STUDIES ON HAIR LOSS (ALOPECIA) ASSOCIATED WITH USE OF COSMETIC HAIR PRODUCTS AND INGREDIENTS IN THESE PRODUCTS

FINAL REPORT

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1 EXECUTIVE SUMMARY

This final report provides an overview of preclinical investigations undertaken to evaluate hair loss (alopecia) and the potential mechanisms of alopecia associated with the use of select commercially available hair care products. The study consisted of two projects; Project 1, the in vivo study using a murine model and Project 2, the in vitro cytotoxicity of test products and ingredients found in these hair care products. A list of priority hair care products and ingredients was provided by the FDA and included four test products and 21 test ingredients.

These investigations were conducted in stages. The initial stage comprised a series of in vivo pilot studies to identify (i) variables and confounding factors that might affect the validity and results of the experiment, (ii) the experimental conditions that minimally interfere with HF cycling, and (iii) an application method as close as possible to the real “in use” situation to enhance the skin accessibility of the test products. The results of these pilot studies helped establish the technical standards and methods, validate the preclinical models used in Project 1, and test the technical feasibility of long-term in vivo alopecia studies, for which there exists no extensive literature to date. In vitro pilot studies were conducted to optimize experimental conditions and determine dose-ranges.

In Project 1, the potential association between the four hair care products and alopecia was evaluated employing the depilation-induced synchronized hair follicle (HF) model in C57BL/6J mice. In Project 2, the potential effects of the test products and the hair care product ingredients on cell viability and growth were investigated using human hair follicle (HF) dermal papilla cells (DPCs) and normal human epithelial keratinocytes (NHEKs). Test ingredients that demonstrated cytotoxicity were further evaluated for apoptosis induction in DPCs.

This report is intended to highlight the design and development of appropriate research approaches and to discuss the research findings. It is organized into two main parts: the first describes the results of Project 1, and the second describes those of Project 2. Each of the projects includes a detailed description of its testing models and methodologies, discusses its challenges, and provides recommendations for future studies.

Key findings Project 1. Alopecia Assessment of Test Hair Care Products Using Synchronized Murine HF Model.

- *A delay in progression to the 2nd anagen phase was observed in mice treated with WEN Sweet Almond Mint Cleansing Conditioner (WEN) or DevaCurl Low-Poo Delight Cleanser (DevaCurl), compared to the mock cohort.*
- *Treatment with either Monat Renew Shampoo (Monat) or WEN substantially increased total mast cell numbers at Day 98.*
- *WEN caused significant increases in mast cell activation.*

Key findings Project 2. In vitro Cytotoxicity Assessments of Ingredients Found in Hair Care Products.

- *All test products demonstrated cytotoxicity within 24h in DPCs.*
- *Compared to the other test products, Monat was the most cytotoxic in both DPCs and NHEKs.*
- *Of the 20 test ingredients, 11 demonstrated varying degrees of cytotoxicity in DPCs within 72h. Of these, 7 induced acute cytotoxicity in DPCs, decreasing cell viability by more than 50% within 24h. These included MCI, MI, CAPB, Lavender oil, Polysorbate 60, CATC, and Pea extract.*
- *Of all ingredients tested, MCI and MI were the most cytotoxic.*
- *Apoptosis was detected in DPCs treated with Guar, Lavender oil, or Rosemary extract, while Calendula extract, CATC, and Pea extract primarily induced necrosis.*

Overall Conclusion, Challenges, and Recommendations for Future Studies:

- The observation of a prolonged duration of the telogen phase in mice treated with DevaCurl or WEN products demonstrates that hair cycle abnormalities could be triggered by repeated applications of hair products. A prolonged telogen may result in a delay in anagen induction and subsequent hair growth.
- While the majority of ingredients differed between these products, five ingredients that were cytotoxic to DPCs were present in both DevaCurl and WEN (i.e., Rosemary extract, Calendula extract, MCI, MI, and Polysorbate 60). The relevance of these ingredients to alopecia warrants further investigation.
- Future in vivo long-term treatment studies to assess for alopecia should include a sufficient number of animals to statistically power the study.
- Transcriptomic and cytokine profiling of DevaCurl- and WEN-treated skin may help identify molecular signatures and inflammatory responses that contribute to aberrant hair cycling associated with these products.

2 PROJECT INFORMATION (Table 1)

Table 1. Project information.

Project Title	Studies on hair loss (alopecia) associated with the use of cosmetic hair products and ingredients in these products
Project Period	August 31, 2018 – August 30, 2022
Name of Grantee	[redacted]
Contract Number	HHSF223201810176P
Performance Site	[redacted]
Project Status	Overall project progress: Completed.* Overall project milestones: Achieved.

*, Ki67/TUNEL staining was not performed based on the assessments of HF morphology and skin pigmentation.

3 PROJECT TIMELINE (Table 2)

Table 2. Project timeline.

		Pilot Study 1: Development of methods technical standards for long-term treatment	Pilot Study 2: Methods validation Selection of HF model and time points (Spontaneous HF Model)	Pilot Study 3: Methods validation Selection of HF model and time points (Synchronized HF model)	Pilot Study 4: Optimization of in vitro experimental conditions and dose-range determination	In Vitro Studies: Cell viability (DPCs, NHEKs) Apoptosis (DPCs)	EXP-1: In vivo Alopecia Assessment	EXP-1: In vivo Alopecia Assessment	EXP-2: In vivo Alopecia Assessment
	Treatment duration	57 days	98 days	70 days	N/A	6 days		85 days	98 days
	No. cohorts	5 cohorts	4 cohorts	4 cohorts	N/A	N/A	5 cohorts	5 cohorts	4 cohorts
	No. mice	40 mice	15 mice	12 mice	N/A	N/A	80 mice	80 mice	64 mice
2018 Sep	Work plan draft								
2018 Dec	Kick-off meeting								
2019 Jan									
2019 Feb	IACUC protocol obtained								
2019 Mar	Final work plan								
2019 Apr									
2019 May	Modifications to IACUC	Pilot Study 1							
2019 Jun		Pilot Study 1							
2019 Jul		Pilot Study 1							
2019 Aug		Pilot Study 1							
2019 Sep		Pilot Study 1							
2019 Oct			Pilot Study 2	Pilot Study 3					
2019 Nov			Pilot Study 2	Pilot Study 3					
2019 Dec			Pilot Study 2	Pilot Study 3					
2020 Jan			Pilot Study 2	Pilot Study 3					
2020 Feb					Pilot Study 4		Mice purchased		
2020 Mar					Pilot Study 4		Study suspended/ mice euthanized*		
2020 Apr	Expanded work plan				Pilot Study 4				
2020 May					Pilot Study 4				
2020 Jun					Pilot Study 4				
2020 Jul						In vitro studies			
2020 Aug	Project setback*								
2020 Sep	Project setback*								
2020 Oct	Project setback*								
2020 Nov	Project setback*								
2020 Dec	Project setback*								
2021 Jan	Project setback*								
2021 Feb	Project setback*								
2021 Mar						In vitro studies			
2021 Apr						In vitro studies			
2021 May						In vitro studies			
2021 Jun						In vitro studies			
2021 Jul						In vitro studies			
2021 Aug						In vitro studies			
2021 Sep								Mice purchased	
2021 Oct								Exp-1 in vivo	
2021 Nov								Exp-1 in vivo	
2021 Dec								Exp-1 in vivo	
2022 Jan									Mice purchased
2022 Feb									Exp-2 in vivo
2022 Mar									Exp-2 in vivo
2022 Apr									Exp-2 in vivo
2022 May									Exp-2 in vivo
2022 Jun									
2022 Jul									
2022 Aug									

* In response to the COVID-19 pandemic, Columbia University implemented a ramp-down of all non-essential on-site laboratory research. Under the Institute of Comparative Medicine's contingency plans, the on-site research activity of Project 1 was deemed non-essential, as C57BL/6 mice used in this study were commercially available and this research activity was not needed for a pending publication or grant application. Therefore, Project-1 Exp-1, which was scheduled to begin on March 17, 2020, was suspended, and mice were euthanized, causing a delay in the project's progress. Project completion was further delayed due to the lack of research personnel and the Institutional hiring freeze.

4 PROJECT 1: Alopecia Assessment of Test Hair Care Products Using Synchronized Murine HF Model

4.1 Project Rationale and Objectives

Sudden, temporary hair loss (alopecia) can occur due to a host of diverse stimuli (e.g., physiologic and psychological stress, parturition, major surgery, hormonal changes, physical trauma), as well as exposure to allergens and chemicals present in hair care products [1-3]. In fact, many ingredients used in the hair care industry are known contact allergens and potential toxicants [4, 5]. These include various fragrances (e.g., fragrance mix (FM)1, FM2, balsam of Peru), surfactants (e.g., cocamidopropyl betaine, lauryl polyglucoside, decyl glucoside), and preservatives (e.g., Kathon CG, formaldehyde, captan) [5-8]. The safety and toxicity data of these ingredients relevant to hair disorders are largely unknown. The objectives of this project were to assess the potential effects of commercially available hair care products on hair loss by utilizing a well-characterized murine model of hair growth and to gain a better understanding of the alopecia mechanisms that are associated with the use of hair care products.

4.2 Test Products

The test products were identified by the FDA based on the information available on the CFSAN Adverse Event Reporting System (CAERS). Several lines of WEN cleansing conditioner products (e.g., Sweet almond mint cleansing conditioner, Lavender cleansing conditioner, Pomegranate cleansing conditioner) and Monat shampoo products (e.g., Renew shampoo, Intense Repair shampoo, Revive shampoo), as well as several lines of DevaCurl hair cleanser products (e.g., Low-poo delight cleanser, No-poo original cleanser, Low-poo original cleanser) have been reported to cause various hair disorders. Alopecia represents the primary adverse event associated with the use of these products. The severity of alopecia associated with WEN products ranges from balding patches to loss of one-quarter to one-third or more of hair, which often continues for weeks, even if the consumer immediately discontinues the use of the product. Alopecia is also indicated in the majority of complaints associated with WEN and Monat products received by the FDA. In addition to excessive hair loss, other adverse events reported in the complaints related to these hair care products include hair breakage, thinning hairs, as well as various scalp problems (e.g., pruritus, irritation, rash, seborrhoeic dermatitis). There also appear to be some differences in the adverse reaction profiles and frequencies between the three brands. For example, abdominal and breast pain, menstrual disorders, and abnormal hormone levels are reported in some of the complaints related to Monat products, albeit in low frequency. Irritation/sensitization symptoms, on the other hand, are more prevalent in DevaCurl complaints. While these differences may be contributed by concomitant genetic/environmental factors, it raises a concern of possible systemic influence. This study evaluated three test products for potential effects with regard to hair loss (i.e., DevaCurl Low-poo delight cleanser, Monat Renew shampoo, WEN Sweet almond mint cleansing conditioner), which had the most adverse event reports from each brand **(Table 3)**. Aquaphor baby wash & shampoo was used as a control product, selected by the FDA **(Table 3)**.

Table 3. Test Products and control drugs/products.

Name	Manufacturer	Vendor	Cat. No.	Lot No.
Aquaphor Baby Wash & Shampoo-Fragrance free *	Aquaphor	https://www.amazon.com	N/A	01065957
DevaCurl Low-Poo Delight Cleanser	DevaCurl	https://www.amazon.com	N/A	0006019A
Monat Renew Shampoo	Monat	https://corp.mymonat.com	N/A	19J0813144
WEN Sweet Almond Mint Cleansing Conditioner	WEN by Chaz Dean	https://chazdean.com	N/A	07022-006
Cyclophosphamide for Injection, USP **	Sandoz	https://www.medline.com/	0781-3233-94	19030125
Rogaine (5% Minoxidil) ***	Johnson & Johnson	https://www.rogaine.com	N/A	0719CP

*, a control product; **, a control drug for hair loss; ***, a control drug for hair growth

4.3 Testing model and Methods

The murine model of synchronized hair growth

The HF undergoes continuous cycles consisting of regeneration (anagen, the actively growing stage), apoptosis-driven organ involution (catagen, the regression stage), and a resting stage (telogen) [9]. In both humans and mice, this process is tightly controlled by numerous stimulatory and inhibitory factors (e.g., hormones, growth factors, cytokines, neuropeptides, transcription factors, adhesion molecules) through signaling interactions between the dermal papilla cells ─a cluster of mesenchymal cells located at the base of the HF─ and HF keratinocytes [10, 11].

The mouse is an excellent model with which to study the hair cycle for several reasons: the first two cycles of the mouse HFs are synchronized, the mouse hair cycle is short (~3 weeks), and the HF stages have been well characterized and can easily be examined at specific time points in the cycle. In addition, various transgenic murine models of hair abnormalities are available for studying the genetic aspects of hair disorders [12, 13]. However, although mouse HFs share the same essential features as human HFs, and HF cycling does not differ structurally between mice and humans, there exist some species-specific differences. For example, the human hair cycle occurs asynchronously in the scalp, and the anagen phase of human HFs lasts from 3−5 years [13]. Furthermore, the capacity for percutaneous absorption likely differs between humans and mice, as the human dermis is substantially thicker than the mouse dermis and contains fewer HFs. Moreover, mice do not suffer from androgenetic alopecia (AGA), the most common form of hair loss in humans, and the key mechanisms controlling androgen-dependent HF miniaturization in the human scalp are not recapitulated in mice [14]. These species-specific differences in HF growth and regulation must be considered carefully when interpreting the outcomes of mouse studies [14, 15].

This study used the inbred C57BL/6 strain, one of the most extensively studied and best-standardized hair research models [16, 17]. In C57BL/6 mice, and other murine strains (e.g., CBA/J, C3H, BALB/c), HFs on dorsal skin at postnatal day 60 (P60) are predominantly in the telogen stage. The removal of telogen hair shafts by depilation immediately initiates synchronized hair growth with all follicles entering the final stage of the growth cycle (anagen VI) on day 9 post depilation. After full anagen development, the consecutive stages, catagen, and telogen, develop spontaneously in a relatively homogeneous pattern. The depilation-induced synchronized HF model is widely used in hair biology research as it allows the evaluation of specific HF stages at specific time points. It also provides an adequate in vivo platform for the preclinical evaluation of both drug efficacy and safety testing for humans [13].

Depilation

To induce synchronized hair growth, the back hairs of P60 mice were shaved using an animal clipper. Nair hair removal cream (Lot no. LL8331, purchased from Amazon) was then applied to the shaved dorsal skin for 3 min to remove the hair shafts. The depilated area was thoroughly washed using a spray of warm water. Depilation was performed under anesthesia.

Sex and age
Animals of either sex are typically used in in vivo hair research [18]. Hair cycle studies customarily begin with the second telogen–anagen transition (7-8 weeks of age after birth) which has shown to be highly reproducible and reliable in C57BL/6 mice [19]. Female C57BL/6J mice, postnatal day 50 (P50), were purchased from Jackson Laboratory (stock no. 000664).

Housing condition
Mice were housed in groups of four animals per cage under pathogen-free conditions in the animal facilities of Columbia University. Mice were kept in 12h light/dark cycles in a temperature-controlled (20–25ºC) room with a 50~60% relative humidity and given a standard rodent diet and water *ad libitum*.

Ethics statement
All animal experiments described in this report and animal procedures including euthanization were performed according to the approved Columbia University Institutional Animal Care and Use Committee (IACUC) protocol (AC-AABM0551).

4.4 Administration of Test Products

Experimental conditions were established based on the results of three pilot studies conducted to identify (i) variables and confounding factors that might affect the validity and results of the experiment, (ii) the experimental conditions that minimally interfere with HF cycling, and (iii) an application method as close as possible to the real “in use” situation to enhance the skin accessibility of the test products. The mice subjected to the following experimental conditions and application methods for 10 -14 weeks presented no acute, as well as chronic, dermal, or systemic toxicity.

Experimental setup
Fig. 1 shows the experimental setup and materials used in the mouse studies.

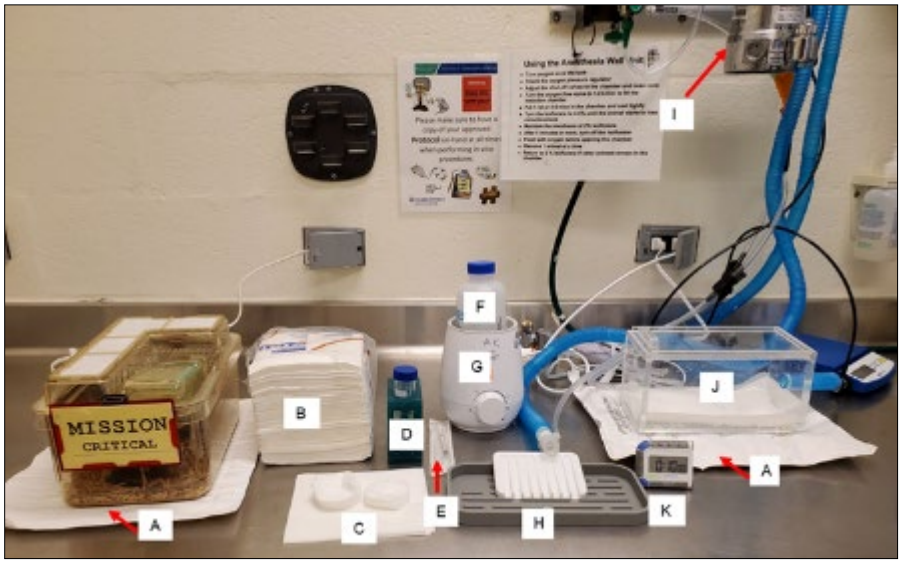


Figure 1. Materials for treatment and cleansing. A. Electric heating pad, **B.** Paper towels, **C.** Cotton pads, **D.** Test product in a 50 ml conical tube, **E.** 1 ml syringe, **F.** Water bottle, **G.** Baby bottle warmer, **H.** Soap trays, **I.** Isoflurane machine, **J.** Anesthesia induction chamber, and **K.** Timer.

