by the Animal Care and Use Committee (approval no. 30258) in Nagoya University. Raspberry ketone (Cas# 5471-51-2; Tokyo Chemical Industry Co., Ltd.) and rhododenol (Cas# 69617-84-1; Wako Pure Chemical Industries, Ltd.) were dissolved in 50% ethanol according to the method previously described (Abe et al., 2016). Each solution was applied to dorsal skin (6 μl/cm^2) and tail skin (6 μl/cm^2).

2.2. Histological analysis

Immunohistochemical analysis was performed according to the method previously described (Iida et al., 2016; Kato et al., 2004). Paraffin sections were treated with 10 mM sodium citrate (pH 6.0) for antigen retrieval as previously reported (Ohgami et al., 2010).

Anti-TRP2 antibody (1:100; Santa Cruz) and alexa Fluor 594 anti-goat IgG (1:1000; Thermo Fisher Scientific Inc.) were used as a first antibody and a secondary antibody, respectively. Fontana-Masson staining was performed according to the protocol of the manufacturer (40592, Muto Pure Chemicals Co., Ltd.).

2.3. Semiquantitative analysis

2.3.1. Evaluation of the skin colors of dorsal skin and tail skin

L*-values (worldwide standard values for lightness) of the dorsal skin were analyzed by using a reflectance spectrophotometer (CR-400; Konica Minolta Sensing, Inc.) according to the method previously described (Kato et al., 2011). Darkness of the tail skin was analyzed by the software program WinROOF (Mitani Corp., Japan) after capturing digital images of the tail skin with a darkness standard because a reflectance spectrophotometer for tail skin with a round shape is not available.

2.3.2. Evaluation of melanin density

Digital images of epidermal melanin detected by Fontana-Masson staining were traced by binarization using the software program WinROOF, and the total amounts of traced dots (digitally traced melanin) in the epidermis were divided by the area of the epidermis in the analyzed field.

2.4. Statistical analysis

Paired Wilcoxon's signed rank test (Fig. 1) and the chi-square test (Fig. 3) were used for comparison between two groups. Comparisons among three groups were performed by using the Steel-Dwass test (Fig. 2). JMP Pro (version 11.0.0; SAS Institute) was used in all

104 statistical analyses in this study.

105

106 **3. Results**

107 Following the usual method for assessing the risk of external treatment with chemicals
108 (Abe et al., 2016; Kuroda et al., 2014), dorsal skin (Fig. 1) was treated with 2% (w/w)
109 rhododenol and the same concentration of raspberry ketone in addition to their solvent in
110 wild-type hairless mice crossed with C57BL/6N previously described (Yajima et al., 2017).
111 Unfortunately, however, no macroscopic hypopigmentation developed in the dorsal skin
112 even 14 weeks after the treatment (Fig. 1b, c). Correspondingly, our microscopic analyses
113 showed comparable amounts of melanin and numbers of tyrosinase-related protein-2
114 (TRP-2)-positive cells (melanocytes) in the epidermis of dorsal skin in mice treated with
115 rhododenol and raspberry ketone and control mice treated with the solvent even 14 weeks
116 after the treatment (Fig. 1d-g). Our macroscopic and microscopic results indicate that
117 sensitive risk assessment for chemical leukoderma by using dorsal skin of mice is difficult.
118 Generally, it is very rare to use tail skin for risk assessment of external treatment with
119 chemicals. However, the tail skin of mice was used to assess the risk of leukoderma caused
120 by 2% rhododenol and the same concentration of raspberry ketone because of our failure in
121 risk assessment using dorsal skin. At 4 weeks after treatment (Fig. S1), mild but
122 macroscopically detectable hypopigmentation (early stage of leukoderma) had developed in
123 tail skin treated with 2% raspberry ketone and rhododenol but not with their solvent. An

advanced stage of hypopigmentation limited to areas treated with 2% raspberry ketone and 2% rhododenol was observed in the tail skin of all mice up to 14 weeks after treatment (Fig. 2a). Semiquantitative analysis of macroscopic images also showed that dark levels in the tail skin treated with raspberry ketone and rhododenol were more than 99% decreased compared to those in tail skin treated with their solvent (Fig. 2b). Microscopic analysis further showed that the level of melanin (Fig. 2c, d) in the epidermis of tail skin treated with 2% raspberry ketone and 2% rhododenol was undetectably low even by Fontana-Masson staining, a sensitive method for histologically detecting melanin (Kato et al., 2011). The number of TRP-2-positive cells (melanocytes) in the epidermis of tail skin treated with 2% raspberry ketone and 2% rhododenol was less than 12% of that in tail skin treated with their solvent (Fig. 2e, f).

Melanin production activities of dorsal skin melanocytes and tail skin melanocytes were analyzed. Morphological analysis (Fig. 3a-h) showed that the melanin production level in TRP-2-positive cells was higher in tail skin than in dorsal skin. Further analysis (Fig. 3i) also showed that 13% and 83% of TRP2-positive cells in the dorsal skin and tail skin were positive for melanin, respectively.

