Nat. Volatiles & Essent. Oils, 2021; 8(5): 1950 - 1959

NVEO
Natural Volatiles & Essential Oils

Transgenic Black Mice Induction model for Human Leucoderma

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Abstract: There has been little research evaluating the efficacy and safety of 4 hydroxyl anisole + TRA (4 HA + TRA) in an animal model for Leucoderma.To test the first model of leucoderma to induce epidermal depigmentation, an attempt has been made to achieve an animal model using transgenic mice 4HA+ TRA applications of 3 and 5 % produced depigmentation, but depigmentation was found to be better in 5 %. The inducted skin in the transgenic mice was fixed in 10 % neutral formalin and processed by the routine histological technique using F/M methods for the presence/absence of melanin. Good compliance, exposure, and rising cumulative dose were all linked to a better response to therapy, which was statistically significant (P < 0.01). In addition, there were just minor side effects. During follow-up, only half of the animals had depigmentation at the sites. Our research shows that 4HA combined with TRA is an effective model for Leucoderma, with good depigmentation of the epidermis occurs within 3-4 weeks in a white patchy pattern similar to patients with vitiligo.

Keywords: Leucoderma, De-pigmentation, Transgenic Mice, 4-Hydroxy anisole, Melanocytes, Re-pigmentation

Introduction

Leucoderma (Vitiligo) is a disease of depigmentation of the skin for unknown causes. It is a challenge to medical science. The chronic form of the autoimmune skin disease is highly resistant to therapy. Given that, the victims are placed under a boycott in society and turn, subjected to psychological upset (Gregg et al., 2010). The Leucoderma, though it is not precisely known, some of the factors such as nutritional, metabolic, hereditary, neurogenic and autoimmune disorders, protozoa infestation, stress, etc., are presumed to have led to these patchy white diseases (Antony et al., 2005).

It is generally considered the defect in tyrosine's metabolism that leads to the failure of being converted into melanin. There are many players like that of optimum skin pH (5.6 to 6.8), ultraviolet light, tyrosinase (a copper-containing enzyme), diphosphatide pyridine nucleotide (DPN), nicotic, pentothinic and ascorbic acids, and melanophore stimulating enzyme in materializing the reactions. Melanin or melanosomes include abundant tyrosinase. Such tyrosine catalyzes the oxidation of tyrosine to melanin (Chatterjee et al., 2014). Melanin is often under the influence of ultraviolet light. In addition to that, copper deficiency also appears to be frequently responsible for such disease. The presence of an unidentified neurohormone is also attributed to be responsible for lightening the melanin in the skin. Despite widespread research on this particular disease, the current understanding of the disease is still far from satisfactory (Wehrle-Haller B 2003) & (Toyonobu Yamashita and Tomohiro Kuwahara 2005).

In humans and experimental animals, phenols and catechols are melanocytotoxic and cause depigmentation of the skin and hair. The most extensively studied group of such depigmenting

compounds is p-hydroxyphenyl derivatives, such as 4-hydroxyanisole. Therefore, the cells with activated tyrosinase are sensitive to the cytotoxic impact of p-hydroxy phenols, based on one hypothesis (Fisher GJ, and Voorhees JJ (1996).

The physiological effects of a new kind of p-hydroxyphenyl, N-acetyl-4-S-cysteaminylphenol, were studied previously. It has demonstrated a robust depigmenting potency in black hair follicles and a considerable growth inhibitory effect in mice with melanoma. It was discovered that the latter impact was dose dependant. Hair follicles from C57BL/6j mice have been identified as a good model for testing medication effects on Leucoderma in vivo (Essien et al., 2014). These mice come in various colours, but the C57BL/6j, a/a strain is black due to the synthesis of eumelanin, a dark pigment. Hair follicle plucking stimulates the hair cycle's anagen phase, followed by follicular melanocyte activation and enhanced tyrosinase activity (Arnold et al., 1975 and Gano SE and Garcia R, (1979). As a result, a significant melanocytotoxic phenol that is a substrate for tyrosinase should cause depigmentation of newly grown hair in black mice during the maturation stage (Picardo et al., 2015).

We investigated the in vivo susceptibilities of black follicular melanocytes to 4-hydroxy anisole in this study. The findings imply that glutathione concentration (tripeptide glutathione) is essential in the depigmenting impact of 4-hydroxy anisole, which increases pheomelanin synthesis via providing cysteine (Nishimura et al., 2002). Furthermore, we found that the depigmenting effectiveness of 4-hydroxy anisole in follicular melanocytes is directly related to the GSH level in the target tissues of black mice, using the GSH-modulating drug vitamin C, which lowers GSH synthesis by blocking gamma-glutamylcysteine synthetase (Welsh et al., 1999).