Application
The depilated dorsal site was first wetted using a water-soaked cotton pad. The test product was applied to the site using a repeating pipette (Bel-Art SP Scienceware). The site was then gently rubbed and left uncovered for 10 min. After 10 min, the site was washed with a spray of warm water. The entire procedure typically took ~ 15 min per mouse and was performed under anesthesia in conjunction with electric heating pads to prevent hypothermia.

Administration volume
0.3 ml (0.05 ml/cm^2) of the test product was applied per mouse. This amount sufficiently covers the entire application site (~ 6 cm^2) and is comparable to that recommended for the WEN products (0.04 ml – 0.07 ml/cm^2 in humans with short hair). The administration volume was increased to 0.5 ml per application when hairs in the depilated area regrew.

Treatment frequency and duration
Once a day, five days/week, until the majority of the mice in the mock cohort have either progressed to the anagen stage or regrown their hair (12 – 14 weeks).

4.5 Study Design

Control drugs and product
The pharmaceutical grade cyclophosphamide (CYP, Baxter) and Rogaine (5% minoxidil solution, Johnson & Johnson) were used as controls for hair loss and hair growth, respectively. Aquaphor baby wash shampoo served as a control product.

CYP-induced disruption of actively growing anagen HFs in C57BL/6 mice is a clinically relevant model that has been extensively used in studying the biology of chemotherapy-induced alopecia [20-22]. Minoxidil (2,4-diamino-6-piperidino-pyrimidine-3-oxide) is the most commonly used drug for the treatment of androgenetic alopecia. It has been shown to shorten the telogen stage, while prolonging the anagen stage through both proliferative and anti-apoptotic effects on the dermal papilla cells of human HFs [23]. A topical minoxidil solution (2-5%) has also been shown to enhance hair growth in mice (e.g., C57BL/6J, CBA/J mice) [24, 25].

Cohorts
The animal study was performed in two sequential experiments, consisting of nine cohorts of 16 mice each **(Table 4)**. Baseline body weight and baseline blood were obtained from all mice. The treatment began two days after depilation. The first experiment (Exp-1, Cohorts 1-5) included nontreated, mock, DevaCurl, Rogaine, and CYP. Exp-2 (Cohorts 6-9) included mock, Aquaphor, Monat, and WEN. Nontreated and CYP cohorts served to validate normal HF cycling in C57BL/6 mice. The mock cohorts of Exp-1 and Exp-2 received water and were subjected to the same experimental conditions as the mice that received the test products. The mock cohorts served as a bridge between the two experiments.

Table 4. Treatment cohorts.

Exp	Group	Cohorts	No. Mice*	Dose or Administration volume	Sample Collection Base	Sample Collection TP1, 2
Exp-1	1	Non-Treatment	16	none	B	B/S/L/H
Exp-1	2	Cyclophosphamide	16	150 mg/kg. *i.p***	B	B/S/L/H
Exp-1	3	Mock Treatment (Water) #	16	0.3 – 0.5 ml*** †	B	B/S/L/H
Exp-1	4	DevaCurl Low-Poo Delight Cleanser	16	0.3 – 0.5 ml*** †	B	B/S/L/H
Exp-1	5	Rogaine	16	0.3 – 0.5 ml*** †	B	B/S/L/H

Exp	Group	Cohorts	No. Mice*	Dose or Administration volume	Sample Collection Base	Sample Collection TP1, 2
Exp-2	6	Mock Treatment (Water) #	16	0.3 – 0.5 ml*** †	B	B/S/L/H
Exp-2	7	Aquaphor Baby Wash & Shampoo	16	0.3 – 0.5 ml*** †	B	B/S/L/H
Exp-2	8	Monat Renew Shampoo	16	0.3 – 0.5 ml*** †	B	B/S/L/H
Exp-2	9	WEN Sweet Almond Mint Cleansing Conditioner	16	0.3 – 0.5 ml*** †	B	B/S/L/H

* n=8 per each time point (TP1, TP2).
** a single i.p injection at 1st anagen.
*** once a day, five days per week, until the majority of the mice in the mock cohort have either progressed to the 2nd anagen stage or regrown their hair (12-14 weeks).
† Administration volume was increased to 0.5 ml when hairs in the depilated area regrew.
# Mock cohort is included as an internal control in both Exp-1 and Exp-2 and served as a bridge between the two experiments.
B, blood; S, skin; L, liver; H, hair.

Timepoints

Half of the mice in each cohort (n=8) were evaluated at the 1st anagen–1st telogen HF transition (Timepoint 1) and the remaining half (n=8) at the 1st telogen–2nd anagen HF transition (Timepoint 2) **(Fig. 2A)**. The anagen-telogen HF transition corresponded to Day 21, and the telogen-anagen HF transition corresponded to Day 85 (Exp-1) and Day 98 (Exp-2).

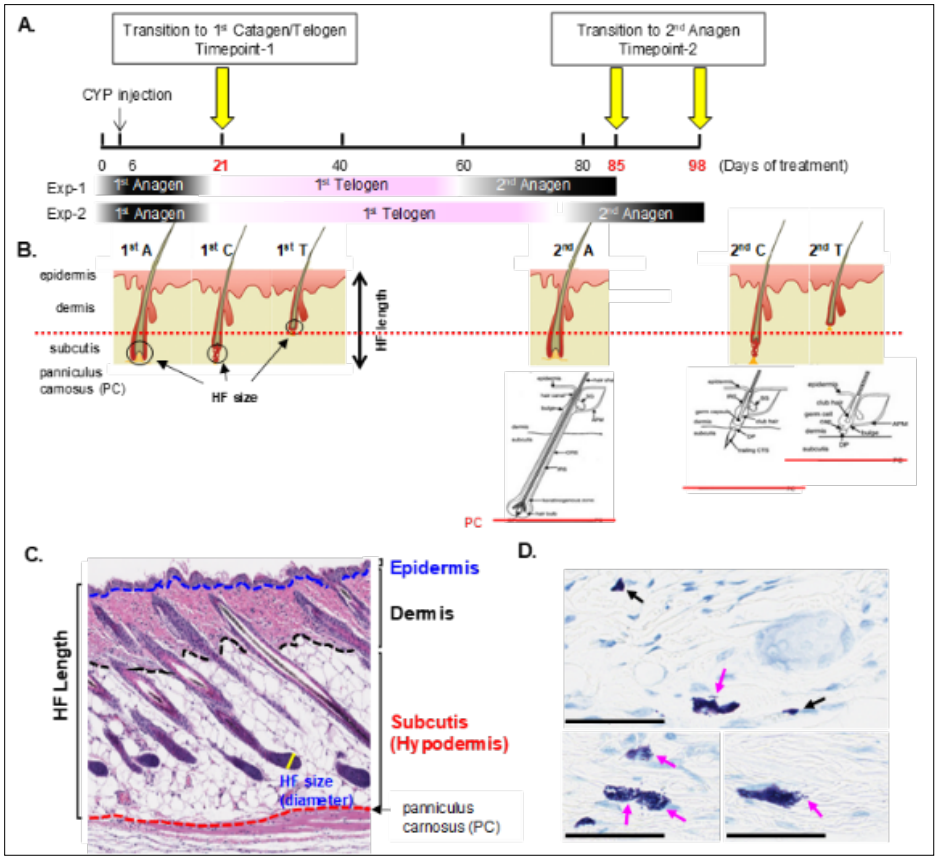


Figure 2. Study overview. A. Treatment timeline. B. Schematic representation of hair cycle associated changes in HF length and size (diameter, arrows) in correlation with the panniculus carnosus (PC) and

the dermis/subcutis border [1]. A, anagen; C, catagen; T, telogen. C. Representative image of mouse skin showing different layers of skin and HFs. HF length and size (diameter) measured in this study are indicated. Blue dotted line, epidermis/dermis border; black dotted line, dermis/subcutis border; red dotted line, subcutis/PC border; solid yellow line, HF diameter. D. Representative images of nondegranulated (nonactivated) mast cells (black arrows) and degranulated (activated) mast cells with extracellular granules (magenta arrows). Scale bar = 50 μm.

Data collection

Data were collected for the following evaluations **(Table 5)**: i) the hair growth pattern and presence of alopecia (Evaluation 1), ii) the correlations between skin pigmentation and HF morphology (Evaluation 2), iii) the extent of hair damage (Evaluation 3), and iv) the presence of mast cells (Evaluation 4) and macrophages (Evaluation 5).

Body weight measurements and liver histology were assessed for possible systemic effects of the test products (Evaluation 6).

Table 5. Data Collected.

Evaluation	Data Collected	Data Collection Time
1. Hair growth	Photo documentation of hair growth	2x/week during anagen; 1x/week during telogen
1. Hair growth	Quantitation of skin pigmentation (gray intensity)	2x/week during anagen; 1x/week during telogen
1. Hair growth	% area of full hair growth	TP2
1. Hair growth	% area of anagen skin	TP2
1. Hair growth	% area of telogen skin	TP2
1. Hair growth	% area of depigmented hairs	TP2
2. HF	HF morphology (H&E)	TP1, TP2
2. HF	Quantitation of catagen and telogen HFs	TP1
2. HF	Quantitation of 1st telogen, 2nd anagen – 2nd telogen HFs	TP2
2. HF	HF diameter and length	TP1, TP2
2. HF	HF dystrophy (H&E)	TP1, TP2
3. Hair structure	Representative photos	TP1, TP2
3. Hair structure	% focal bulge	TP1, TP2
3. Hair structure	% pigment clumping	TP1, TP2
3. Hair structure	% structural weakness	TP1, TP2
4. Mast cells	Toluidine blue staining	TP1, TP2
4. Mast cells	Quantitation infiltrating/degranulated mast cells	TP1, TP2
5. Macrophages	Immunohistochemical staining for F4/80	TP1, TP2
5. Macrophages	Quantitation of F4/80+ macrophages	TP1, TP2
6. Systemic effects	Liver histology	TP1, TP2
6. Systemic effects	Weight measurement	1x/week

TP, timepoint; H&E, hematoxylin and eosin

4.6 Tissue Collection and Storage

Blood: Blood was collected from the submandibular vein at baseline and by cardiac puncture at timepoints 1 and 2. The blood was processed to obtain serum, which was then stored at -80 °C for future studies.

Skin: Full-thickness dorsal skin (1 x 2 cm) was collected at timepoints 1 and 2 from the same area on all mice. A longitudinal section was fixed in 10% buffered formalin and paraffin-embedded for histological analyses. A portion was snap-frozen and stored at -80 °C for future studies.

Hair: Hair samples were obtained at timepoints 1 and 2 by plucking them lightly from the unshaved location on the dorsal skin. The collected hairs were placed in a microcentrifuge tube and stored at room temperature.

Liver: Liver samples were collected at timepoints 1 and 2. One piece of liver tissue (1 x 1 cm) was taken from the right lobe, formalin-fixed, and paraffin-embedded for histological evaluation.

4.7 Analyses

Body weight measurement
Mouse body weight was obtained at baseline and every week.

Photo documentation
Images of mouse dorsal skin were acquired twice a week during anagen progression, and once a week during the telogen phase. A light-equipped photo box that provides consistent lighting and prevents shadow and reflection in the photo was used for photo documentation **(Fig. 3A)**. The distance of the light source to the subject (23 cm) was kept constant for all imaging.

Measurement of skin pigmentation and hair growth
Melanin pigmentation in C57BL/6J black skin is tightly coupled to the HF, which makes it easy to correlate skin colors with the underlying hair cycle stage (e.g., gray-to-black colored anagen skin of active hair growth and pink colored skin of the resting telogen stage). Grayscale values were used to determine the level of skin pigmentation. Using ImageJ software (https://imagej.nih.gov/ij/download.html), regions of interest (ROI) (e.g., entire depilated area, gray area, pink area) were drawn over the photographic images to obtain the mean grayscale value (average counts per pixel) **(Fig. 3B.a)** and the area of ROI **(Fig. 3B.b)**. Image J displays values ranging from 0 to 255, where zero represents black and 255 represents white. For data interpretation purposes, the gray value is represented by subtracting the original value from 255. “Area of visible hair growth" and "area of gray pigmentation and hair growth" are presented as percent relative to the entire depilated area (i.e., treatment area) (**Fig. 3B.b**). To better observe changes in skin pigmentation, entire dorsal hairs except for the upper region of the application site were shaved when the majority of the mice has transitioned to catagen/telogen (D20).

Figure 3. Image acquisition and measurement of skin pigmentation. A. Photo box used in study. B. Measurement of gray values (a) and % gray area (b).

Histological analysis of hair follicles

5-µm thick paraffin sections of dorsal skin tissues obtained at timepoints 1 and 2 **(Fig. 2A)** were stained with hematoxylin and eosin (H&E). H&E-stained sections were imaged at 40x magnification using the Aperio AT2 DX System (Leica Biosystems). The digital images were visualized using Aperio ImageScope software (Leica Biosystems), and the HF stage was evaluated manually according to the previously published guidelines for hair cycle staging and morphological characteristics of the murine hair cycle [19, 26]. At least 50 longitudinally cut HFs per mouse were analyzed for the following three parameters of HF growth **(Fig. 2B, 2C)**: skin thickness corresponding to HF length, HF size corresponding to the average diameter of the hair bulb, and the HF stage percentage. Skin thickness was defined as the distance from the epidermal granular layer to the top edge of the panniculus carnosus [26, 27].

Histological analysis of liver

5-µm thick paraffin sections of liver tissues obtained at timepoints 1 and 2 were stained with H&E. H&E-stained sections were imaged at 40x magnification using the Aperio AT2 DX System (Leica Biosystems). The digital images generated were visualized using Aperio ImageScope software (Leica Biosystems), and signs of hepatic damage (e.g., necrosis, inflammation, etc.) were assessed. Liver tissues were not evaluated by a pathologist. A pathologist is consulted for further analysis only if any forms of changes in the liver are observed or suspected.

Assessment of mast cell infiltration and degranulation

Mast cells and their characteristic metachromatic granules were visualized histochemically by staining the deparaffinized skin sections in freshly prepared toluidine blue solution (0.5%, pH 2) for 1 min at room temperature [28]. Toluidine blue-stained sections were imaged at 40x magnification and evaluated using the Aperio AT2 DX System and Aperio ImageScope software, respectively, as described in the previous section. The number of positively stained cells (with and without granules) in the dermis and subcutis was counted. Mast cells with more than five granules located outside of the cell membrane were counted as degranulated (activated) [29] **(Fig. 2D)** and the ratio of degranulated mast cells to all mast cells was determined. Four to seven mice per cohort (at least 3 mm^2 total tissue area per mouse) were evaluated.

Immunohistochemistry

Immunohistochemical staining was performed on 5-µm thick paraffin-embedded skin sections. Following deparaffinization and rehydration through xylene and graded alcohols, sections were boiled in citric acid antigen unmasking solution (Vector Laboratories). Endogenous peroxidase activity was quenched by incubating sections for 10 min in 3% hydrogen peroxide solution. Staining was performed using ImmPRESS® Horse Anti-Rabbit IgG PLUS Polymer Kit and ImmPACT (TM) DAB HRP Substrate (Vector Laboratories) according to the manufacturer's protocols. Staining for mast cells was carried out using Mast Cell Tryptase Rabbit anti-Human/Mouse/Rat antibody (1:200 dilution, clone ARC2328, Invitrogen), and F4/80 Rabbit anti-Mouse antibody (1:250 dilution, clone SP115, Invitrogen) was used to stain macrophages. Sections were imaged at 40x magnification and evaluated using the Aperio AT2 DX System and Aperio ImageScope software, respectively, as described in the previous section. Five sections per cohort were evaluated for mast cells. Four to five mice per cohort (at least 3 – 4 mm^2 total tissue area per mouse) were evaluated for F4/80+ macrophages.

Hair structure assessment

Hairs were mounted on glass slides using Permount mounting media. Hair shafts (n=25-35 per mouse) were examined using an inverted microscope (Axioplan 2, Zeiss). Hair shaft anomalies (e.g., hair breakage, focal bulge, structural weakness, and hair depigmentation) were imaged and quantified.

Statistical methods
Statistical analysis was performed using GraphPad (version 9.4.1.681, GraphPad Software, Inc.). Data were analyzed using two-way ANOVA with Bonferroni multiple comparison test. Data are presented as mean ± SD. Adjusted *p* values are included in the tables. $p \leq 0.05$ was considered statistically significant.

4.8 Results and Discussion

4.8.1 Alopecia assessment of the test products

Alopecia was assessed using multiple hair growth parameters.

Skin pigmentation and hair recovery: Melanogenic truncal skin melanocytes in pigmented mice are confined to HFs, where they become melanogenically active during the anagen III phase of the hair growth cycle and are directly involved in hair shaft pigmentation. As no melanin is synthesized in telogen skin, changes in skin pigmentation from unpigmented (pink) to pigmented (gray to black) indicate active hair growth [30-32], which can be tracked and measured during the entire treatment period and correlated with hair growth pattern.

HF morphology and stages of HF growth: HF disorders and abnormalities in HF cycling that affect the duration of the anagen and telogen phases are key mechanisms underlying the pathogenesis of alopecia. From a clinical perspective, both premature termination of the anagen phase, as seen in androgenetic alopecia (AGA), and premature entry into the telogen phase, increase the percentage of HFs in the telogen phase [33]. In AGA, HF miniaturization is known to impair hair growth in the anagen phase and lead to a shortening of this phase, thereby prolonging the telogen phase [34]. A prolonged telogen phase that delays the onset of anagen has also been observed in mice in response to the topical application of glucocorticoids, prototypic stress hormones, or estrogen [33]. HF morphology and stages of HF growth were assessed in H&E-stained longitudinal skin sections.

HFs maintain their maximal lengths between the anagen VI and catagen II phases. During this time, the dermal papilla is located close to the panniculus carnosus, and anagen and catagen HFs are not easily distinguishable using morphologic criteria and a light microscope [19]. Therefore, catagen I-II HFs were excluded from the analysis.