4. Discussion

Generally, there is a limited number of dermal melanocytes in humans (Hoerter et al.,

143 2012). It has been reported that rhododenol-induced leukoderma in humans was caused by
144 depletion of epidermal melanocytes (Tokura et al., 2015). Since the main purpose of this
145 study was to develop a novel system to sensitively assess the risk for leukoderma, we
146 focused on epidermal melanocytes.

147 There are several possible reasons why tail skin is more suitable than dorsal skin for
148 risk assessment of chemical leukoderma. We first showed that melanin density and number
149 of melanocytes in the epidermis of tail skin were >50-fold and >4.5-fold greater than those
150 in dorsal skin, respectively. These results suggest that there are quantitative differences for
151 melanin production and melanocyte number between dorsal skin and tail skin. We also
152 showed that the number of melanin-producing melanocytes in tail skin was 6.2-fold larger
153 than that in dorsal skin. Melanocytes with dendrites in tail skin morphologically seem to be
154 more differentiated than those with a limited number of dendrites in dorsal skin, because
155 fully differentiated melanocytes are characterized by melanin-producing activity as well as
156 by well-developed dendrites (Hirobe, 2005). In addition, the proportion of
157 TRP2-positive/Ki67-positive cells in dorsal skin was shown to be only 3% in our previous
158 study, in which the same hairless mice were used, indicating that most melanocytes in dorsal
159 skin are TRP2-positive/Ki67-negative cells with limited melanin production (Yajima et al.,
160 2017). These results suggest that there are qualitatively different melanocytes with different
161 degrees of differentiation in dorsal skin and tail skin. Since previous studies showed that

rhododenol-induced melanocyte cytotoxicity is dependent on tyrosinase activity that regulates melanin production in melanocytes (Giebel et al., 1990; Kasamatsu et al., 2014; Sasaki et al., 2014), the qualitative differences as well as the quantitative differences between dorsal skin and tail skin may result in the different sensitivities of dorsal skin and tail skin to rhododenol. On the other hand, the thicknesses of dorsal skin and tail skin of the hairless mice used in this study were about 30 μm and about 90 μm, respectively (data not shown). However, leukoderma was induced by rhododenol in tail skin but not in dorsal skin, although tail skin is about 3-fold thicker than dorsal skin. These results suggest that different levels of chemical penetration based on the different thicknesses of dorsal skin and tail skin have a limited effect on sensitivity to leukoderma.

To our knowledge, rhododenol is the only chemical for which a leukoderma-inducible concentration (2%) has been detected in a large number (>16,000) of people (Nishigori et al., 2015; Sasaki et al., 2014). In this study, we chose rhododenol as a concentration-known leukoderma inducer and raspberry ketone as a concentration-unknown leukoderma inducer in humans. The same concentrations (2%) of rhododenol and raspberry ketone induced leukoderma in all cases in our risk assessment system of tail skin. Further study of higher concentrations of chemicals showed that an early stage of leukoderma was already detectable in tail skin treated with 30%, but not 10%, raspberry ketone and rhododenol at 2 weeks after treatment (Fig. S2). Leukoderma caused by 30% raspberry ketone and

rhododenol was more severe than that caused by 10% raspberry ketone and rhododenol at 2 weeks and 10 weeks after treatment (Fig. S2). Our results suggest that these chemicals have comparable risks for leukoderma. The results are reasonable since raspberry ketone and rhododenol are analog chemicals. Thus, our system can sensitively assess the risk for the development of leukoderma in only 2% of users of skin-lightning agents including 2% rhododenol (Nishigori et al., 2015). Our results encourage us to assess the risks of various previously reported chemical inducers for leukoderma by our assessment system in the future.

In this study, we demonstrated for the first time a unique technique that can assess the risk of chemical leukoderma induced by 2% rhododenol by using murine tail skin. Our risk assessment system does not require either ultraviolet light irradiation or gene modification. Our system has the potential to prevent chemical leukoderma due to its convenience as well as sensitivity. Further study to clarify the mechanism of chemical leukoderma by using our system *in vivo* might lead to the development of a novel *in vitro* system for sensitively assessing the risk for leukoderma.

Declarations of interest

None.

Acknowledgements

This study was supported in part by Grants-in-Aid for Scientific Research on Innovative Areas (16H01639 and 18H04975), Research Activity start-up (18H06125) and Scientific Research (A) (15H01743, 15H02588 and 19H01147), (B) (16H02962 and 17KT0033) and (C) (16K08343, 16K11177, 16K10152 and 17K09156) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT), Mirai-Program Small Start Type from the Japan Science and Technology Agency (JST), Kobayashi International Scholarship Foundation, Daiko Foundation, Foundation for the Vitamin & Biofactor Society and Research Foundation from Center for Advanced Medical and Clinical Research Nagoya University Hospital.

References

Abe, Y., Okamura, K., Kawaguchi, M., Hozumi, Y., Aoki, H., Kunisada, T., Ito, S., Wakamatsu, K., Matsunaga, K., Suzuki, T., 2016. Rhododenol-induced leukoderma in a mouse model mimicking Japanese skin. J. Dermatol. Sci. 81, 35–43. https://doi.org/10.1016/j.jdermsci.2015.10.011

Fukuda, Y., Nagano, M., Futastuka, M., 1998. Occupational Leukoderma in Workers Engaged in 4-(p-Hydroxyphenyl)-2-Butanone Manufactunng. J. Occup. Health 40, 118–122. https://doi.org/10.1539/joh.40.118

Giebel, L.B., Strunk, K.M., King, R.A., Hanifin, J.M., Spritz, R.A., 1990. A frequent tyrosinase gene mutation in classic, tyrosinase-negative (type IA) oculocutaneous albinism. Proc. Natl. Acad. Sci. U. S. A. 87, 3255–3258.