Because of such necessity, a search to better cure for Leucoderma is attempted in alternative medicine. The dire need for complementary alternative therapy forms an essential component of the present research. The alternative system of medicine of traditional Chinese Indians and homoeopathy propounds large varieties of treatment. Siddha Medicine, rich heritage and ancient tradition of Tamilnadu, India, has been treating ailments like Leucoderma for more than five thousand years (Zavadskii VN 1973 & 1976). The present Siddha practitioners have come out successfully, bringing a complete cure for this significant disorder. While attempting to go for globalizing this success story, a necessity of pre-clinical study is warranted. Like any other disease, animal models are needed. It isn't easy to propose an animal model for this Leucoderma. Earlier studies have projected the usage of hydroquinone, corticosteroids, retinoic acid, 4 hydroxyanisole, Azelaic acid, Kojic acid for depigmentation. Depigmenting agents are commonly prescribed to treat disorders of hyper pigmentation (Rashighi et al., 2014a).While reviewing the earlier reports, several notable depigmenting agents have noticed their own merits and demerits.

Materials and methods

Animals: The five-week-old female black C57BL/6j. a/a mice were obtained from the Centre for Cellular and Molecular Biology (CCMB), Animal House, Hyderabad. Mice are kept in a polystyrene cage

with rice husk bedding and are fed a regular laboratory diet (Pet Care Division, Bangalore) and free access to tap water. The Institutional Animal Ethical Committee (9/SASTRA/IAEC/RPP) has given their clearance to the research. The animals are treated with the utmost care to ensure that they are treated most humanely and ethically. Olympus Digital Camera was used to take all of the photos. All fluids, equipment, and medicines used on living animals must be sterile, and aseptic technique should be employed wherever possible

Drug Treatment

The mice were given a chloroform anaesthetic, and their hair follicles were manually removed from a small area on the backside (below the neck). 4-hydroxy anisole + 0.01 % TRA is incorporated with simple ointment for the use of external applications. After three days, after plucking, the 4HA ointment was smoothly applied over the specific spot chosen using cotton buds. Each time necessary care was taken, an equal amount of ointment was applied twice a day on each animal daily for seven weeks (Kasrace et al., 2005)

Depigmentation: Black mice were placed into four groups at random and given the following treatments: Group I Topical application of 1 % 4HA alone (No depigmentation); group II 2 % 4HA +0.01 % TRA (more effective depigmentation); group III 0.01 % TRA alone (no depigmentation); group VI simple ointment (control group). The assay was completed on day 7 weeks after plucking. Group II (4HA/TRA (2 % /0.01 %) animals are having no signs of local irritation such as erythema or scaling (Xina Nair et al., 1993). We evaluated the usefulness of various compound combinations for improved efficacy and reduced toxicity.

Melanin content

The eumelanin and pheomelanin contents of newly grown hair from previously plucked areas on the backs of black mice were examined using a method established by Ito and jimbow (1983). Briefly, the eumelanin content was determined by the KMnO4-induced chemical breakdown of eumelanin. Then, HPLC with a UV detector was used to examine the product, pyrrole-2, 3, 5-tricarboxylic acid (PTCA) (Prota G 1972 and 1980).

The samples were each measured three times. The data are given in nanograms of PTCA per milligram of wet tissue. One nanogram of PTCA equals around 50 nanograms of eumelanin. After the chemical degradation of pheomelanin by hydroiodic acid into amino hydroxy-phenylamine (AHP), the pheomelanin content was determined by HPLC. The results are reported in nanograms of AHP per milligram of tissue, with one nanogram of AHP equaling five nanograms of pheomelanin (Prota G, 1972 and 1980).

GHS Assay

Hair follicles were extracted from black mice and supplied to the mice via an external treatment. The mice were divided into four groups: one control group and three experimental groups treated with

different doses of 4HA. The animals were sacrificed via cervical dislocation after 21 days of ointment administration.

The skin was peeled out and rinsed in ice-cold saline before being preserved in 10% neutral formalin and processed using standard histology techniques. The H/E and F/M methods were used to stain the cross-sections.

Tietze and Griffith (1980) describe a method for measuring total GSH content that is later adopted. First, the frozen tissues were weighed and then homogenized using a glass-glass homogenizer in 5.0 ml of 1 per cent picric acid on ice. Next, the homogenates were centrifuged, and the supernatants were collected and employed in an oxidized GHS reductase test to estimate the total GHS level in the tissue.

Fig1.Topical application .