HF size and length: HF lengths increase during anagen phases I–VI and decrease during catagen phases I–VIII. These hair cycle-associated fluctuations in HF lengths correlate with changes in skin thickness [19]. Therefore, HF sizes and lengths were measured as additional parameters of HF cycling.

4.8.1.1 Evaluation of DevaCurl Low-Poo Delight Cleanser (Exp-1)

Hair growth cycle and Hair recovery
The removal of the hair shafts triggered synchronized HF cycling. Except for the mice that received cyclophosphamide (CYP) injection, the majority of the mice in all cohorts initiated 1st anagen by Day 7, as evidenced by changes in skin pigmentation from pink (telogen) to gray (anagen onset), with the hair regrowing fully over entire depilated areas by Day 17. The hair cycle then progressed through a period of regression and resting phases, as changes in skin color from black to gray were identifiable at Day 20 **(Figs. 4, 5, Table 6)**.

In nontreated mice, the hair cycle progressed to the catagen/telogen stage, entering the 1st telogen stage by Day 24, **(Figs. 4A, 5)**. The HFs remained in telogen for about 4-5 weeks before transitioning to the second anagen stage at about Day 57, with all animals having fully regrown their hair by Day 77.

In some animals, spots of pink skin were noticeable at about this time **(Fig. 4A)**, suggesting a transition to the 2nd telogen stage. At Day 84, the mean gray value was similar to that at Day 77 **(Fig. 5, Table 6)**, and 99.02% of the depilated areas displayed a normal hair coat **(Fig. 4A, Table 7)**.

A single administration of CYP at the onset of the 1st anagen stage significantly impaired hair growth. While the mice eventually recovered and regrew their hair, achieving 93.02% hair recovery at Day 84 **(Fig. 4B, Table 7)**, 36.54% of the hairs that regrew in the depilated areas were depigmented and displayed a rough texture **(Table 8)**.

Compared to the nontreated cohort, the mice treated with water and exposed to the same experimental conditions (mock) as those in the DevaCurl and Rogaine cohorts showed a delay in HF cycling. The transition to catagen/telogen occurred at about Day 30, followed by entry into the second anagen stage around Day 60 **(Fig. 5, Table 6)**, and reaching 77.63% hair growth at Day 84 **(Fig. 4B, Table 7)**.

Minoxidil (Rogaine) has been shown to shorten telogen, causing premature entry of resting HFs into anagen. Consistent with the stimulatory effect of minoxidil on HFs, in mice treated with Rogaine, the progression to the anagen stage was accelerated after Day 60 **(Fig. 5, Table 6)**. At Day 84, the extent of hair recovery in this cohort was significantly higher than in the mock (99.26% vs. 77.63% in mock) and was comparable to that in the nontreated mice (vs. 99.02% in nontreated) **(Fig. 4E, Table 7)**.

In contrast, a significant delay in HF cycling was observed in mice treated with DevaCurl, resulting in 56.11% hair recovery at Day 84 (*p=0.0034* vs. mock) **(Fig. 4D, Table 7)**. While four mice in the mock cohort fully regrew their hair, a full coat was visible only in one mouse in the DevaCurl cohort **(Fig. 4D)**. The area of gray skin, which represents the active growth phase, was smaller in the DevaCurl cohort than in the mock control **(Table 9)**. Notably, the percent area of pink telogen skin in the DevaCurl cohort was higher than that in the mock cohort (16.68% vs. 1.21% in mock) **(Table 10)**. However, these data were not statistically significant.

HF morphology

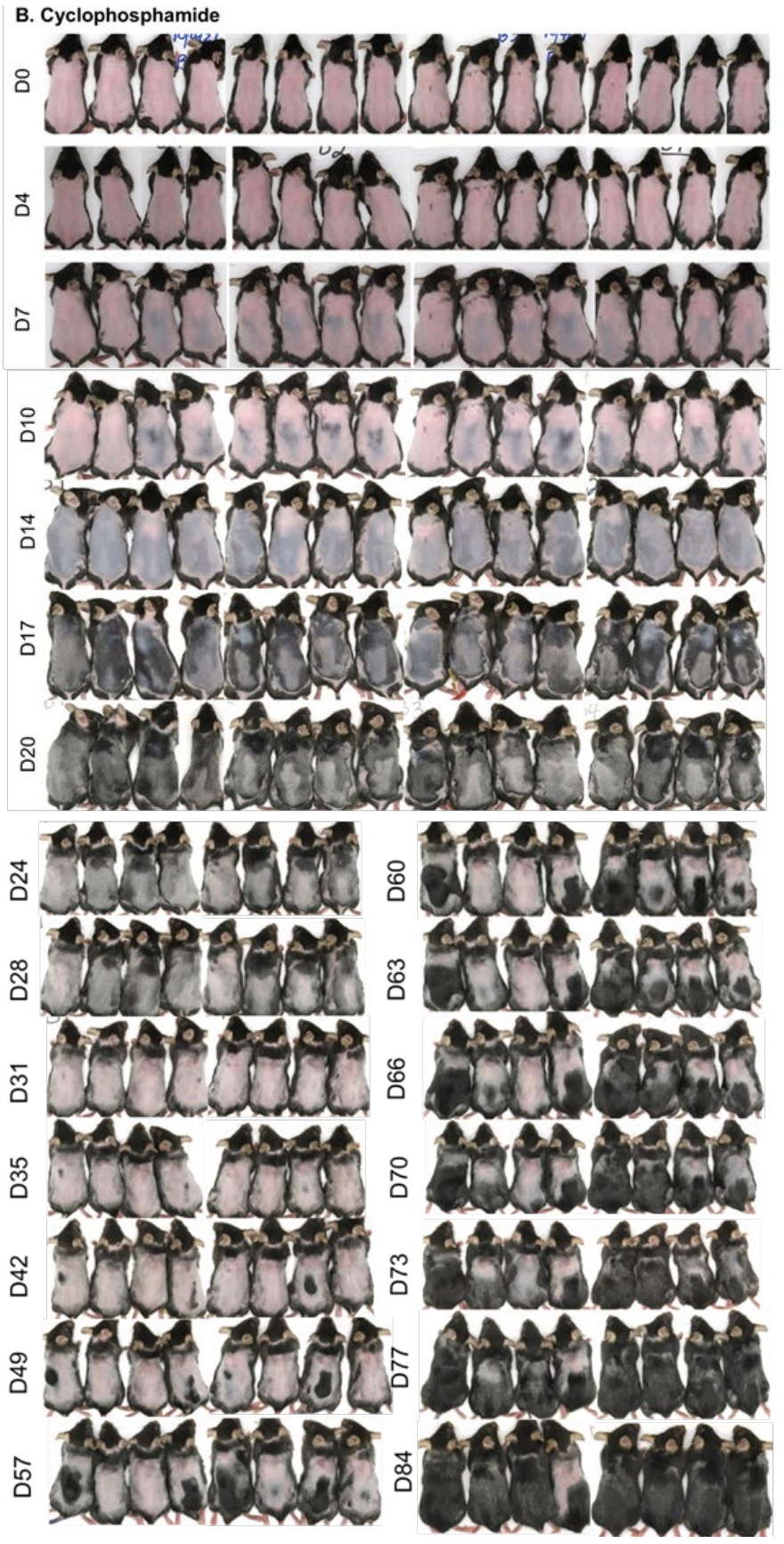
Because premature entry into the telogen phase contributes to hair loss, telogen HFs were quantified in skin sections at the anagen–telogen transition. Consistent with gray skin color, the HFs at Day 21 were predominantly in the catagen stage in all cohorts, and albeit not statistically significant, small fractions of HFs in the nontreated (5.77%) and Rogaine (10.76%) cohorts progressed to the telogen phase **(Fig. 6A, 6B; Table 11)**.

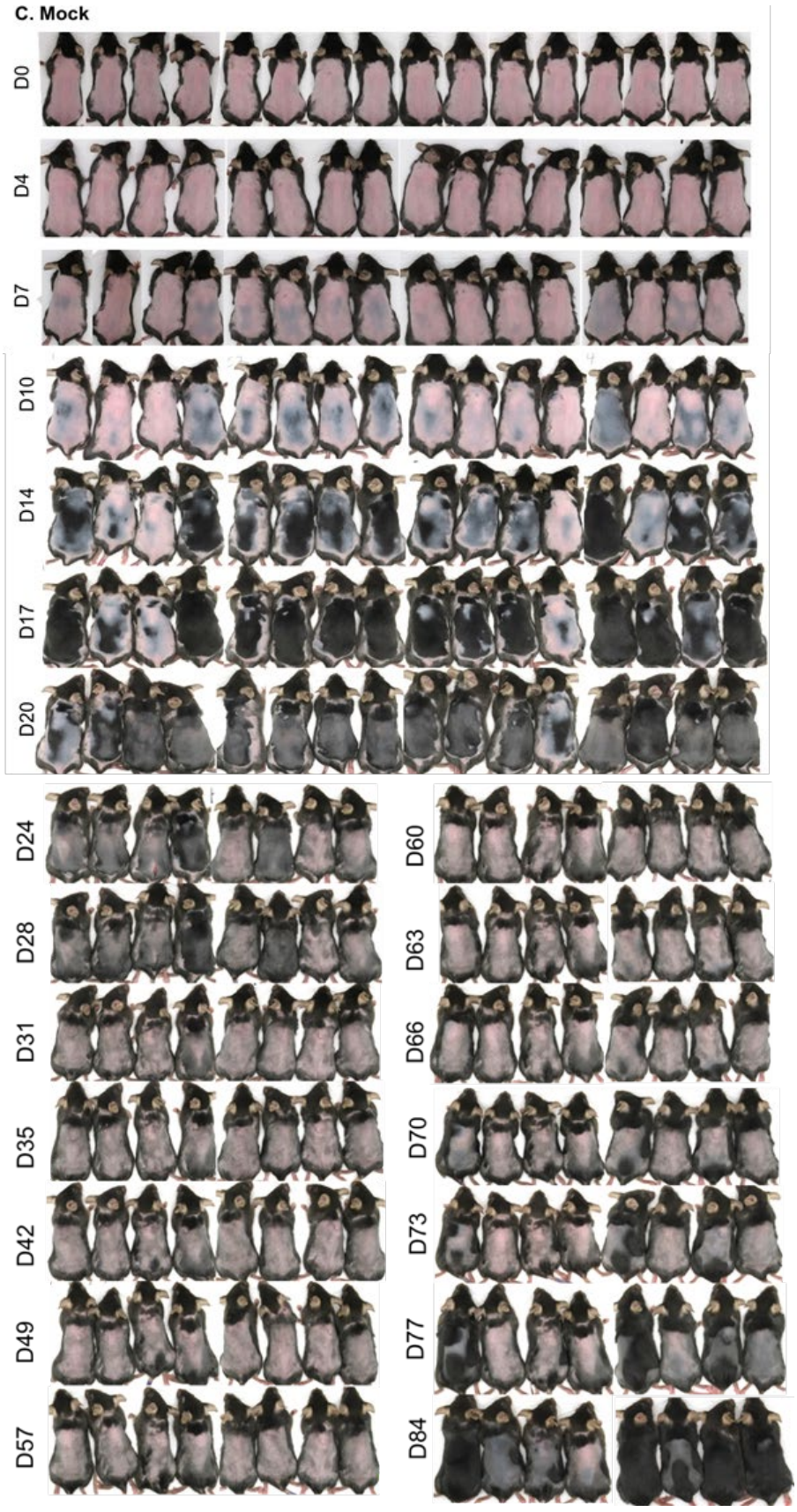
CYP has been shown to prematurely induce the catagen phase and increase telogen HFs [35]. Furthermore, a higher dose of CYP (150 mg/kg, the same dose used in this study) has been shown to induce dystrophic catagen and telogen in C57BL/6 mice [16]. In this experiment, the percentage of telogen HFs was substantially higher in the CYP cohort (29.65% vs. 5.77% in nontreated, *p=0.005*) **(Fig. 6B, Table 11)**, and although not present in every HF, some features of dystrophic catagen and telogen were detectable (e.g., ectopic melanin clumps, abnormal widening of the hair canal, remnants of the hair shaft) **(Fig. 7)**. Given the relatively synchronous hair cycling at Day 21, we found no substantial differences in HF size or length among different cohorts **(Fig. 6C, 6D)**.

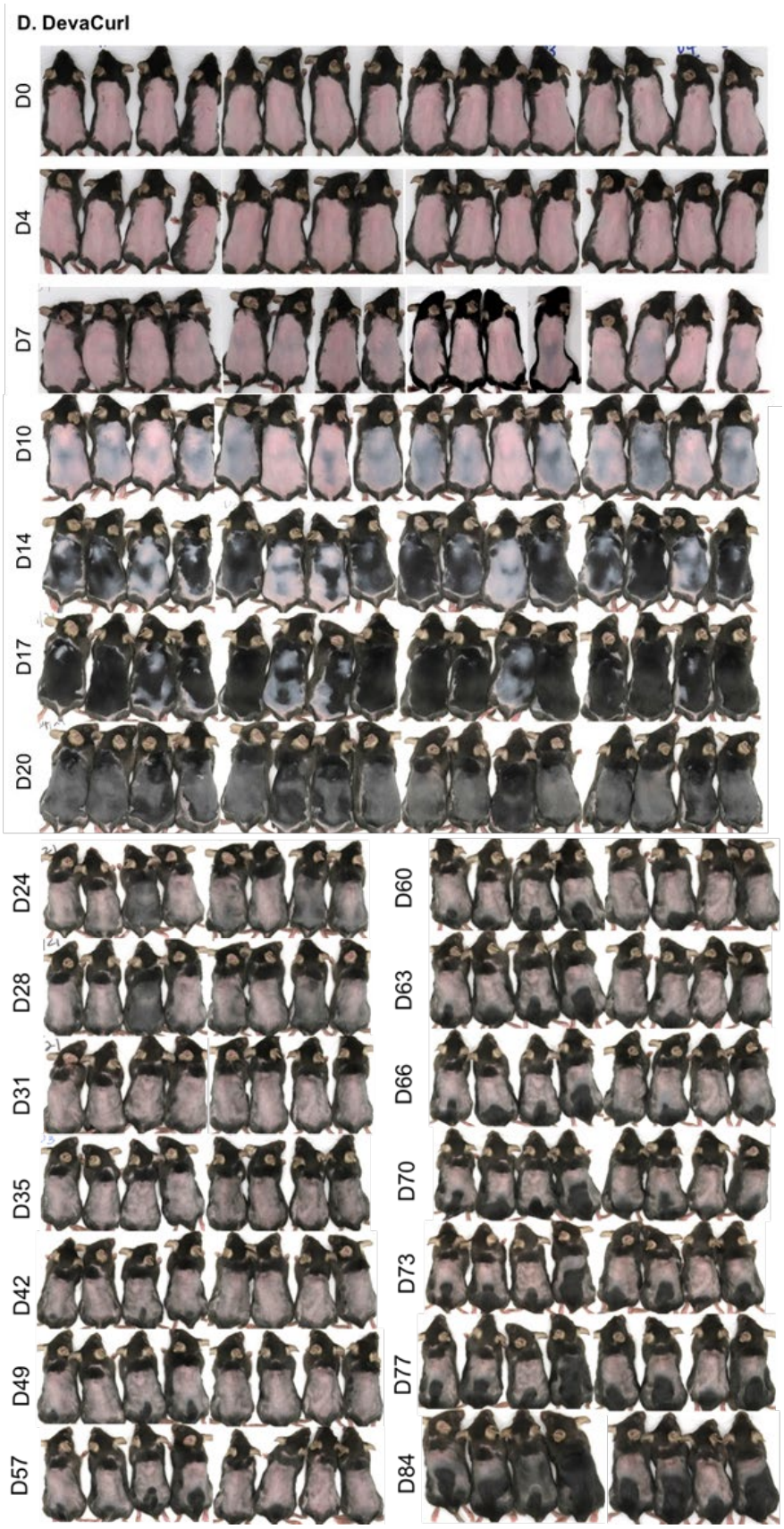
At Day 85, hair recovery in the CYP and Rogaine cohorts was similar to that observed in nontreated mice (99.02% in nontreated, 93.02% in CYP, and 99.26% in Rogaine). It is important to note that mouse club hairs from the telogen phase and growing hairs typically share a hair follicle, and that healthy murine HFs often retain old hair shafts from preceding cycles [36]. For these reasons, hair loss may not be readily detectable by visual assessment. This is likely the case with these cohorts, as evidenced by the predominance of HFs in the telogen phase in haired skin sections on these mice, indicating progression to the 2nd telogen phase **(Fig. 8A, 8B; Table 12)**. Compared to the nontreated

cohort, a delay in HF cycling was observed in the mock cohort, in which 30.71% of HFs were in the 2nd anagen phase and 60% were in the 2nd catagen phase. Consistent with the delayed hair recovery observed in mice treated with DevaCurl, the majority of DevaCurl HFs were distributed between the 1st telogen and 2nd anagen phases. Although 47.06% of HFs entered the 2nd anagen phase—corroborated by larger HF sizes and lengths **(Fig. 8C, 8D; Table 12)**, 46.39% of DevaCurl HFs remained in the 1st telogen phase at Day 85 **(Fig. 8B, Table 12**). Although the differences in the 1st telogen phase and the 2nd anagen phase were not statistically significant, the percentage of HFs in the 2nd catagen phase was significantly lower in mice treated with DevaCurl (6.55% vs. 60% in Mock, *p=0.0173*), suggesting overall delay in the telogen—anagen transition.