Hariharan, V., Klarquist, J., Reust, M.J., Koshoffer, A., McKee, M.D., Boissy, R.E., Caroline Le Poole, I., 2010. Monobenzyl Ether of Hydroquinone and 4-Tertiary Butyl Phenol Activate Markedly Different Physiological Responses in Melanocytes: Relevance to Skin Depigmentation. J. Invest. Dermatol. 130, 211–220. https://doi.org/10.1038/jid.2009.214

Harris, J.E., 2017. Chemical-Induced Vitiligo. Dermatol. Clin. 35, 151–161. https://doi.org/https://doi.org/10.1016/j.det.2016.11.006

Hirobe, T., 2005. Role of keratinocyte-derived factors involved in regulating the

232 proliferation and differentiation of mammalian epidermal melanocytes. Pigment Cell
233 Res. 18, 2–12. https://doi.org/10.1111/j.1600-0749.2004.00198.x
234 Hoerter, J.D., Bradley, P., Casillas, A., Chambers, D., Denholm, C., Johnson, K.,
235 Weiswasser, B., 2012. Extrafollicular dermal melanocyte stem cells and melanoma.
236 Stem Cells Int. 2012, 407079. https://doi.org/10.1155/2012/407079
237 Iida, M., Nakano, C., Tamaki, M., Hasegawa, M., Tsuzuki, T., Kato, M., 2016. Different
238 biological effects of a constant dose for single UVB irradiation with different intensities
239 and exposure times. Exp. Dermatol. 25, 386–388. https://doi.org/10.1111/exd.12969
240 Kasamatsu, S., Hachiya, A., Nakamura, S., Yasuda, Y., Fujimori, T., Takano, K., Moriwaki,
241 S., Hase, T., Suzuki, T., Matsunaga, K., 2014. Depigmentation caused by application of
242 the active brightening material, rhododendrol, is related to tyrosinase activity at a
243 certain threshold. J. Dermatol. Sci. 76, 16–24.
244 https://doi.org/https://doi.org/10.1016/j.jdermsci.2014.07.001
245 Kato, M., Iida, M., Goto, Y., Kondo, T., Yajima, I., 2011. Sunlight exposure-mediated DNA
246 damage in young adults. Cancer Epidemiol. Biomarkers Prev. 20, 1622–1628.
247 https://doi.org/10.1158/1055-9965.EPI-11-0228
248 Kato, M., Takeda, K., Kawamoto, Y., Tsuzuki, T., Hossain, K., Tamakoshi, A., Kunisada, T.,
249 Kambayashi, Y., Ogino, K., Suzuki, H., Takahashi, M., Nakashima, I., 2004.
250 c-Kit-Targeting Immunotherapy for Hereditary Melanoma in a Mouse Model. Cancer

Res. 64, 801 LP – 806. https://doi.org/10.1158/0008-5472.CAN-03-2532

Kuroda, Y., Takahashi, Y., Sakaguchi, H., Matsunaga, K., Suzuki, T., 2014. Depigmentation of the skin induced by 4-(4-hydroxyphenyl)-2-butanol is spontaneously re-pigmented in brown and black guinea pigs. J. Toxicol. Sci. 39, 615–623. https://doi.org/10.2131/jts.39.615

Lengagne, R., Le Gal, F.-A., Garcette, M., Fiette, L., Ave, P., Kato, M., Briand, J.-P., Massot, C., Nakashima, I., Rénia, L., Guillet, J.-G., Prévost-Blondel, A., 2004. Spontaneous Vitiligo in an Animal Model for Human Melanoma. Cancer Res. 64, 1496 LP – 1501. https://doi.org/10.1158/0008-5472.CAN-03-2828

Nishigori, C., Aoyama, Y., Ito, A., Suzuki, K., Suzuki, T., Tanemura, A., Ito, M., Katayama, I., Oiso, N., Kagohashi, Y., Sugiura, S., Fukai, K., Funasaka, Y., Yamashita, T., Matsunaga, K., 2015. Guide for medical professionals (i.e. dermatologists) for the management of Rhododenol-induced leukoderma. J. Dermatol. 42, 113–128. https://doi.org/10.1111/1346-8138.12744

Ohgami, N., Ida-Eto, M., Shimotake, T., Sakashita, N., Sone, M., Nakashima, T., Tabuchi, K., Hoshino, T., Shimada, A., Tsuzuki, T., Yamamoto, M., Sobue, G., Jijiwa, M., Asai, N., Hara, A., Takahashi, M., Kato, M., 2010. c-Ret-mediated hearing loss in mice with Hirschsprung disease. Proc. Natl. Acad. Sci. U. S. A. 107, 13051–13056. https://doi.org/10.1073/pnas.1004520107