Fig 2. Topical application of 4HA+ TRA

Fig 3. Pure white depigmented black mice

Results

4HA can induce depigmentation

Black hair follicles: When 4HA was applied topically during the anagen phase of hair regrowth, it had a dose-dependent effect on follicular melanocytes, resulting in depigmenting alterations in their coat colour. A dose of 4HA did not produce any visible depigmentation of the black follicles (Fig1.). In contrast, a dose of 2 % 4HA +0.01 % TRA was partially effective in the newly growing hair follicles that were brownish-grey colour (Fig 2.) 3 % 4 HA + 0.01 % TRA was the most effective, resulting in pure white depigmented hair follicles (Fig 3.) The black follicles did not show any obvious depigmentation after receiving a dosage of 4HA (Fig1.) However, a dose of 2% 4HA +0.01% TRA was only partially

effective in freshly emerging hair follicles that were brownish grey (Fig 2.) 3 % 4 HA + 0.01 % TRA was the most effective in pure white depigmented hair follicles (Fig 3.) Each skin site is given a score between 0 and 5, with 0 indicating no evidence of depigmentation and 5 indicating complete depigmentation at that site. As a result, each mouse's maximum score is 20. A blinded yet trained investigator who is not aware of the experimental set up information should rate the vitiligo mice for unbiased scoring. The results can be seen in Table 1.

Table 1: Mice develop epidermal depigmentation over the course of 7 weeks.

Leucoderma Scoring Point System						
SCORE	***0***	***1***	***2***	***3***	***4***	***5***
Below the Head & neck	0	<10%	10–25%	>25–50%	>50–75%	80-100%
Tail	0	<10%	10–25%	>25–50%	>50–75%	80 -100%

After treatment with 4 HA/TRA and GSH-modulating agents, the amount of melanin in hair follicles changes

4-Hydroxy anisole/Trans Retinoic acid (Gregg et al., 2010)

Table 2 summarizes the amounts of eumelanin and pheomelanin in hair samples from black mice treated with various concentrations of 4HA. 2 % 4 HA/TRA treatment with 10, 25, and 50 mg/kg in black mice resulted in a dose-dependent reduction in eumelanin content of hair follicles to 98 %, 28 %, and 5% of the control group, respectively. In the hair follicles of black mice, only trace quantities of pheomelanin were found.

Table 2. Amount of Eumelanin and Pheomelanin in Black Mice with 4HA/TRA

Animals	**4HA/TRA mg/kg**	**PTCA ng/mg**	**AHP ng/mg**	**PTCA/AHP ratio**
C57BL/6j, a/a	control	1077.16±7.88	57.86±0.57	18.61
	10 mg	1056.50±5.04	48.02±1.55	22.0
	25 mg	371±2.54	54.40±0.81	6.82
	50 mg	44.95±0.65	57.18±0.77	0.77

Data expressed as sample mean±SD (n=6)

GSH-Modulating agents

Table 3 shows the findings of eumelanin and pheomelanin content measurements in the hair follicles of black mice given three different medication regimens. Grey hair follicles of mice treated with 4HA/TRA contained 29 % of the eumelanin content of controls expressed as its PTCA derivative in black mice. The amount of eumelanin in black hair follicles from mice given 50 mg/kg was reduced to 14 % of that in controls.

GSH content in skin changes after drug treatment

In the tissues of black mice, the 4 HA/TRA treatments resulted in a dose-dependent depletion of GSH. In black mice, dosages of 4HA of 10, 25, and 50 mg/kg lowered GSH content in the skin to 95 %, 85 %, and 76 % of control levels, respectively, as shown in Table II. GSH depletion was also observed in the lung and liver at the exact dosages of 4HA/TRA.

Table 2. GSH concentration of black mice's skin, lungs, and liver

Animals	**4HA/TRA**	**GSH content (µmol/g of tissue**		
C57BL/6j, a/a	**mg/kg**	**Skin**	**Lung**	**Liver**
	control	0.545±0.006	0.55±0.007	5.02±0.004
	10	0.510±0.006	0.51±0.006	5.01±0.003
	25	0.468±0.003	0.47±0.003	4.40±0.019
	50	0.3710±0.002	0.37±0.002	3.22±0.009

Data expressed as mean±SD (n=6)

The cross-section of the **F/M method** is displaced here from Fig 4 to 11. Fig 4 confirms polygonal and spindle-shaped cells with abundant melanin content in the control sample, whereas Fig 5 picturizes the complete absence of melanin in the melanocytes (11). Fig 6 shows the absence of melanin in the hair shafts. However, the right top in the boundary of treatment and non-treatment presence of melanin in the hair shaft is observed. Similarly, Fig 7 puts up the absence of melanin in the hair roots and the few presences of melanin content at the base of the hair roots in the bottom of Fig 16. Fig 8 shows the presence of melanin in the hair shafts, whereas Fig 9 projects the absence of the same in the treated portions but for few streaks here and there. Fig 10 & 11 show the complete absence of the melanin content in the treated layer of the skin.