A. Nontreated
D0
D4
D7
D10
D14
D17
D20
D24
D28
D31
D35
D42
D49
D57
D60
D63
D66
D70
D73
D77
D84


B. Cyclophosphamide
D0
D4
D7
D10
D14
D17
D20
D24
D28
D31
D35
D42
D49
D57
D60
D63
D66
D70
D73
D77
D84


C. Mock
D0
D4
D7
D10
D14
D17
D20
D24
D28
D31
D35
D42
D49
D57
D60
D63
D66
D70
D73
D77
D84


D. DevaCurl
D0
D4
D7
D10
D14
D17
D20
D24
D28
D31
D35
D42
D49
D57
D60
D63
D66
D70
D73
D77
D84

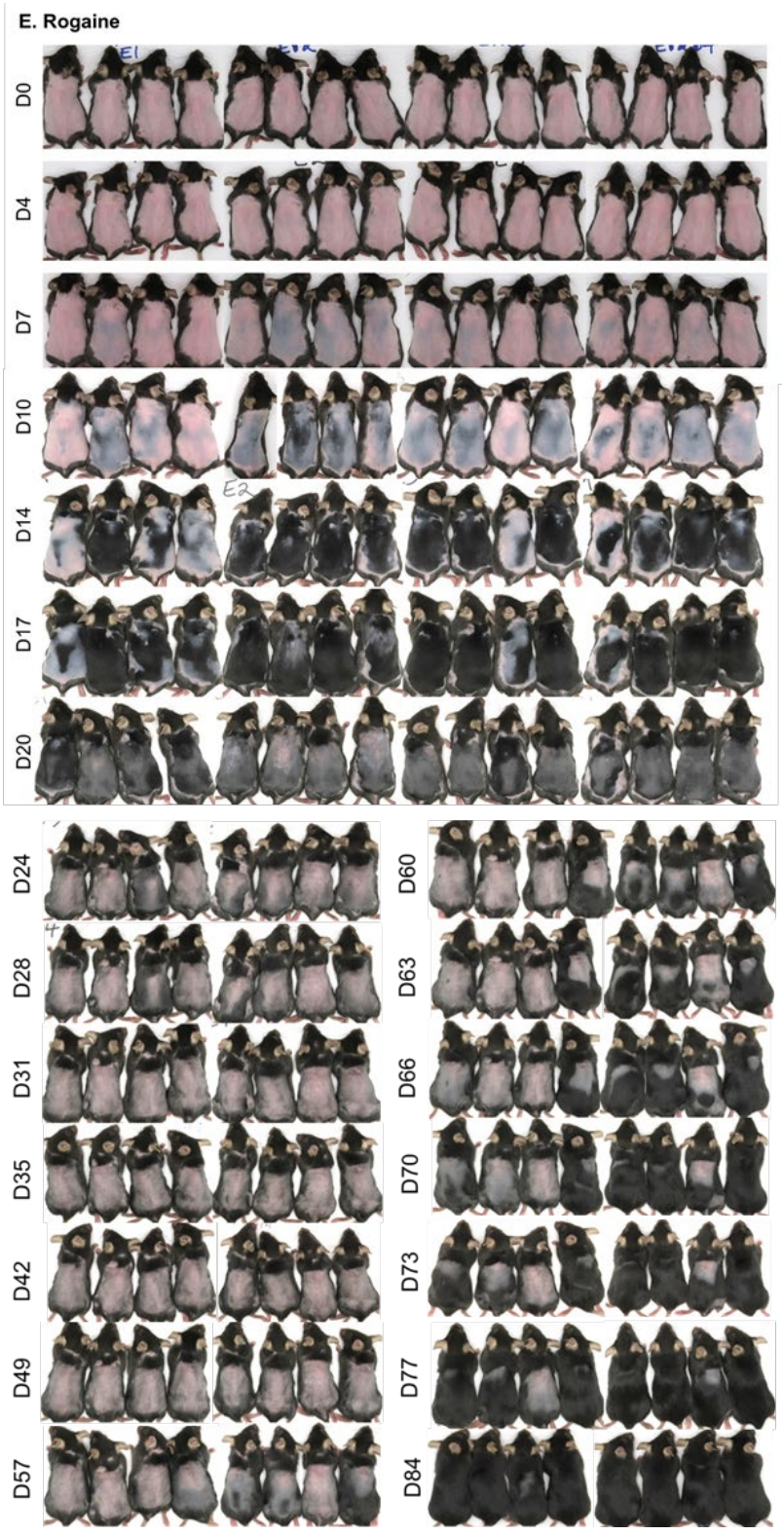


Figure 4. Visual assessment of skin pigmentation and hair growth from Day 0 (D0) to Day 84 (D84) after treatment. D0−D20: 16 mice per cohort. D24–D84: 8 mice per cohort. At D21, 8 mice from each cohort were euthanized and tissue samples were collected for analyses.

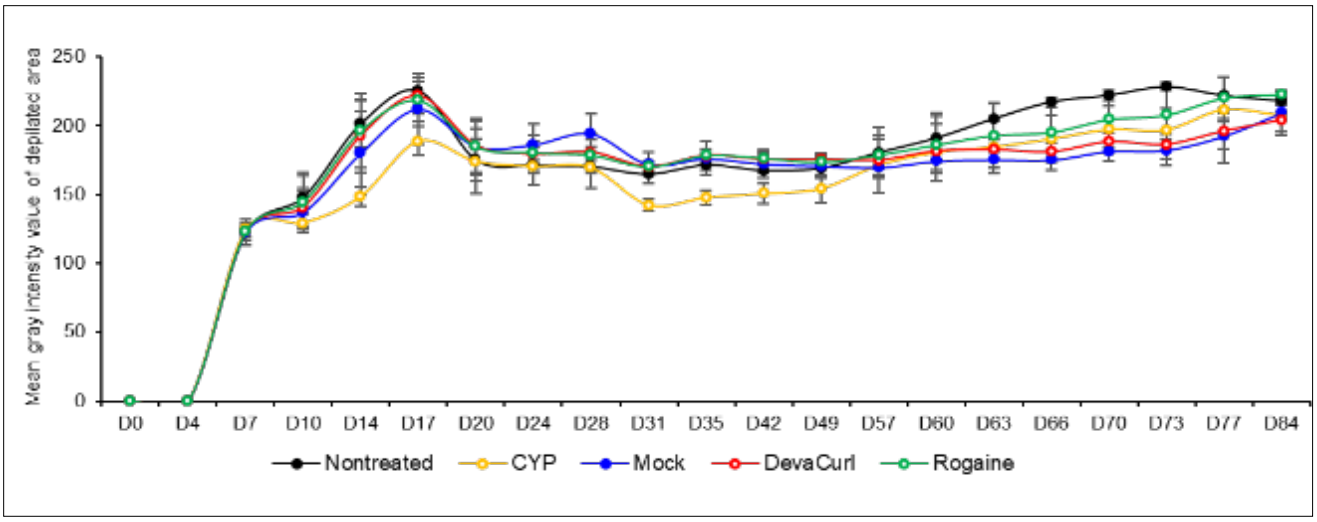


Figure 5. Mean gray intensity values of depilated areas quantified using ImageJ. Refer to Table 6 for the adjusted (adj.) p values for all data points.

Table 6. Mean gray values of depilated areas (Exp-1).

Day		Nontreated	CYP	Mock	DevaCurl	Rogaine
D0	**mean ± SD**	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	adj. p-value (vs. NT or Mock)		>0.9999	>0.9999	>0.9999	>0.9999
D4	**mean ± SD**	0 ± 0	0 ± 0	0 ± 0	0 ± 0	0 ± 0
	adj. p-value (vs. NT or Mock)		>0.9999	>0.9999	>0.9999	>0.9999
D7	**mean ± SD**	122.23 ± 6.13	124.69 ± 4.82	122.36 ± 9.66	122.95 ± 8.94	123.04 ± 6.03
	adj. p-value (vs. NT or Mock)		>0.9999	>0.9999	>0.9999	>0.9999
D10	**mean ± SD**	147.93 ± 18.08	129.32 ± 6.78	137.11 ± 15.37	140.78 ± 13.72	144.64 ± 19.02
	adj. p-value (vs. NT or Mock)		**0.0007**	0.2086	>0.9999	>0.9999
D14	**mean ± SD**	201.54 ± 17.15	148.66 ± 6.75	180.14 ± 30.29	192.66 ± 26.67	196.38 ± 26.77
	adj. p-value (vs. NT or Mock)		**<0.0001**	**<0.0001**	0.0753	**0.0054**
D17	**mean ± SD**	225.39 ± 7.1	188.81 ± 10.86	212.17 ± 25.45	221.62 ± 13.08	218.49 ± 16.02
	adj. p-value (vs. NT or Mock)		**<0.0001**	**0.0478**	0.435	>0.9999
D20**	**mean ± SD**	175.24 ± 15.43	174.08 ± 23.8	185.11 ± 18.84	185.49 ± 19.41	184.95 ± 20.56
	adj. p-value (vs. NT or Mock)		>0.9999	0.349	>0.9999	>0.9999
D24	**mean ± SD**	171.16 ± 3.69	170.49 ± 13.68	186.36 ± 14.92	179.4 ± 13.77	180.49 ± 8.17
	adj. p-value (vs. NT or Mock)		>0.9999	0.217	>0.9999	>0.9999
D28	**mean ± SD**	170.26 ± 5.2	169.44 ± 15.16	194.39 ± 13.92	180.71 ± 13.72	178.8 ± 11.09
	adj. p-value (vs. NT or Mock)		>0.9999	**0.0028**	0.3885	0.1858
D31	**mean ± SD**	164.91 ± 6.75	142.35 ± 4.23	172 ± 8.75	170.16 ± 3.52	170.24 ± 4.26
	adj. p-value (vs. NT or Mock)		**0.0067**	>0.9999	>0.9999	>0.9999
D35	**mean ± SD**	171.58 ± 6.73	147.7 ± 5.42	176.14 ± 4.74	178.29 ± 10.25	178.46 ± 4.74
	adj. p-value (vs. NT or Mock)		**0.0032**	>0.9999	>0.9999	>0.9999
D42	**mean ± SD**	167.61 ± 5.58	150.64 ± 7.64	172.25 ± 6.59	175.51 ± 4.87	176.5 ± 5.61
	adj. p-value (vs. NT or Mock)		0.1038	>0.9999	>0.9999	>0.9999
D49	**mean ± SD**	169.29 ± 7.08	154.24 ± 9.99	170.81 ± 7.29	175.6 ± 4.45	173.56 ± 4.87
	adj. p-value (vs. NT or Mock)		0.2303	>0.9999	>0.9999	>0.9999
D57	**mean ± SD**	180.64 ± 17.7	171.18 ± 19.5	169.71 ± 7.6	174.86 ± 6	178.86 ± 14.46

Day		Nontreated	CYP	Mock	DevaCurl	Rogaine
	adj. p-value (vs. NT or Mock)		>0.9999	0.9875	>0.9999	>0.9999
D60	**mean ± SD**	191.18 ± 18.09	180.65 ± 20.78	174.25 ± 6.2	181.41 ± 5.42	186.19 ± 20.59
	adj. p-value (vs. NT or Mock)		>0.9999	0.1061	>0.9999	0.7128
D63	**mean ± SD**	205.18 ± 11.18	184.53 ± 19.37	175.25 ± 5.7	182.56 ± 7.24	192.91 ± 22.91
	adj. p-value (vs. NT or Mock)		**0.0184**	**<0.0001**	>0.9999	0.0767
D66	**mean ± SD**	217.43 ± 3.64	190.35 ± 17.3	175.13 ± 7.46	180.89 ± 6.38	195.29 ± 20.72
	adj. p-value (vs. NT or Mock)		**0.0005**	**<0.0001**	>0.9999	**0.0235**
D70	**mean ± SD**	222.19 ± 3.64	197.14 ± 17.3	181.5 ± 7.24	188.18 ± 6.38	204.59 ± 20.72
	adj. p-value (vs. NT or Mock)		**0.0016**	**<0.0001**	>0.9999	**0.005**
D73	**mean ± SD**	228.43 ± 2.66	197.04 ± 15.85	182.38 ± 11.64	186.34 ± 10.36	207.84 ± 18.4
	adj. p-value (vs. NT or Mock)		**<0.0001**	**<0.0001**	>0.9999	**0.0012**
D77	**mean ± SD**	222.01 ± 1.55	211.61 ± 8.83	192.54 ± 20.04	195.99 ± 13.17	220.23 ± 14.93
	adj. p-value (vs. NT or Mock)		>0.9999	**<0.0001**	>0.9999	**0.0003**
D84	**mean ± SD**	218.2 ± 2.46	207.44 ± 11.86	209.34 ± 16.05	203.96 ± 11.06	222.63 ± 2.26
	adj. p-value (vs. NT or Mock)		>0.9999	>0.9999	>0.9999	0.4472

Two-way ANOVA with Bonferroni multiple comparison test was used for statistical analysis. Adjusted (adj.) p values are included. Bold type: $p \leq 0.05$. CYP, cyclophosphamide. Gray value = pure white value (255) - gray value from Image J. **Dorsal hairs were shaved at D20.

Table 7. % area of full hair growth at Day 84.

	Nontreated	CYP	Mock	DevaCurl	Rogaine
mean ± SD	99.02 ± 2.24	93.02 ± 17.27	77.63 ± 25.13	56.11 ± 19.69	99.26 ± 2.08
adj. p-value		>0.9999	**0.0037**	**0.0034**	**0.0032**

Two-way ANOVA with Bonferroni multiple comparison test was used for statistical analysis. Adjusted (adj.) p values are included. Bold type: $p \leq 0.05$. CYP, cyclophosphamide. Gray skin without visible hairs was excluded. % area of full hair growth = {area of visible hair growth ÷ total depilated area} x 100.

Table 8. % area with depigmented hairs at Day 84.

	Nontreated	CYP	Mock	DevaCurl	Rogaine
mean ± SD	0 ± 0	36.54 ± 17.02	0 ± 0	0 ± 0	0 ± 0
adj. p-value		**<0.0001**	>0.9999	>0.9999	>0.9999

Two-way ANOVA with Bonferroni multiple comparison test was used for statistical analysis. Adjusted (adj.) p values are included. Bold type: $p \leq 0.05$. CYP, cyclophosphamide.

Table 9. % anagen skin at Day 84.

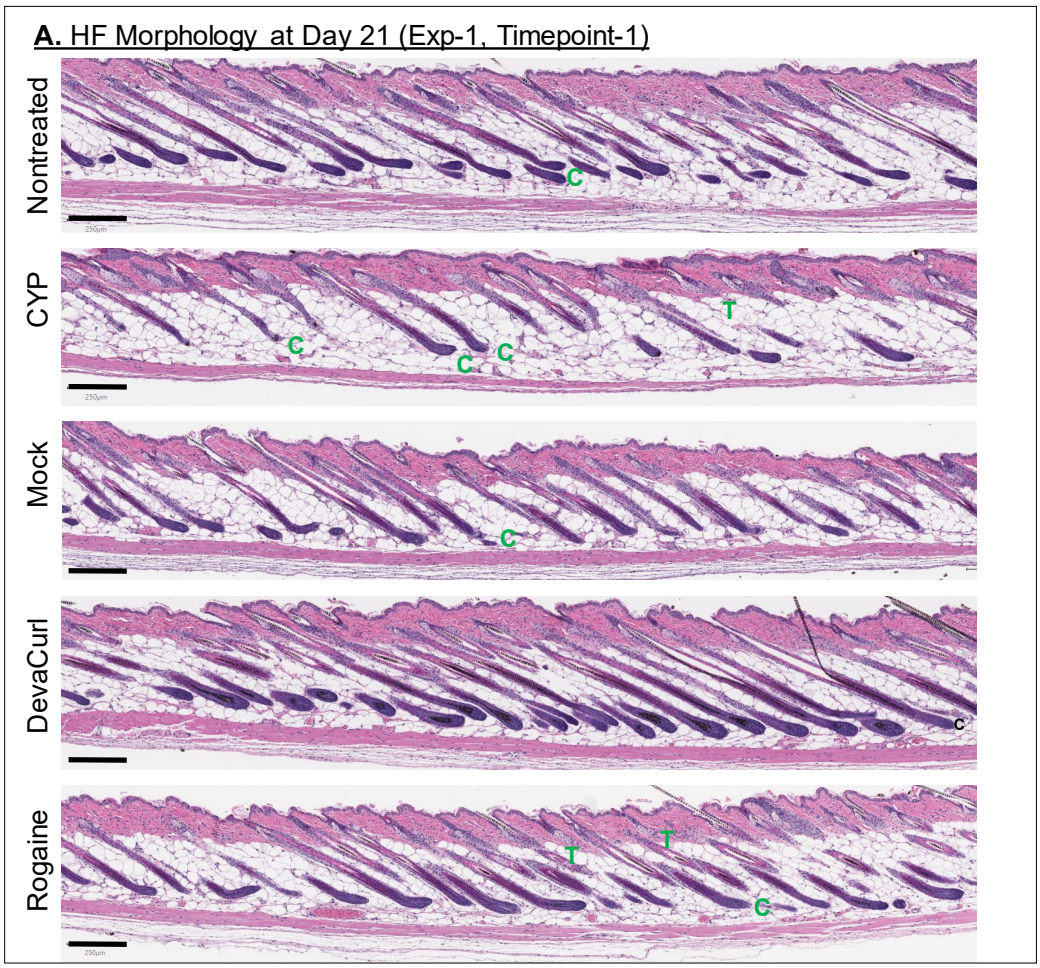
	Nontreated	CYP	Mock	DevaCurl	Rogaine
mean ± SD	0.05 ± 0.14	0 ± 0	18.75 ± 23.27	12.13 ± 14.72	0.92 ± 2.6
adj. p-value		>0.9999	**0.0176**	>0.9999	**0.0282**

Two-way ANOVA with Bonferroni multiple comparison test was used for statistical analysis. Adjusted (adj.) p values are included. Bold type: $p \leq 0.05$. CYP, cyclophosphamide, % area of anagen skin = {area of gray skin without visible hairs ÷ total depilated area} x 100.

Table 10. % telogen skin at Day 84.

	Nontreated	CYP	Mock	DevaCurl	Rogaine
mean ± SD	0 ± 0	2.99 ± 8.46	1.21 ± 3.42	16.68 ± 16.86	0 ± 0
adj. p-value		>0.9999	>0.9999	0.0926	>0.9999

Two-way ANOVA with Bonferroni multiple comparison test was used for statistical analysis. Adjusted (adj.) p values are included. CYP, cyclophosphamide. % telogen skin = {area of pink skin without visible hairs ÷ total depilated area} x 100.