Sasaki, M., Kondo, M., Sato, K., Umeda, M., Kawabata, K., Takahashi, Y., Suzuki, T., Matsunaga, K., Inoue, S., 2014. Rhododendrol, a depigmentation-inducing phenolic compound, exerts melanocyte cytotoxicity via a tyrosinase-dependent mechanism. Pigment Cell Melanoma Res. 27, 754–763. https://doi.org/10.1111/pcmr.12269

Tokura, Y., Fujiyama, T., Ikeya, S., Tatsuno, K., Aoshima, M., Kasuya, A., Ito, T., 2015. Biochemical, cytological, and immunological mechanisms of rhododendrol-induced leukoderma. J. Dermatol. Sci. 77, 146–149. https://doi.org/10.1016/j.jdermsci.2015.02.001

Watabe, A., Yamasaki, K., Asano, M., Kanbayashi, Y., Nasu-Tamabuchi, M., Terui, H., Furudate, S., Kakizaki, A., Tsuchiyama, K., Kimura, Y., Ito, Y., Kikuchi, K., Aiba, S., 2018. Efficacy of oral cholecalciferol on rhododendrol-induced vitiligo: A blinded randomized clinical trial. J. Dermatol. 45, 456–462. https://doi.org/10.1111/1346-8138.14244

Yajima, I., Kumasaka, M.Y., Iida, M., Oshino, R., Tanihata, H., Al Hossain, A., Ohgami, N., Kato, M., 2017. Arsenic-mediated hyperpigmentation in skin via NF-kappa B/endothelin-1 signaling in an originally developed hairless mouse model. Arch. Toxicol. 91, 3507–3516. https://doi.org/10.1007/s00204-017-1975-0

289 **Figure legends**

290 **Fig. 1. Limited effect of chemicals on pigmentated level of dorsal skin**

291 **(a)** Chemical structures of raspberry ketone (RK) and rhododenol (RD) are presented. **(b-g)**
292 Macroscopic and microscopic results of the dorsal skin of 24-week-old hairless mice treated
293 with 2% (w/w) RK, 2% (w/w) RD and their solvent (Sol) of 50% ethanol once a day for 5
294 days per week for a period of 14 weeks are presented. **(b)** Representative macroscopic
295 images of murine dorsal skin. Dots in the skin indicate the corners of the area treated with
296 the chemicals. **(c)** L*-values (mean ± standard deviation) of 9 dorsal skin areas (3 skin areas
297 in 3 mice) treated with Sol, 2% RK and 2% RD according to the previously described
298 method (Kato et al., 2011). L*-values analyzed by using a reflectance spectrophotometer
299 (CR-400; Konica Minolta Sensing, Inc.) show digitalized levels of pigmentation in dorsal
300 skin (Kato et al., 2011). A representative result is shown **(b, c)** after the same experiments
301 were independently repeated 3 times. **(d)** Level of melanin in the epidermis of dorsal skin
302 evaluated by Fontana-Masson staining is shown. Nuclei were stained with nuclear fast red
303 (pink). White arrowheads indicate melanin. **(e)** Melanin density (melanin-containing area per
304 analyzed field) of 15 sections (5 sections in 3 mice) in the dorsal skin (mean ± standard
305 deviation) treated with Sol, 2% RK and 2% RD was analyzed by WinROOF. **(f)**
306 Representative results of immunohistochemical analyses of TRP2-positive cells (red
307 immunofluorescent cells indicated by white arrowheads) in the epidermis. White broken
308 lines show the border between the epidermis and dermis. Nuclei were stained with DAPI
309 (blue). **(g)** Number of TRP2-positive cells per analyzed field (600 μm along the border
310 between the epidermis and dermis) in the epidermis of dorsal skin (mean ± standard
311 deviation) in 12 sections (4 sections in 3 mice) treated with Sol, 2% RK and 2% RD is
312 shown. ns, not significant. Bars, 10 μm.

313

Fig. 2. Hypopigmented effect of chemicals on the tail skin

(a-f) Macroscopic and microscopic results of the tail skin of 24-week-old hairless mice treated with 2% (w/w) raspberry ketone (RK), 2% (w/w) rhododenol (RD) and their solvent (Sol) of 50% ethanol once a day for 5 days per week for a period of 14 weeks are presented. **(a)** Representative macroscopic images of murine tail skin. Green tattoos (white arrowheads) in the tail skin indicate the ends of the areas treated with the chemicals. A representative result is shown after the same experiments were independently repeated 3 times. **(b)** Dark levels (mean ± standard deviation) of 9 tail skin areas (3 skin areas in 3 mice) treated with Sol, 2% RK and 2% RD were analyzed by the software program WinROOF (Mitani Corp.). **(c)** Melanin levels in the epidermis of tail skin evaluated by Fontana-Masson staining are shown. Nuclei were stained with nuclear fast red (pink). White arrowheads indicate melanin. **(d)** Melanin density (melanin-containing area per analyzed field) of 15 sections (5 sections in 3 mice) in the tail skin (mean ± standard deviation) treated with Sol, 2% RK and 2% RD was analyzed by WinROOF. **(e)** Representative results of immunohistochemical analyses of TRP2-positive cells (red immunofluorescent cells indicated by white arrowheads) in the epidermis. White broken lines show the border between the epidermis and dermis. Nuclei were stained with DAPI (blue). **(f)** Number of TRP2-positive cells per analyzed field (600 μm along the border between the epidermis and dermis) in the epidermis of 12 sections (4 sections in 3 mice) in the tail skin (mean ± standard deviation) treated with Sol, 2% RK and

333 2% RD is shown. **p < 0.01, *p < 0.05. Bars, 50 μm.