The cross section of F/M method

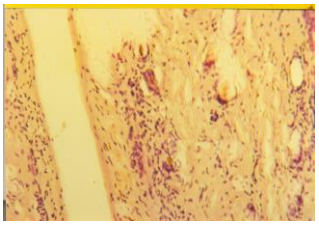

Fig.4 (160x)

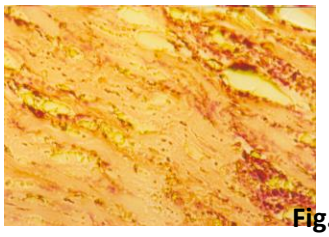

Fig.5 (160x)

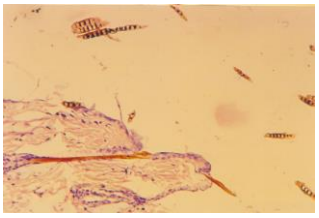

Fig. 6 (160x)

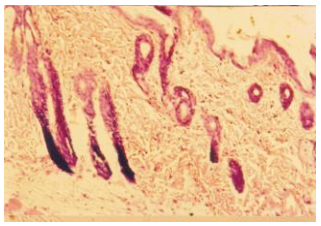

Fig. 7 (160x)

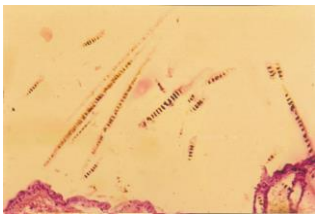

Fig. 8 (250x)

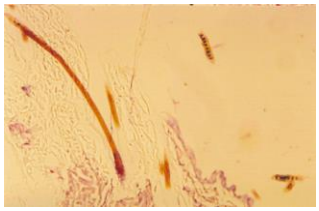

Fig. 9 (250x)

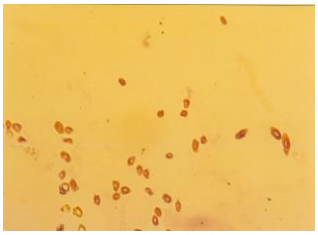

Fig. 10 (160x)

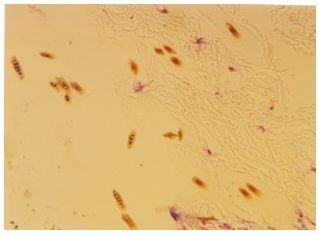

Fig. 11 (160x)

Discussion

Inactivated follicular melanocytes, in vivo feeding of 4HA/TRA to black C57BL/6j mice revealed exceptional depigmenting potential. Depigmentation was shown to be dose-dependent, ranging from no obvious depigmentation to various degrees of grey and pure white depigmentation of newly growing hair. The depigmenting effect of phenolic compounds on melanocytes is thought to be due to melanocytotoxicity caused by the interaction of these chemicals with tyrosinase (Denton et al., 1952 and Ito S. Jimbow K 1983). Tyrosinase is expected to convert phenols into highly reactive orthoquinones, leading to semiquinone radicals and derivatives. When given at the start of the melanogenic phase of hair growth in black mice, even an external application of 4HA/TRA was capable of causing total depigmentation of new hair. Spontaneous models of leucoderm provide an opportunity to investigate how vitiligo is initiated, and the genetic contributions to disease. The black mice appears to exhibit multiple characteristics of vitiligo that parallel human disease. (Kligman AM, and Willis I 1975 Sanquer et al., 1998). Future studies in this model may help to clarify interactions between melanocyte stress and autoimmunity that drive depigmentation. Induced models of leucoderma have been primarily conducted in black mice, which are less expensive to breed and maintain, and provide a large number of tools to study mechanistic contributions to vitiligo pathogenesis. These models are well suited to study mechanisms that drive disease progression, which are particularly relevant to therapeutic intervention. In summary, animal models provide an opportunity for mechanistic studies to define leucoderma pathogenesis (Subasini et al., 2009). This induction model has its strengths and weaknesses, and should be selected based on the experimental questions being addressed.

Conclusion

Vitiligo is a severely disfiguring autoimmune skin disease that affects about 1% of the population. Vitiligo patients develop patchy white spots on the skin that result from the loss of pigment-producing

cells in the epidermis known as melanocytes. Mouse models of vitiligo have been critical to the understanding of vitiligo pathogenesis and research using these models has revealed key immune pathways responsible for human vitiligo pathogenesis. More broadly, there is also considerable overlap between the pathways responsible for vitiligo and those of other organ-specific autoimmune diseases. Therefore, vitiligo mice are not only an excellent tool for the investigation of vitiligo disease pathogenesis, but also reveal basic scientific principles that lead to the identification of new targets for therapeutic use in autoimmunity.

Acknowledgement

The author express their gratitude to Vice-Chancellor R. Sethuraman and his capable administrative colleagues for allowing us to accomplish this effort. We would also like to express our gratitude to the Drugs and Pharmaceuticals Division of the Department of Science and Technology, Government of India, New Delhi, and M/S Citadel Fine Chemicals, Chennai, for their financial support project.

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