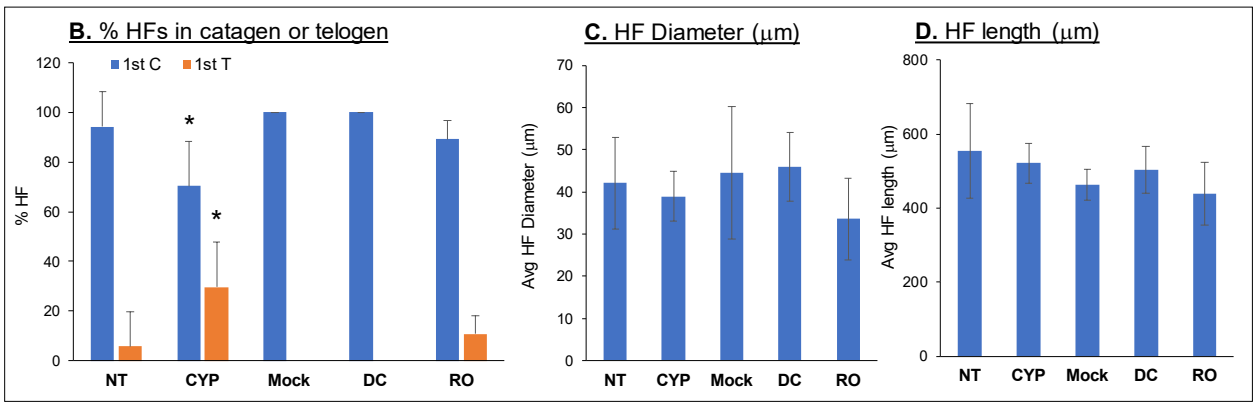


Figure 6. HF analysis at the anagen-telogen transition (Exp-1, Timepoint-1). A. Representative images of H&E stained longitudinal sections of mouse dorsal skin. C, catagen HF; T, telogen HF. B. % catagen (C) and telogen (T) HFs. % catagen HFs = {No. of catagen HFs÷Total No. of HFs} x 100. % telogen HFs = {No. of telogen HFs÷Total No. of HFs} x 100. C. Average HF diameter. D. Average HF length, corresponding to the distance from the epidermal granular layer to the top edge of the panniculus carnosus. The graph represents the mean ± SD. n = 4-7 mice per cohort. *, $p \leq 0.05$. Refer to Table 11 for the adj. p values for all data points. NT, nontreated; CYP, cyclophosphamide; DC, DevaCurl, RO, Rogaine. Scale bar = 250 µm.

Table 11. HF analysis at Day 21 (Exp-1, Timepoint-1).

Cohorts	Values	1st C (%)	1st T (%)	HF Diameter (µm)	HF Length (µm)
NT	**mean ± SD**	94.23 ± 14.13	5.77 ± 14.13	42.04 ± 10.82	554.26 ± 127.5
	adj. p-value				
CYP	**mean ± SD**	70.35 ± 18.03	29.65 ± 18.03	38.87 ± 5.92	522 ± 53.97
	adj. p-value (vs. NT)	**0.0050**	**0.0050**	>0.9999	>0.9999
Mock	**mean ± SD**	100 ± 0	0 ± 0	44.49 ± 15.66	462.65 ± 42
	adj. p-value (vs. NT)	>0.9999	>0.9999	>0.9999	0.0736
DC	**mean ± SD**	100 ± 0	0 ± 0	45.95 ± 8.15	503.65 ± 62.31
	adj. p-value (vs. Mock)	>0.9999	>0.9999	>0.9999	>0.9999
RO	**mean ± SD**	89.24 ± 7.5	10.76 ± 7.5	33.6 ± 9.67	439.16 ± 84.36
	adj. p-value (vs. Mock)	>0.9999	>0.9999	>0.9999	>0.9999

Two-way ANOVA with Bonferroni multiple comparison test was used for statistical analysis. Adjusted (adj.) p values are included. Bold type: $p \leq 0.05$.

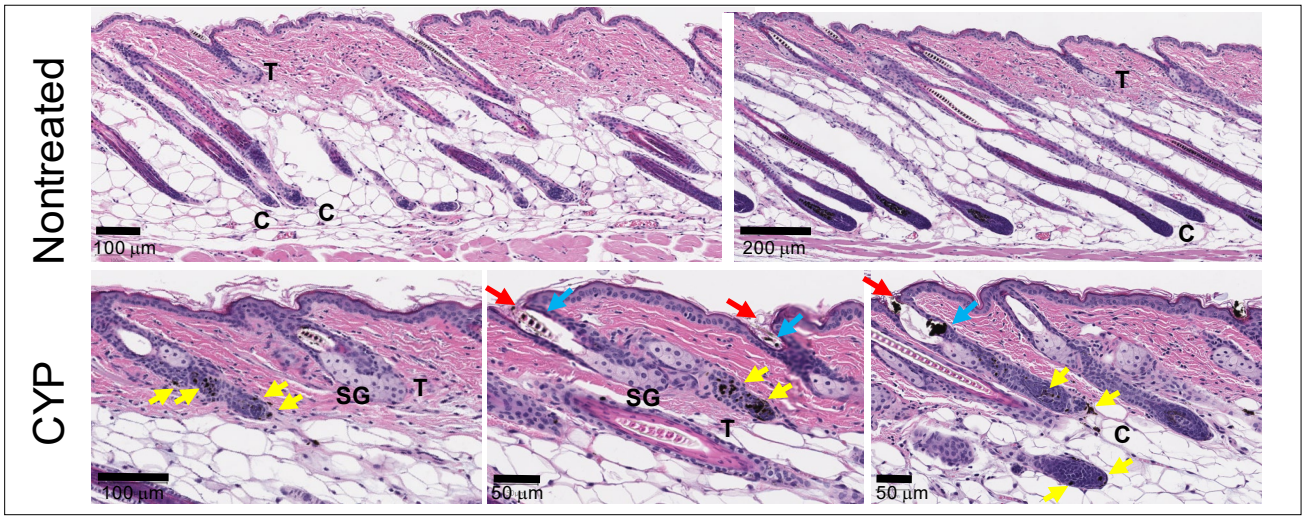
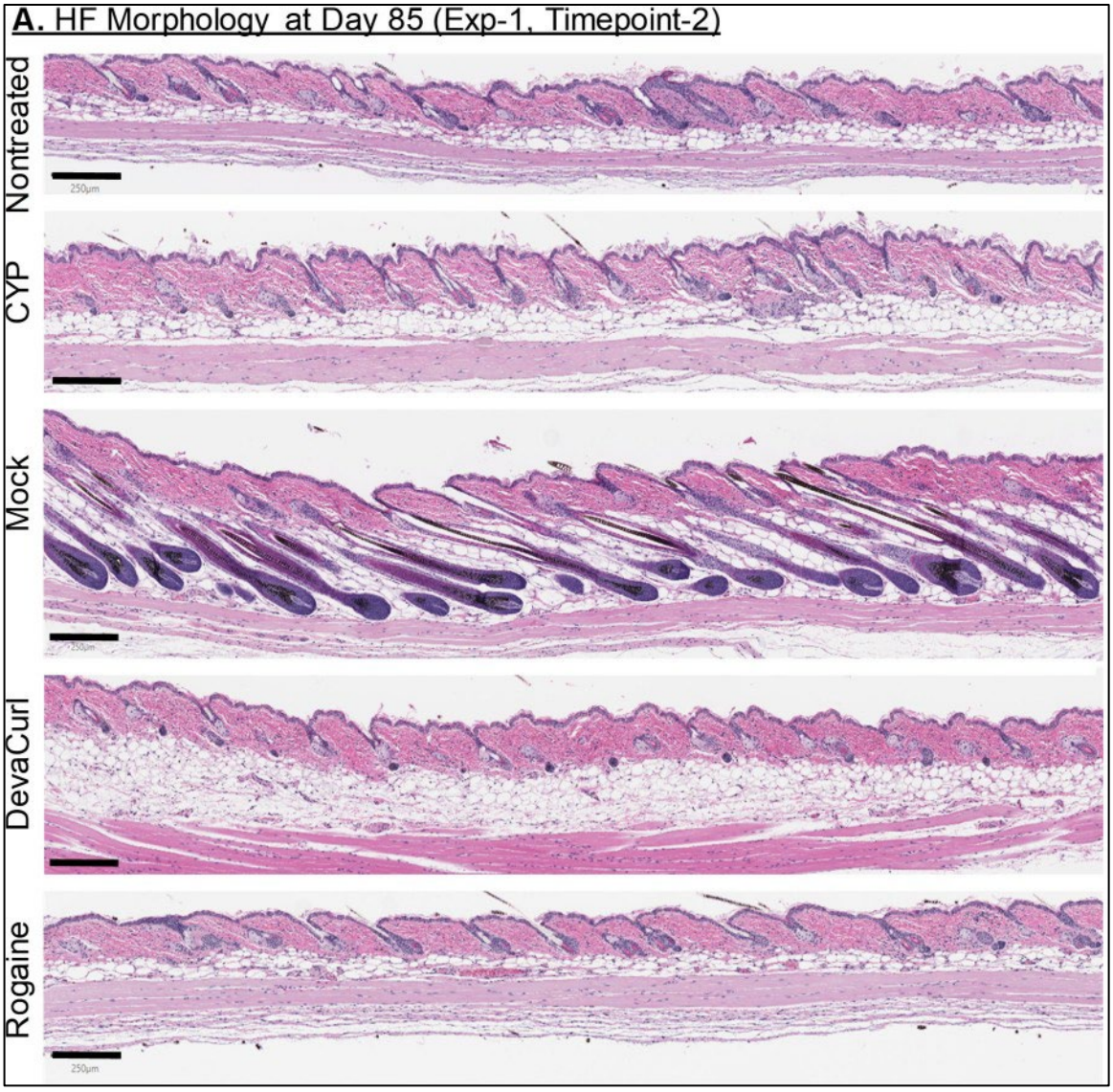
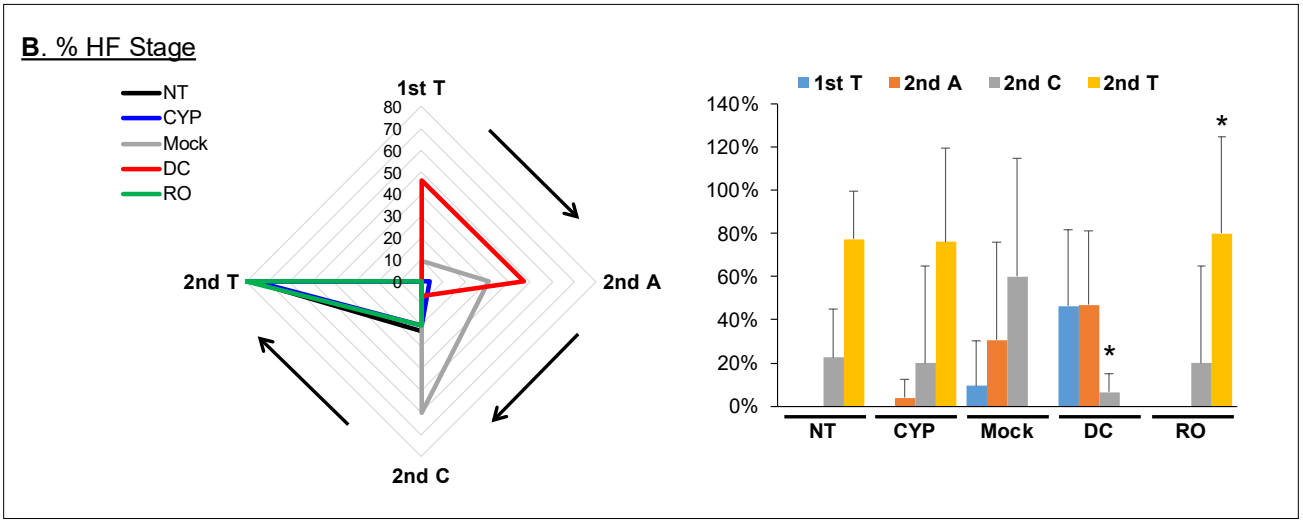


Figure 7. Dystrophic catagen and telogen in CYP-treated mice. Representative images of H&E-stained longitudinal sections of mouse dorsal skin. CYP, cyclophosphamide; C, catagen HF; T, telogen HF; SG, sebaceous gland; yellow arrows, melanin clumps; blue arrows, abnormal widening of hair canal; red arrows, remnants of the hair shaft. Images are presented at multiple magnifications for better visualization.


A. HF Morphology at Day 85 (Exp-1, Timepoint-2)
Nontreated
CYP
Mock
DevaCurl
Rogaine
250µm


B. % HF Stage
NT
CYP
Mock
DC
RO
1st T
2nd A
2nd C
2nd T
80
70
60
50
40
30
20
10
0
1st T
2nd A
2nd C
2nd T
140%
120%
100%
80%
60%
40%
20%
0%
*
*
NT
CYP
Mock
DC
RO

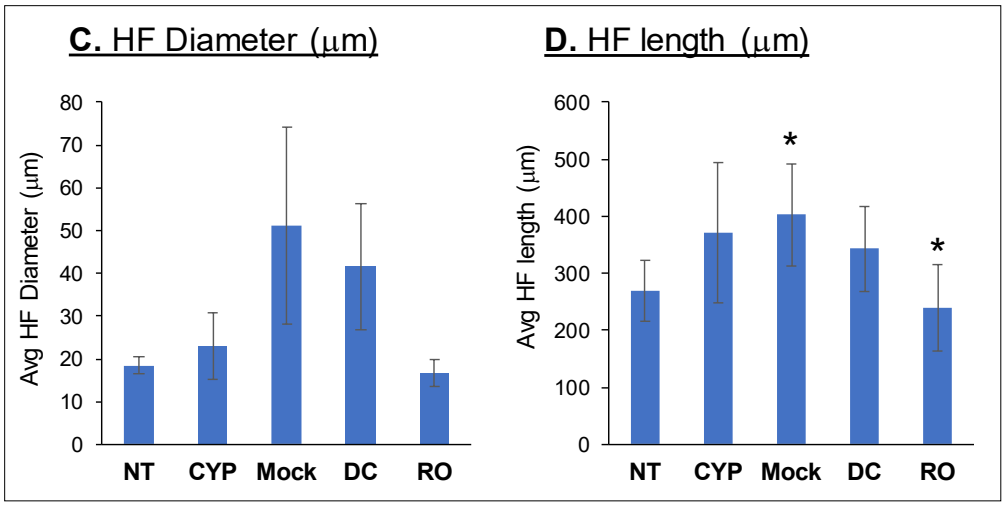


Figure 8. HF analysis at the telogen-anagen transition (Exp-1, Timepoint-2). A. Representative images of H&E stained longitudinal sections of mouse dorsal skin. B. % stage-specific HFs presented in radial (left) and column (right) charts. A, anagen HF; C, catagen HF; T, Telogen HF. % stage-specific HFs = {No. of stage-specific HFs÷ No. of total HFs} x 100. C. Average HF diameter. D. Average HF length, corresponding to the distance from the epidermal granular layer to the top edge of the panniculus carnosus. The graph represents the mean ± SD. n = 4-7 mice per cohort. *, $p \leq 0.05$. Refer to Table 12 for adj. *p* values for all data points. NT, nontreated; CYP, cyclophosphamide; DC, DevaCurl, RO, Rogaine. Scale bar = 250 μm.

Table 12. HF analysis at Day 85 (Exp-1, Timepoint-2).

Cohorts		1st T (%)	2nd A (%)	2nd C (%)	2nd T (%)	HF Diameter (μm)	HF Length (μm)
NT	**mean ± SD**	0 ± 0	0 ± 0	22.53 ± 22.19	77.47 ± 22.19	18.52 ± 1.9	268.59 ± 53.65
	adj. p-value						
CYP	**mean ± SD**	0 ± 0	3.78 ± 8.46	20 ± 44.72	76.22 ± 43.39	23 ± 7.68	371.17 ± 121.93
	adj. p-value (vs. NT)	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	0.1647
Mock	**mean ± SD**	9.29 ± 20.76	30.71 ± 45.15	60 ± 54.77	0 ± 0	51.06 ± 22.99	402.33 ± 88.97
	adj. p-value (vs. NT)	>0.9999	>0.9999	0.5738	**0.0014**	>0.9999	**0.0221**
DC	**mean ± SD**	46.39 ± 35.5	47.06 ± 33.95	6.55 ± 8.67	0 ± 0	41.54 ± 14.72	342.74 ± 74.27
	adj. p-value (vs. Mock)	0.2733	>0.9999	**0.0173**	>0.9999	>0.9999	0.9529
RO	**mean ± SD**	0 ± 0	0 ± 0	20 ± 44.72	80 ± 44.72	16.61 ± 3.14	239.89 ± 76.03
	adj. p-value (vs. Mock)	>0.9999	0.9768	0.3192	**0.0004**	>0.9999	**0.0013**

Two-way ANOVA with Bonferroni multiple comparison test was used for statistical analysis. Adjusted (adj.) *p* values are included. Bold type: $p \leq 0.05$.