334

335 **Fig. 3. Different morphologies and different melanin production activities of dorsal**

336 **skin melanocytes and tail skin melanocytes**

337 **(a-i)** Results of morphologic **(a-h)** and quantitative analyses **(i)** of TRP2-positive cells

338 (melanocytes) in dorsal skin and tail skin from 24-week-old hairless mice are presented.

339 Results of immunohistochemical analyses of TRP2-positive cells (red immunofluorescent

340 cells indicated by arrowheads) in the epidermis of dorsal skin **(a, b)** and tail skin **(c, d)** are

341 shown. Nuclei were stained with DAPI (blue) **(a-d)**. Representative merged images of bright

342 fields showing melanin (brown) and immunofluorescent fields showing TRP2-positive cells

343 (orange) in the epidermis of dorsal skin **(e, f)** and tail skin **(g, h)** are presented. Magnified

344 images **(b, d, f, h)** of TRP2-positive cells in the presence **(d, h)** or absence **(b, f)** of dendrites

345 in the dotted boxed areas **(a, c, e, g)** are shown. A limited amount and a large amount of

346 melanin production in TRP2-positive cells were obtained in dorsal skin (white arrowheads)

347 and tail skin (yellow arrowheads), respectively. **(i)** Percentages (n=60) of melanin-negative

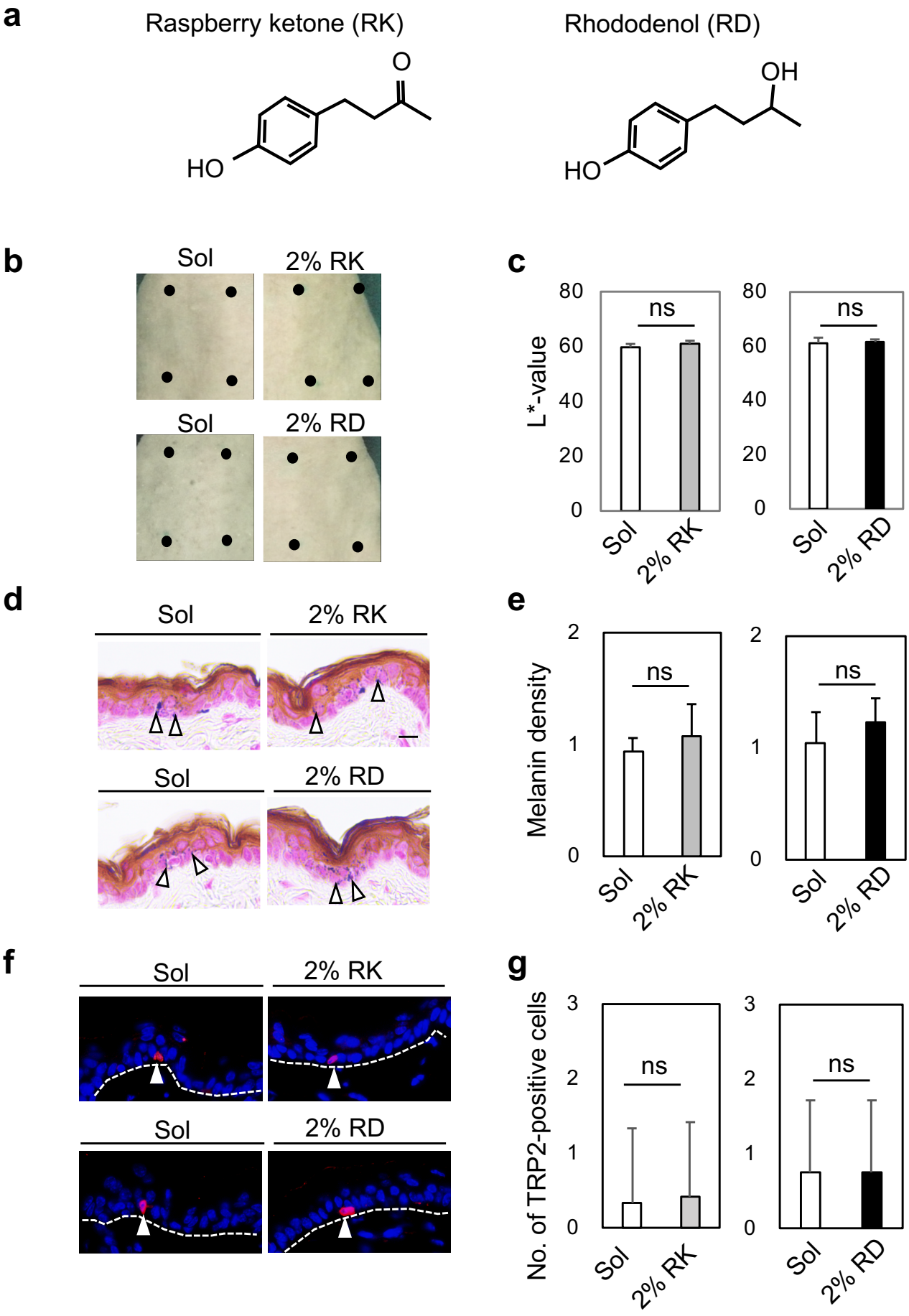
348 and melanin-positive TRP2-positive cells in the epidermis of dorsal skin and tail skin from 3