4.8.1.2 Evaluation of Aquaphor Baby Wash & Shampoo, Monat Renew Shampoo, and WEN Sweet Almond Mint Cleansing Conditioner (Exp-2)

Hair growth cycle and Hair recovery

The removal of the hair shafts triggered synchronized HF cycling, and complete hair regrowth over the entire depilated areas in all experimental animals by Day 17 **(Fig. 9)**. While the extent of the hair growth and overall hair density at Day 17 were comparable in all cohorts, the onset of the anagen phase was accelerated in the mice treated with either Aquaphor Baby Wash & Shampoo or Monat Renew Shampoo. At Day 7, the mean intensities of the gray values of the depilated areas were significantly higher in Aquaphor and Monat cohorts (173.91 in Aquaphor, $p < 0.0001$ and 177.59 in Monat, $p < 0.0001$ vs. 156.73 in mock) **(Fig. 9; Table 13, D10)**. The hair growth patterns of the mice treated with WEN

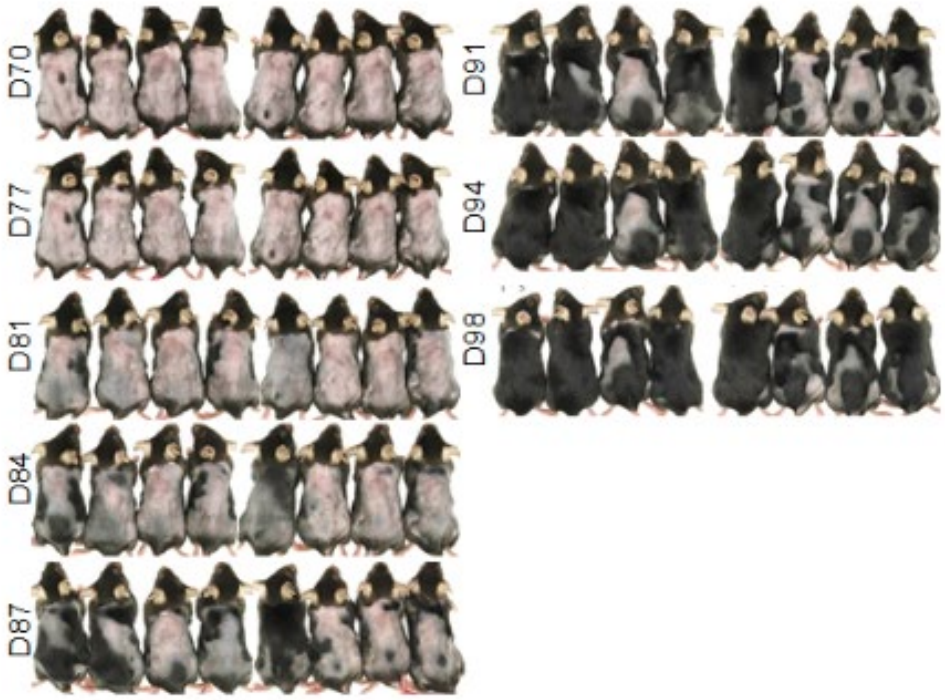
sweet Almond Mint Cleansing Conditioner were similar to those of the mock cohort **(Figs. 9D & 10; Tables 13 and 14)**. The hair cycle in the mock cohort progressed to catagen-telogen, entering the 1st telogen stage around Day 24 and Day 28. The hair growth cycle in the mock cohort remained predominantly in the telogen stage for about 7.5 weeks (from Day 24 to Day 77) and then progressed to the 2nd anagen phase around Day 81, reaching 87.28% hair regrowth at Day 98 **(Figs. 9A, 10; Tables 13 & 14)**. In contrast, the transition to the 2nd anagen stage was observed at around Day 42 in mice treated with Monat **(Fig. 9C)**, and the mean gray value was substantially higher in this cohort compared to the mock cohort at Day 66 (210.83 Monat vs. 177.63 mock, *p<0.0001*) **(Fig. 10, Table 13)**. A slight increase in the mean gray value was also noticed in mice treated with Aquaphor at Day 66 (184.99 Aquaphor vs. 177.63 mock). However, these increases were statistically insignificant **(Figs. 9B & 10, Tables 13 & 14)**. WEN also caused a slight increase in the mean gray value at Day 66 (186.64 WEN vs. 177.63 in mock, *p=0.1673*) **(Fig. 10, Table 13)**. However, this increase was not statistically significant, and the hair cycling pattern of the WEN-treated mice was comparable to that of the mock mice **(Fig. 9D)**.

At Day 98, the hair growth in mice treated with Aquaphor was comparable to that observed in the mock mice (88.21% Aquaphor vs. 87.28% mock) **(Fig. 9B, Table 14)**. Compared to the mock cohort, in mice treated with Monat, the transition to the 2nd anagen stage was observed at around Day 42 with all animals having fully regrown their hair by Day 77 **(Figs. 9C, 10; Table 14)**. At Day 98, these mice displayed 99.48% of hair recovery (vs. 87.28% in mock, *p<0.05*) in the depilated areas **(Table 14)**. In contrast, a substantial delay in HF cycling was observed in mice treated with WEN, resulting in 70.41% hair recovery at Day 98 (*p=0.003*) **(Fig. 9D, Table 14)**. While four mice in the mock cohort fully regrew their hair, a full coat was visible only in one mouse in the WEN cohort **(Fig. 9D)**, and anagen progression and hair growth were observed in 84.70% of the depilated areas in the WEN cohort at Day 98 (vs. 99.87% in mock, *p=0.0086*) **(Table 15)**. While the mean gray values did not differ substantially among cohorts **(Fig. 10, Table 13)**, the pink telogen skin was observed in 15.30% of the depilated area in the WEN cohort at Day 98 (vs. 0.13% mock, *p=0.0086*) **(Table 16)**, indicating a delay in the 1st telogen-to-2nd anagen transition. These results demonstrate hair cycle abnormalities in mice treated with WEN and suggest that the impaired hair growth associated with WEN may involve a prolonged duration of the telogen stage, thereby delaying anagen induction and subsequent hair growth.

HF morphology

At Day 21, HF cycling progression in the test cohorts (i.e., Aquaphor, Monat, WEN cohort) appeared to be faster than that in the mock cohort **(Fig. 11A, 11B; Table 17)**. Compared to the catagen phase observed in the mock cohort, the test cohorts displayed higher proportions of telogen HFs and smaller and shorter HFs **(Fig. 11A-D, Table 17)**, suggesting progression to the subsequent telogen phase. At Day 98, the % area of anagen skin and hair growth was >99% for the mock, Aquaphor, and Monat cohorts, whereas 84.70% for the WEN cohort **(Table 15)**. These data indicated that the majority of animals in the mock, Aquaphor, and Monat cohorts had transitioned through the 2nd anagen phase and regrown their hair, whereas, in the WEN cohort, a full coat was visible in only one mouse **(Fig. 9D)**. Consistent with this observation, the majority of HFs in Aquaphor (60%, *p<0.0001*) and Monat (96.12%, *p<0.0001*) proceeded to the 2nd telogen phase (vs. 0% in mock), indicating accelerated hair growth in these mice compared to the mock cohort **(Fig. 12A, 12B; Table 18)**. In contrast, in the WEN cohort, the telogen–anagen transition was significantly delayed. Compared to 97.37% of mock HFs that progressed to the 2nd anagen, only 28.80% of HFs progressed to the 2nd anagen phase (*p<0.0001*) in mice treated with WEN, while 71.20% (*p<0.0001*) remained in the 1st telogen phase **(Table 18)**. Compared to the mock cohort, the HF sizes and lengths were smaller in the Aquaphor, Monat, and WEN cohorts due to the predominance of HFs in the telogen phase **(Fig. 12C, 12D; Table 18)**.

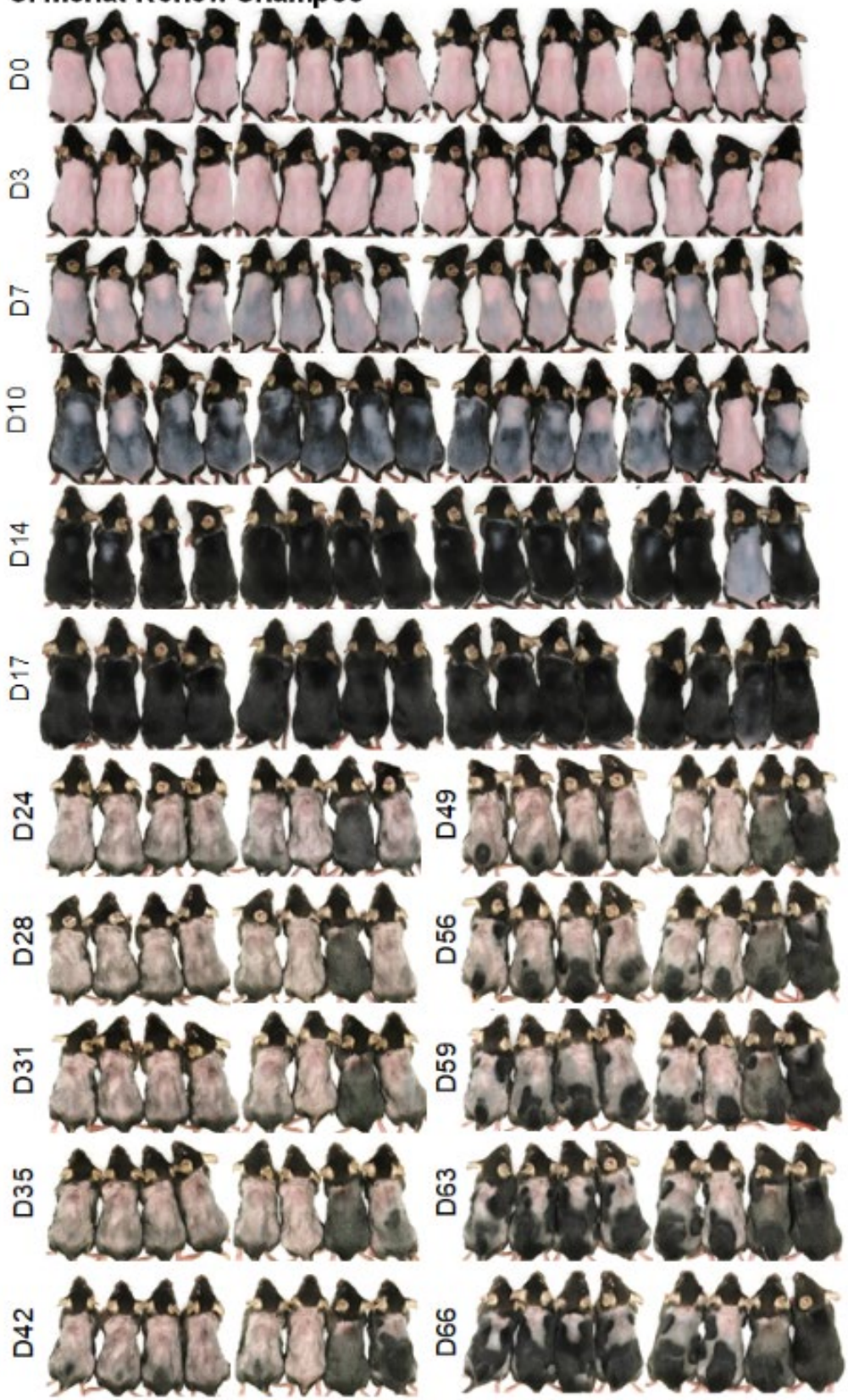

A. Mock
D0
D3
D7
D10
D14
D17
D24
D28
D31
D35
D42
D49
D56
D59
D63
D66

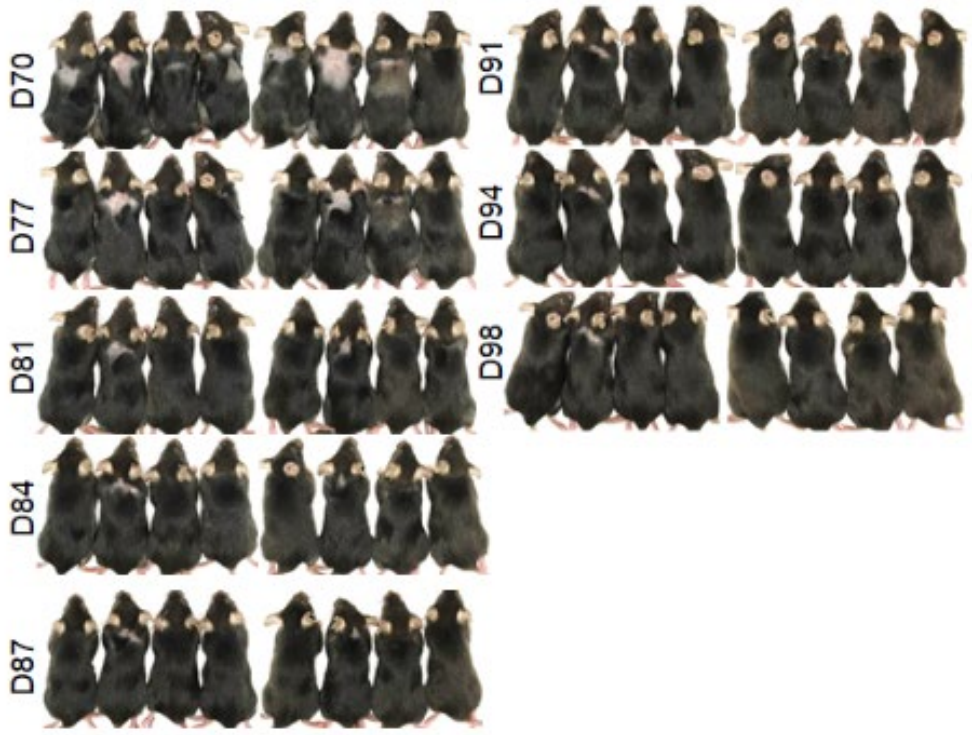


B. Aquaphor Baby Wash & Shampoo


D24
D49
D28
D56
D31
D59
D35
D63
D42
D66
D70
D91
D77
D94
D81
D98
D84
D87

C. Monat Renew Shampoo





D. WEN Sweet Almond Mint Cleansing Conditioner

Figure 9. Visual assessment of skin pigmentation and hair growth from Day 0 (D0) to Day 98 (D98) after treatment. Dorsal hairs were shaved at D20 to better observe changes in skin pigmentation. One mouse in the Aquaphor cohort died at D26 (blue box). A WEN-treated mouse with weight loss (red box); this mouse was included in the timepoint 1 and sacrificed at Day 21.

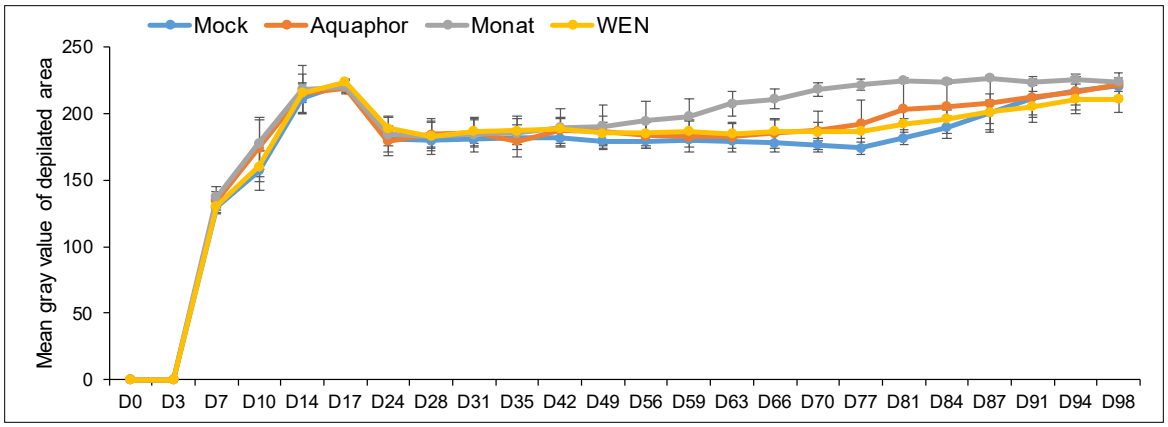


Figure 10. Mean gray values of depilated areas quantified using Image. Refer to Table 13 for the adjusted (adj.) p values for all data points. Gray value = pure white value (255) - gray value from ImageJ.

Table 13. Mean gray values of depilated areas (Exp-2).

Day		Mock	Aquaphor	Monat	WEN
D0	**mean ± SD**	0 ±0	0 ±0	0 ±0	0 ±0
	p-value		>0.9999	>0.9999	>0.9999
D3	**mean ± SD**	0 ±0	0 ±0	0 ±0	0 ±0
	p-value		>0.9999	>0.9999	>0.9999
D7	**mean ± SD**	129.56 ± 5.31	134.38 ± 7.15	137.26 ± 7.81	130.49 ± 4.91
	p-value		0.4941	0.0794	>0.9999
D10	**mean ± SD**	156.73 ± 14.92	173.91 ± 21.13	177.59 ± 19.84	160.01 ± 11.66
	p-value		**<0.0001**	**<0.0001**	>0.9999
D14	**mean ± SD**	211.11 ± 10.95	215.19 ± 14.69	217.91 ± 18.52	215.47 ± 7.24
	p-value		0.7136	0.1486	0.7825
D17	**mean ± SD**	222.16 ± 2.79	219.06 ± 3.1	219.88 ± 4.77	223.71 ± 2.1
	p-value		>0.9999	>0.9999	>0.9999
D24	**mean ± SD**	181.19 ± 5.96	179 ± 11.16	184.29 ± 13.1	189.08 ± 9.09
	p-value		>0.9999	>0.9999	0.3324
D28	**mean ± SD**	179.58 ± 5.15	184.1 ± 10.38	182.71 ± 13.46	182.76 ± 10.8
	p-value		>0.9999	>0.9999	>0.9999
D31	**mean ± SD**	180.71 ± 5.29	185.17 ± 10.75	184.11 ± 13.21	186.41 ± 8.7
	p-value		>0.9999	>0.9999	0.8408
D35	**mean ± SD**	181.43 ± 4.51	179.36 ± 12.01	185.36 ± 12.54	187.26 ± 9
	p-value		>0.9999	>0.9999	0.7649
D42	**mean ± SD**	181.61 ± 5.91	187.36 ± 9.53	189.24 ± 14.27	188.31 ± 7.59
	p-value		0.7713	0.3588	0.5766
D49	**mean ± SD**	179.08 ± 5.9	186.71 ± 11.01	190.19 ± 16.41	185.06 ± 8.25
	p-value		0.3958	0.0703	0.6791
D56	**mean ± SD**	179.18 ± 5.03	183.51 ± 10.07	194.61 ± 14.05	185.1 ± 9
	p-value		>0.9999	**0.005**	0.7029
D59	**mean ± SD**	180.16 ± 5.5	182.21 ± 11.56	197.84 ± 13.49	186.68 ± 8.02
	p-value		>0.9999	**0.001**	0.4882
D63	**mean ± SD**	179.3 ± 5.43	182.43 ± 11.3	207.4 ± 9.08	184.85 ± 7.5
	p-value		>0.9999	**<0.0001**	0.7334
D66	**mean ± SD**	177.63 ± 6.45	184.99 ± 11.07	210.83 ± 7.6	186.64 ± 8.68
	p-value		0.4398	**<0.0001**	0.1673
D70	**mean ± SD**	176.36 ± 5.1	186.93 ± 14.35	218.13 ± 5.22	186.3 ± 7.17
	p-value		0.1119	**<0.0001**	0.1478
D77	**mean ± SD**	174.01 ± 4.81	192.17 ± 18.18	221.41 ± 3.94	186.8 ± 5.49
	p-value		**0.0011**	**<0.0001**	**0.0243**
D81	**mean ± SD**	181.31 ± 4.36	203.3 ± 20.13	224.64 ± 2.01	191.98 ± 4.52
	p-value		**<0.0001**	**<0.0001**	0.0878

Day		Mock	Aquaphor	Monat	WEN
D84	**mean ± SD**	189.35 ± 8.34	205.2 ± 20.26	223.53 ± 1.79	195.94 ± 6.35
	p-value		**0.0054**	**<0.0001**	0.5237
D87	**mean ± SD**	200.28 ± 14.31	207.51 ± 19.73	226.41 ± 2.01	201.13 ± 8.77
	p-value		0.4601	**<0.0001**	>0.9999
D91	**mean ± SD**	211.79 ± 13.35	212 ± 15.19	223.44 ± 1.96	205 ± 11.59
	p-value		>0.9999	0.0525	0.4975
D94	**mean ± SD**	216.94 ± 10.73	216.14 ± 13.79	225.48 ± 1.97	210.84 ± 11.34
	p-value		>0.9999	0.2443	0.6762
D98	**mean ± SD**	220.54 ± 4.4	221.66 ± 9.2	223.8 ± 2.8	210.5 ± 9.79
	p-value		>0.9999	>0.9999	0.1549

Two-way ANOVA with Bonferroni multiple comparison test was used for statistical analysis. Adjusted (adj.) *p* values are included. Bold type: $p \leq 0.05$. Gray value = pure white value (255) - gray value from Image J. Dorsal hairs were shaved at D20.

Table 14. % area of full hair growth at Day 98.

		Mock	Aquaphor	Monet	WEN
D98	**mean ± SD**	87.28 ± 13.95	88.21 ± 19.82	99.48 ± 1.28	70.41 ± 19.67
	adj. p-value		>0.9999	0.1508	**0.0222**

Two-way ANOVA with Bonferroni multiple comparison test was used for statistical analysis. Adjusted (adj.) p values are included. Bold type: $p \leq 0.05$. Gray skin without visible hairs was excluded. % area of full hair growth = {area of visible hair growth ÷ total depilated area} x 100.

Table 15. % anagen skin at Day 98.

		Mock	Aquaphor	Monet	WEN
D98	**mean ± SD**	12.58 ± 13.82	11.60 ± 19.89	0.06 ± 0.18	14.29 ± 13.80
	adj. p-value		>0.9999	0.134	>0.9999

Two-way ANOVA with Bonferroni multiple comparison test was used for statistical analysis. Adjusted (adj.) p values are included. % area of anagen skin = {area of gray skin without visible hairs ÷ total depilated area} x 100.

Table 16. % telogen skin at Day 98.

		Mock	Aquaphor	Monat	WEN
D98	**mean ± SD**	0.13 ± 0.25	0.19 ± 0.34	0.46 ± 1.29	15.3 ± 10.1
	adj. p-value		>0.9999	>0.9999	**0.0467**

Two-way ANOVA with Bonferroni multiple comparison test was used for statistical analysis. Adjusted (adj.) p values are included. Bold type: $p \leq 0.05$. % telogen skin = {area of pink skin without visible hairs ÷ total depilated area} x 100.

Table 17. HF analysis at Day 21 (Exp-2, Timepoint-1).

		1st C (%)	1st T (%)	HF Diameter (µm)	HF Length (µm)
Mock	**mean ± SD**	100 ± 0	0 ± 0	39.54 ± 5.26	401.99 ± 26.36
	adj. p-value				
AQ	**mean ± SD**	59.47 ± 32.39	40.53 ± 32.39	28.42 ± 3.28	273.35 ± 46.45
	adj. p-value (vs. Mock)	0.9008	0.9008	>0.9999	**0.0017**
MO	**mean ± SD**	83.00 ± 21.64	17.00 ± 21.64	30.45 ± 2.84	308.01 ± 45.4
	adj. p-value (vs. Mock)	>0.9999	>0.9999	>0.9999	**0.0295**
WEN	**mean ± SD**	61.54 ± 52.74	36.92 ± 50.85	30.60 ± 1.3	326.60 ± 76.98
	adj. p-value (vs. Mock)	0.9183	>0.9999	>0.9999	0.0944

Two-way ANOVA with Bonferroni multiple comparison test was used for statistical analysis. Adjusted (adj.) p values are included. Bold type: $p \leq 0.05$.

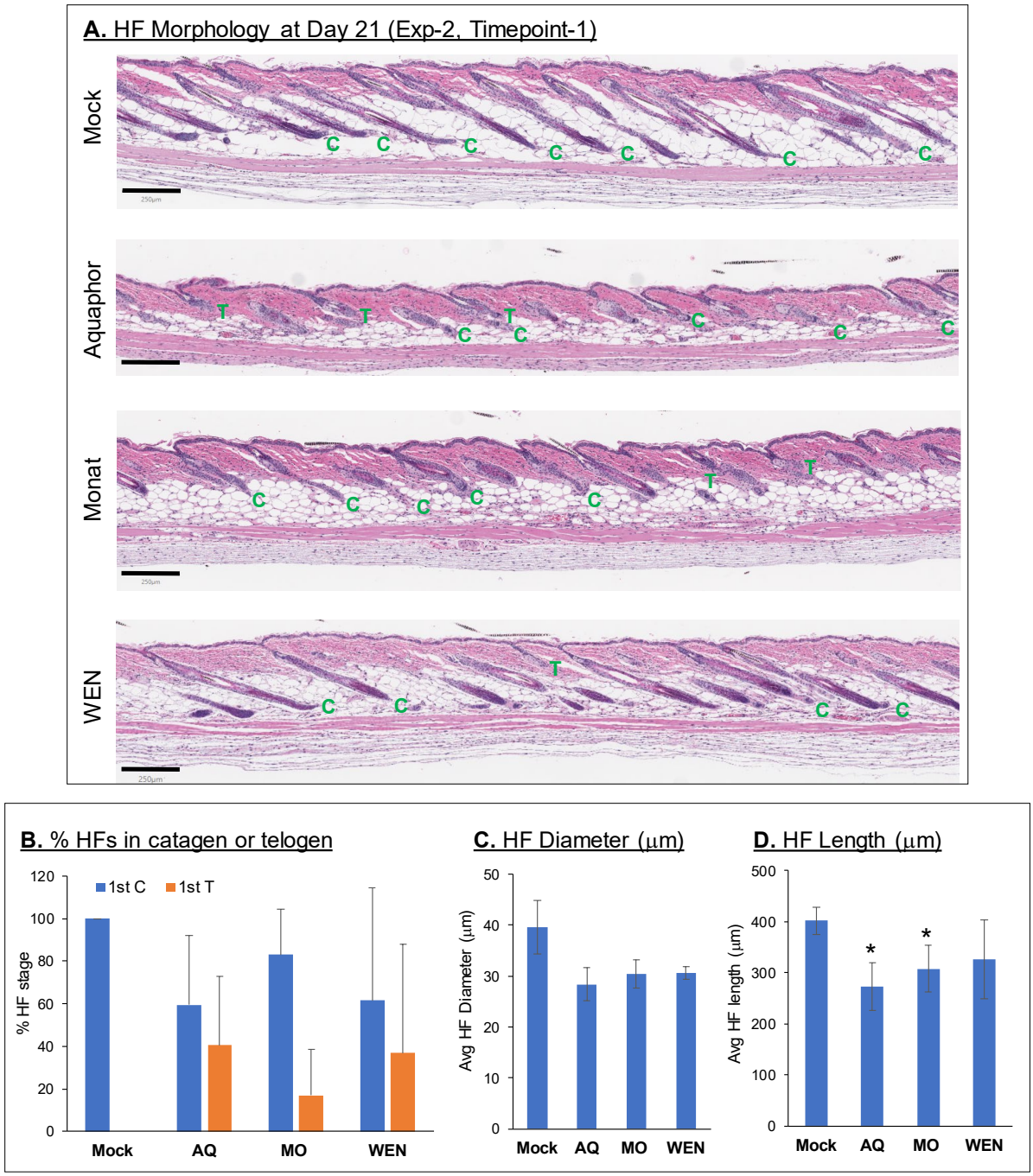
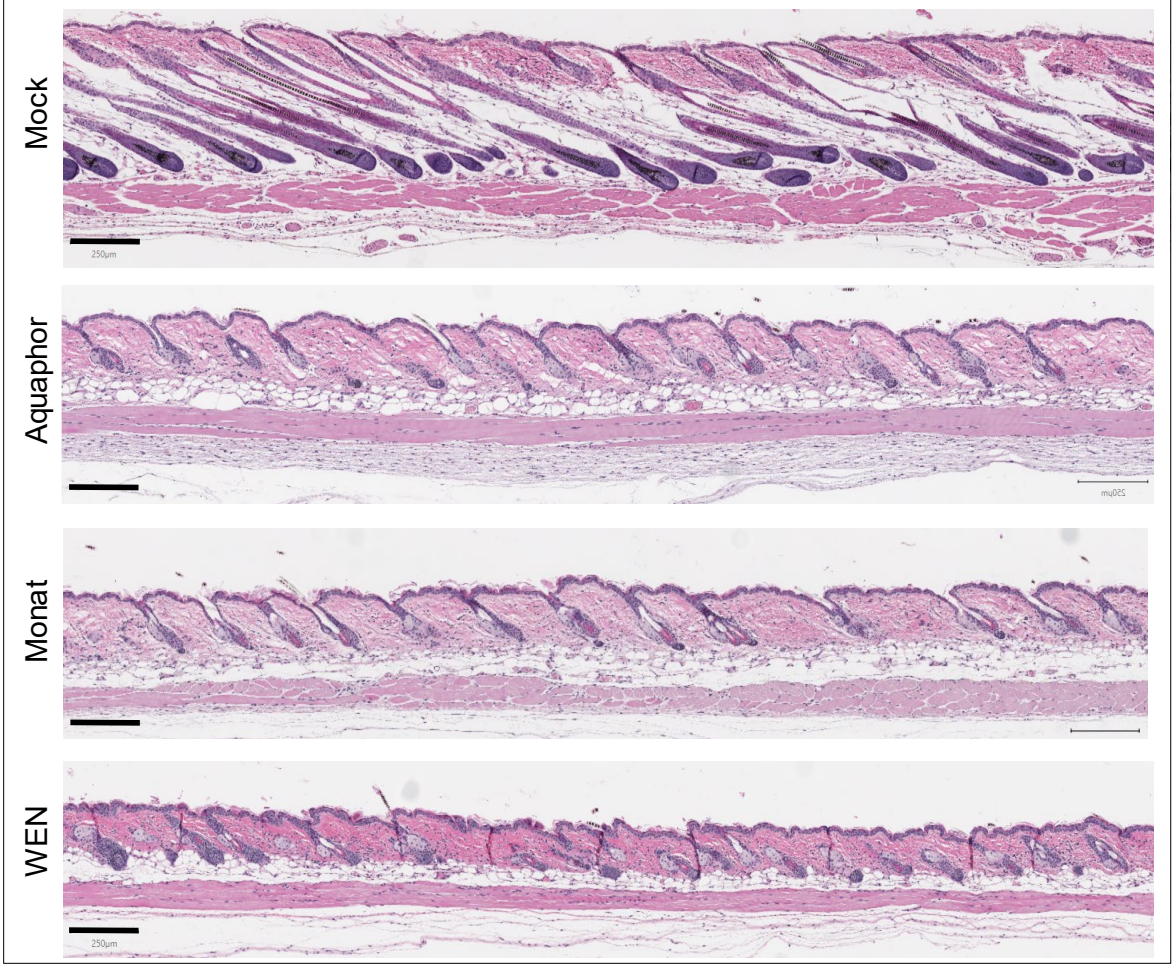
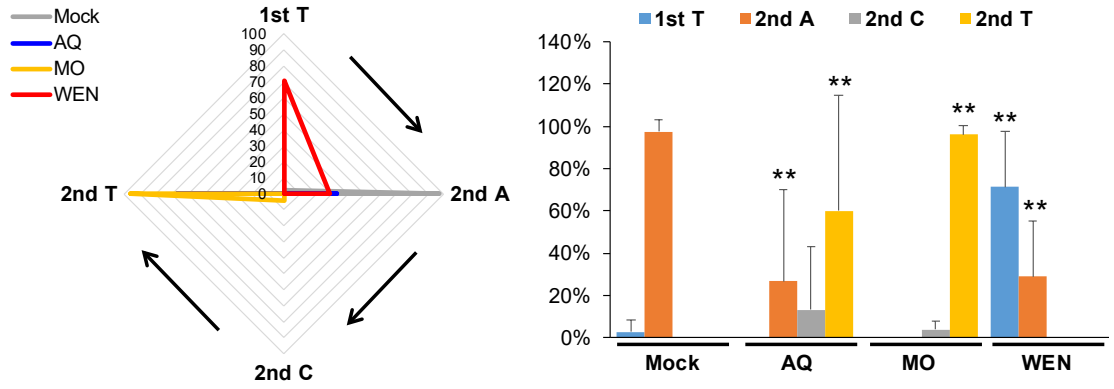


Figure 11. HF analysis at the anagen-telogen transition (Exp-2, Timepoint-1). A. Representative images of H&E stained longitudinal sections of mouse dorsal skin. B. % catagen (C) and telogen (T) HFs. % catagen HFs = {No. of catagen HFs÷Total No. of HFs} x 100. % telogen HFs = {No. of telogen HFs÷Total No. of HFs} x 100. C. Average HF diameter. D. Average HF length, corresponding to the distance from the epidermal granular layer to the top edge of the panniculus carnosus. The graph represents the mean ± SD. n = 4-7 mice per cohort *, $p \leq 0.05$. Refer to Table 17 for the adjusted (adj.) p values for all data points. AQ, Aquaphor; MO, Monat. Scale bar = 250 μm.

A. HF Morphology at Day 98 (Exp-2, Timepoint-2)



B. % HF Stage



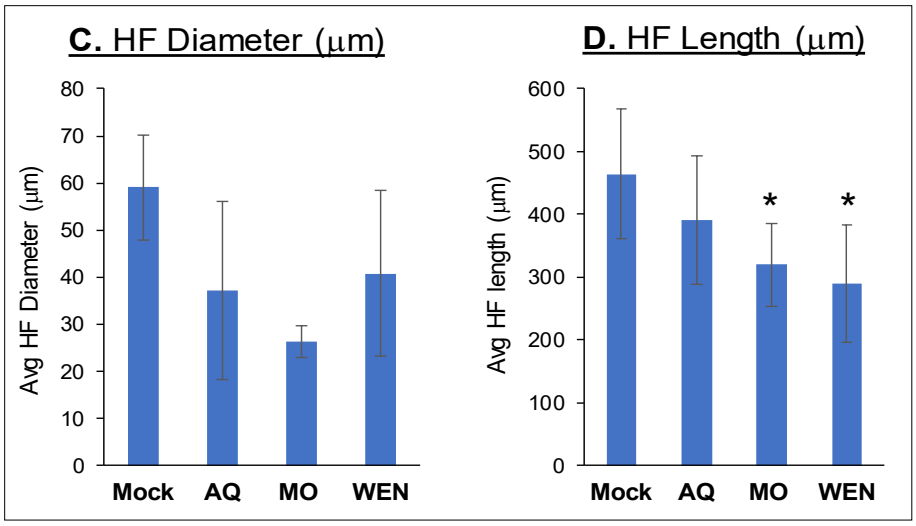


Figure 12. HF analysis at the telogen-anagen transition (Exp-2, Timepoint-2). A. Representative images of H&E stained longitudinal sections of mouse dorsal skin. B. % stage-specific HFs presented in radial (left) and column (right) charts. A, anagen HF; C, catagen HF; T, Telogen HF. % stage-specific HFs = {No. of stage-specific HFs÷ No. of total HFs} x 100. C. Average HF diameter. D. Average HF length, corresponding to the distance from the epidermal granular layer to the top edge of the panniculus carnosus. The graph represents the mean ± SD. n = 4-7 mice per cohort *, $p \leq 0.05$; **, $p \leq 0.0001$. Refer to Table 18 for the adjusted (adj.) p values for all data points. AQ, Aquaphor; MO, Monat. Scale bar = 250 μm.

Table 18. HF analysis at Day 98 (Exp-2, Timepoint-2).