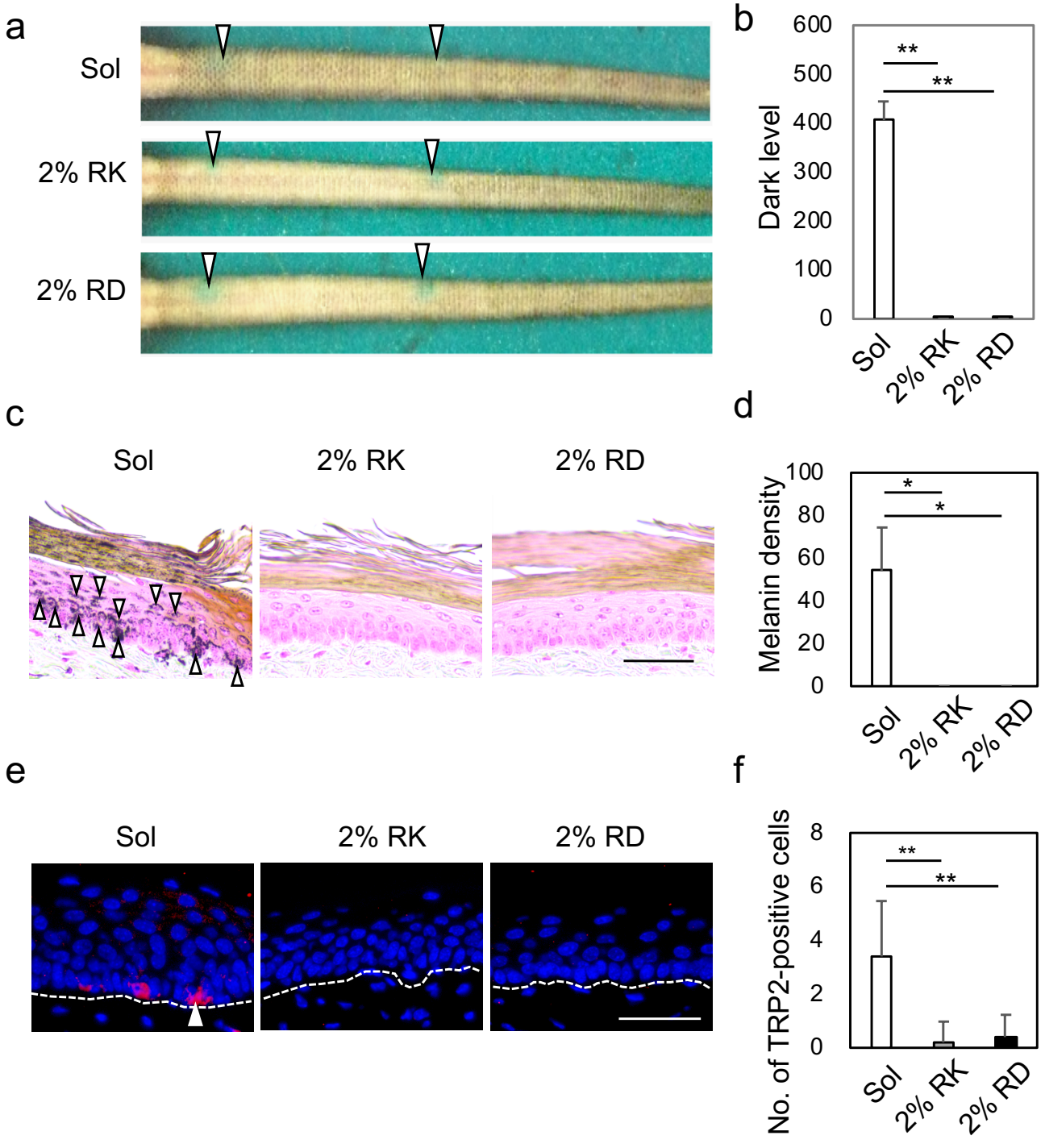
349 mice were analyzed. *p < 0.01. Bars, 25 μm.

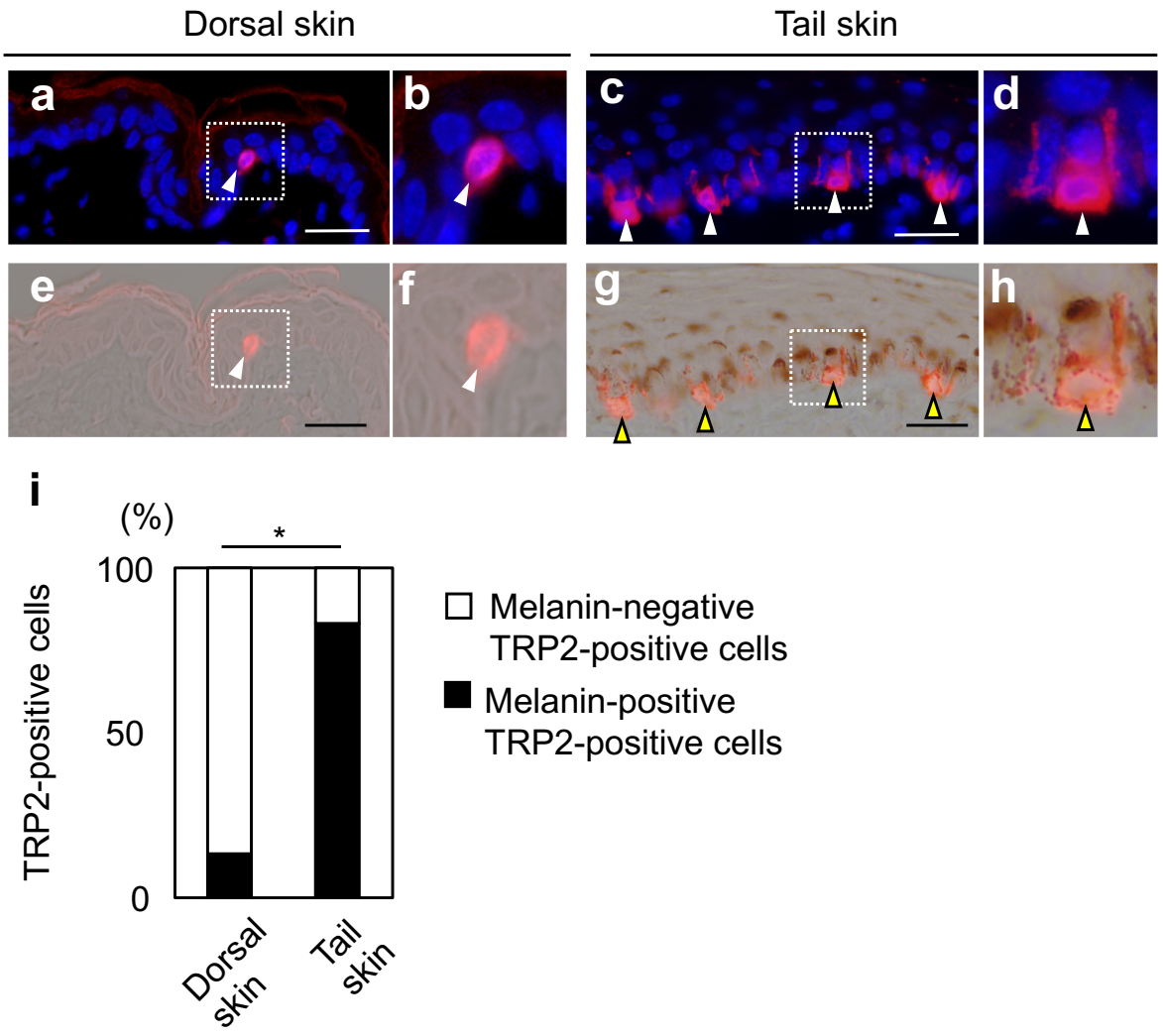
350

351

Figure 1


a
Raspberry ketone (RK)
O
HO
Rhododenol (RD)
OH
HO
b
Sol
2% RK
Sol
2% RD
c
L*-value
80
60
40
20
0
ns
Sol
2% RK
Sol
2% RD
d
Sol
2% RK
Sol
2% RD
e
Melanin density
2
1
0
ns
Sol
2% RK
Sol
2% RD
f
Sol
2% RK
Sol
2% RD
g
No. of TRP2-positive cells
3
2
1
0
ns
Sol
2% RK
Sol
2% RD


a
Sol
2% RK
2% RD
b
Dark level
600
500
400
300
200
100
0
**
**
Sol
2% RK
2% RD
c
Sol
2% RK
2% RD
d
Melanin density
100
80
60
40
20
0
*
*
Sol
2% RK
2% RD
e
Sol
2% RK
2% RD
f
No. of TRP2-positive cells
8
6
4
2
0
**
**
Sol
2% RK
2% RD


Dorsal skin
Tail skin
a
b
c
d
e
f
g
h
i
(%)
*
100
50
0
TRP2-positive cells
Dorsal skin
Tail skin
Melanin-negative TRP2-positive cells
Melanin-positive TRP2-positive cells

Supplementary material

A unique system that can sensitively assess the risk of chemical leukoderma by using murine tail skin

Machiko Iida[a], Akira Tazaki[a], Yuqi Deng[a], Wei Chen[a], Ichiro Yajima[a], Lisa Kondo-Ida[a], Kazunori Hashimoto[a], Nobutaka Ohgami[a], Masashi Kato[a]*

[a]Department of Occupational and Environmental Health, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi, 466-8550, Japan.

*__Corresponding author:__ Masashi Kato M.D., Ph.D. Department of Occupational and Environmental Health, Nagoya University Graduate School of Medicine, 65 Tsurumai-cho, Showa-ku, Nagoya, Aichi 466-8550, Japan. **E-mail:** katomasa@med.nagoya-u.ac.jp

Number of pages: 3

Number of figures: 2

Figure S1

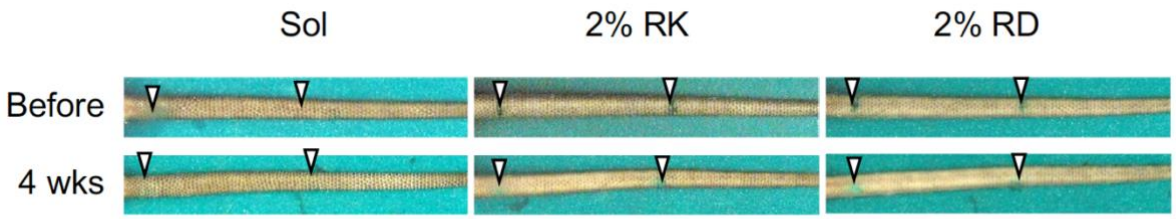


Fig. S1. Development of leukoderma caused by 2% chemicals

Macroscopic results of tail skin treated with 2% raspberry ketone (RK) and rhododenol (RD) and their solvent (Sol) of 50% ethanol. Representative images of tails before and 4 weeks (wks) after treatments are presented. Green tattoos with white arrowheads indicate the areas treated with solvent and 2% chemicals. Representative results are shown after the same experiments were independently repeated 3 times.

Figure S2

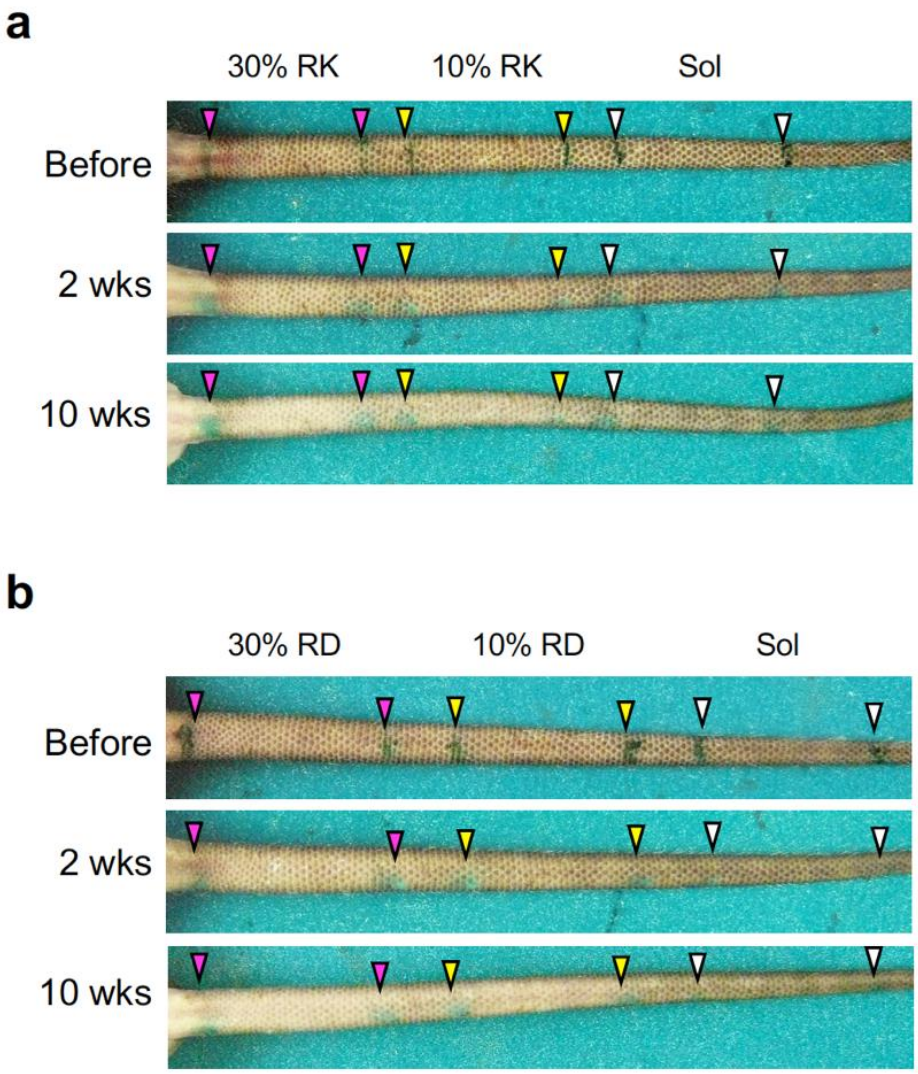


Fig. S2. Kinetics of leukoderma development caused by 10% and 30% chemicals

(**a**, **b**) Macroscopic results of the tail skin of 12-week-old (top), 14-week-old (intermediate) and 22-week-old (bottom) hairless mice treated with 10% and 30% raspberry ketone (RK) (**a**), 10% and 30% rhododenol (RD) (**b**) and their solvent (Sol) of 50% ethanol three times a day for 5 days per week for a period of 10 weeks are presented. Green tattoos with white, yellow and magenta arrowheads indicate the areas treated with the solvent and 10% and 30%

58 chemicals, respectively. Representative results are shown after the same experiments were

59 independently repeated 3 times.

60