		1st T (%)	2nd A (%)	2nd C (%)	2nd T (%)	HF Diameter (μm)	HF Length (μm)
Mock	**mean ± SD**	2.63 ± 5.88	97.37 ± 5.88	0 ± 0	0 ± 0	59.02 ± 11.17	464.1 ± 103.78
	adj. p-value						
AQ	**mean ± SD**	0 ± 0	26.67 ± 43.46	13.34 ± 29.81	60.00 ± 54.77	36.34 ± 18.02	411.26 ± 84.7
	adj. p-value (vs. Mock)	>0.9999	**<0.0001**	0.9696	**<0.0001**	>0.9999	0.5860
MO	**mean ± SD**	0 ± 0	0 ± 0	3.88 ± 4.06	96.12 ± 4.06	26.31 ± 3.44	319.56 ± 65.5
	adj. p-value (vs. Mock)	>0.9999	**<0.0001**	>0.9999	**<0.0001**	>0.9999	**0.0029**
WEN	**mean ± SD**	71.20 ± 26.52	28.80 ± 26.52	0 ± 0	0 ± 0	40.75 ± 17.62	290.56 ± 93.43
	adj. p-value (vs. Mock)	**<0.0001**	**<0.0001**	>0.9999	>0.9999	>0.9999	**0.0002**

Two-way ANOVA with Bonferroni multiple comparison test was used for statistical analysis. Adjusted (adj.) *p* values are included. Bold type: $p \leq 0.05$.

4.8.1.3 Comparison between the mock cohorts in Exp-1 and Exp-2

The rates of hair cycling and subsequent hair recovery differed between the mock cohorts, with overall delay observed in the Exp-2 mock cohort.

The Exp-2 mock cohort remained predominantly in the telogen stage for about 7.5 weeks before transitioning to the 2nd anagen phase, as compared to the 5-6 weeks observed for the Exp-1 mock cohort. Given the identical study design (e.g., experimental conditions, housing, researcher, etc.), the reason for this discrepancy in HF cycling between the two mock cohorts is uncertain. As in humans, mouse HF cycling can be influenced by various genetic and nongenetic conditions. For example, mouse hair growth is highly susceptible to environmental factors, such as season, humidity, cage type, and diet, which can cause stress in rodents, leading to various abnormal physiological responses, including alopecia. Indeed, psychoemotional stress has been shown to alter HF cycling and prematurely terminate the anagen stage in C57BL/6 mice [37]. Seasonal variation in response to

stressful situations has also been reported in C57BL/6 mice [38], and a higher incidence of hair loss and dermatitis in the winter has been observed in the mouse colony at Jackson Laboratory. In addition, mice are sensitive to various scents, and certain chemosignals or pheromones have been shown to invoke stress responses in mice [39]. Peppermint oil, menthol, and red clover can act as effective mouse deterrents. Menthol is present in WEN Sweet almond mint cleansing conditioner and red clover extract is one of the ingredients found in Monat Renew shampoo. The relevance of these confounding variables and the extent of their effects on our study require further investigation. Nevertheless, the hair cycle in the mock cohort eventually progressed to the 2nd anagen phase around Day 81, reaching 87.28% hair regrowth at Day 98 **(Table 14)**. Despite this difference in the hair growth cycle, there were no statistically significant differences in mock cohorts in body weight or skin pigmentation **(Table 19)**.

Table 19. Comparison between mock cohorts.

	Weight	Weight	Skin pigmentation	Skin pigmentation
	D28	**D85 or D98**	**D24**	**D85 or D98**
Exp-1 Mock	18.60 ± 0.6	20.70 ± 0.9	186.36 ± 14.92	209.34 ± 16.05
Exp-2 Mock	18.70 ± 0.6	21.53 ± 0.91	181.19 ± 5.96	220.54 ± 4.4
adj. p value	>0.9999	0.0786	0.7569	0.1258

Two-way ANOVA with Bonferroni multiple comparison test was used for statistical analysis. Adjusted (adj.) p values are included. D85 for Exp-1 mock, and D98 for Exp-2 mock.

4.8.2 Mast cells infiltration and distribution

Mast cells are crucial immunomodulatory cells that tend to be preferentially localized in certain skin regions, such as the perivascular, perifollicular, and perineural dermis [40]. Mast cells release proinflammatory mediators from cytoplasmic granules by degranulation, which is indicative of their activation. Even in the absence of visible signs of skin inflammation, increases in the numbers of total and activated mast cells are detectable in the dermis of non-scarring and non-inflammatory alopecia, including telogen effluvium and alopecia areata (AA) [41], and in AA, perifollicular mast cells have been implicated in facilitating cross-talk with CD8+ T cells [29].

Skin tissue sections were evaluated for the presence of mast cells using toluidine blue stain, one of the most frequently used metachromatic stains for the detection of mast cells [42]. Infiltrating and degranulating mast cells in the dermis and subcutis were quantified, as were degranulating perifollicular and follicular mast cells.

4.8.2.1 Evaluation of DevaCurl Low-Poo Delight Cleanser (Exp-1)

The numbers of total and degranulated mast cells did not differ substantially among all cohorts at Day 21 **(Fig. 13A, 13B; Table 20)**. However, the proportion of degranulated active mast cells was significantly higher in the CYP and DevaCurl cohorts (35.88% in CYP vs. 18.41% in nontreated, *p <0.0001*; 36.33% in DevaCurl vs. 26.71% in mock, *p=0.0224*) **(Fig. 13C, Table 20)**. Furthermore, in the CYP cohort, follicular mast cell activity was substantially higher (12.64% follicular vs. 3.41% perifollicular) **(Fig. 13D, 13E; Table 20)**. At Day 85, the Rogaine cohort showed a slight increase in the total number of mast cells (75.28 in Rogaine vs. 53.6 in mock, *p=0.0204*) **(Fig. 14A, 14B; Table 21)**. Given Rogaine's positive effects on hair growth, the reasons for this increase in mast cells are unclear. In humans, chronic administration of Rogaine has been associated with several adverse cutaneous effects, including scalp pruritus [43], a condition in which mast cells are considered the main conductors of itch [44, 45]. Nevertheless, the relevance of mast cells to pruritus in this study and its link to Rogaine warrant further investigations. Except for the Rogaine cohort, no significant differences in mast cell activation and distribution were observed among all cohorts **(Fig. 14C-E, Table 21)**.

4.8.2.2 Evaluation of Aquaphor Baby Wash & Shampoo, Monat Renew Shampoo, and WEN Sweet Almond Mint Cleansing Conditioner (Exp-2)

At Day 21, although the total number of mast cells was higher in Aquaphor and WEN cohorts **(Fig. 15A, 15B; Table 22)**, no statistically significant differences were observed in mast cell activation and distribution **(Fig. 15C-E, Table 22)**. At Day 98, mice in the Monat and WEN cohorts showed substantial increases in the total number of mast cells (63.02 in Monat, $p<0.0001$; 61.13 in WEN, $p<0.0001$ vs. 36.58 in mock) **(Fig. 16A, 16B; Table 23)**. WEN also caused significant increases in mast cell activation (44.66% in WEN vs. 23.36% in mock, $p<0.0001$) **(Fig. 16C, Table 23)**.

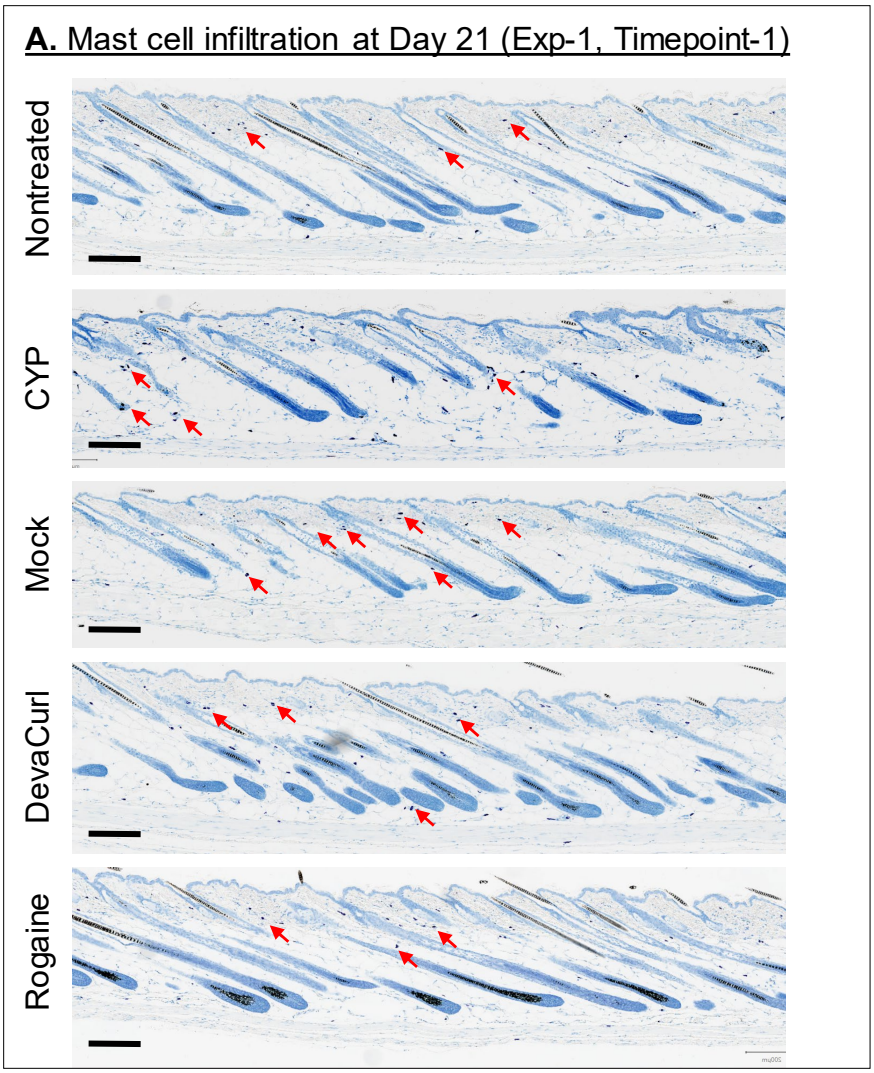
Notably, mast cell activity appeared to be preferentially localized within the HFs in mice treated with WEN, as the percentage of degranulated follicular mast cells was higher compared to those in the mock cohort (7.80% in WEN vs. 0.25% mock) **(Fig. 16A, 16C, 16D; Table 23)**. This difference, however, was not statistically significant **(Table 23)**.

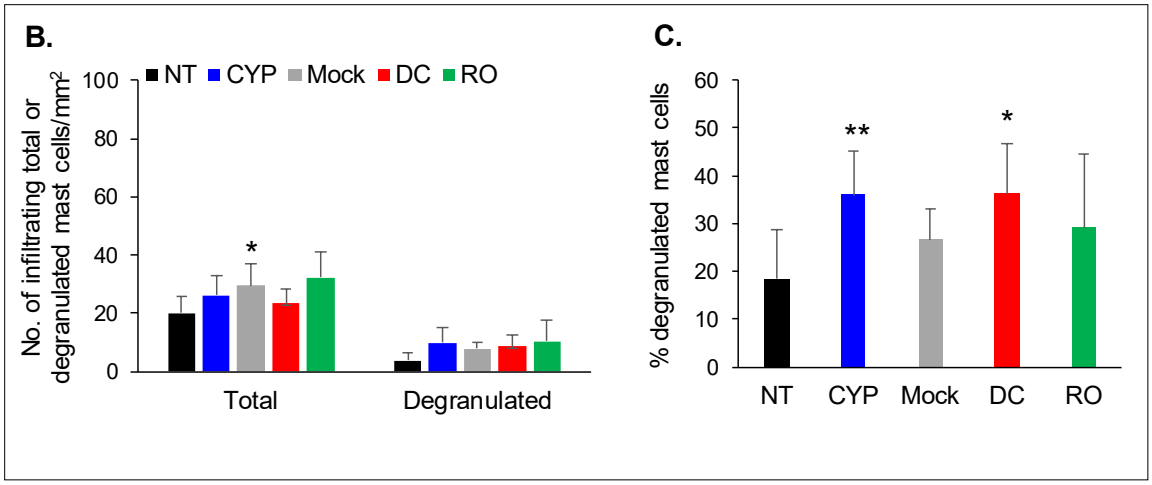
4.8.2.3 Validation of toluidine blue method

Mast cell proteases, including carboxypeptidase, chymase, and tryptase, represent the major protein components of secretory granules [42]. Immunohistochemical staining of select skin sections using mast cell tryptase antibody (Clone ARC2328, Invitrogen) showed a level of mast cell detection similar to that of metachromatic staining, validating the toluidine blue method used in this study (48.7±28 mast cells/mm^2 by immunohistochemical staining vs. 46.3±15.03 by toluidine blue staining, n=5 skin sections from Exp-2, timepoint-2) **(Fig. 17)**.

4.8.2.4 Conclusion

WEN caused significant increases in mast cell degranulation compared to the mock cohort at Day 98, suggesting the possible involvement of mast cell activity in delayed progression to the anagen phase. DevaCurl also increased mast cell activity; however, this increase was detectable only at the anagen-telogen transition (Day 21).


A. Mast cell infiltration at Day 21 (Exp-1, Timepoint-1)
Nontreated
CYP
Mock
DevaCurl
Rogaine


B.
NT
CYP
Mock
DC
RO
No. of infiltrating total or degranulated mast cells/mm²
100
80
60
40
20
0
*
Total
Degranulated
C.
% degranulated mast cells
60
50
40
30
20
10
0
**
*
NT
CYP
Mock
DC
RO

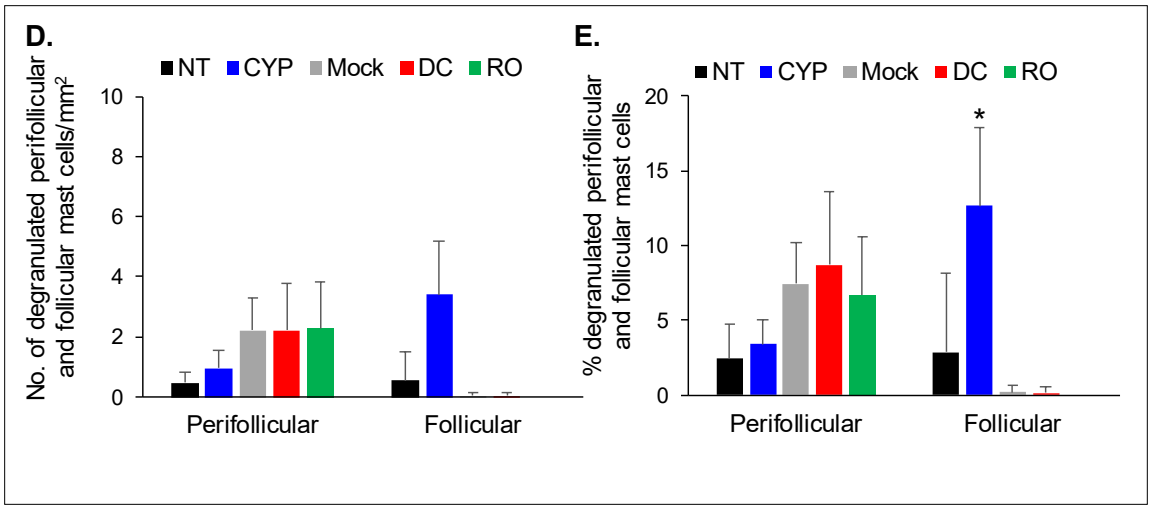
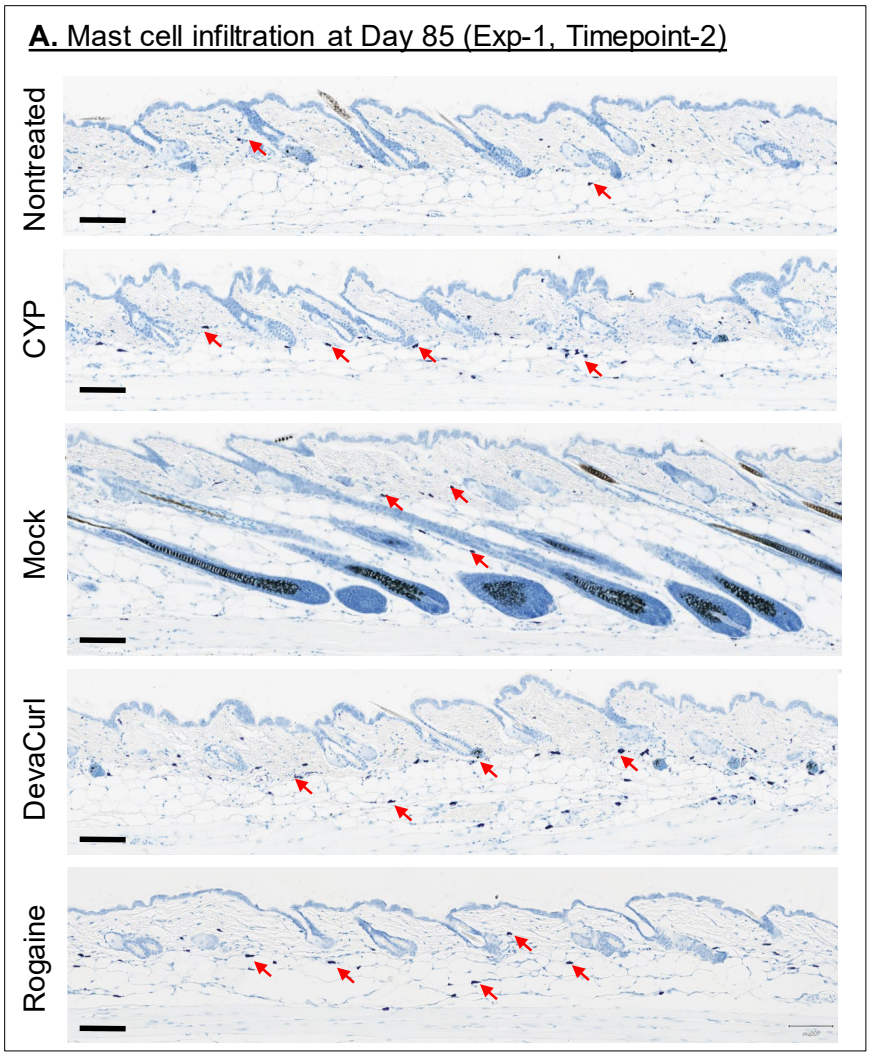


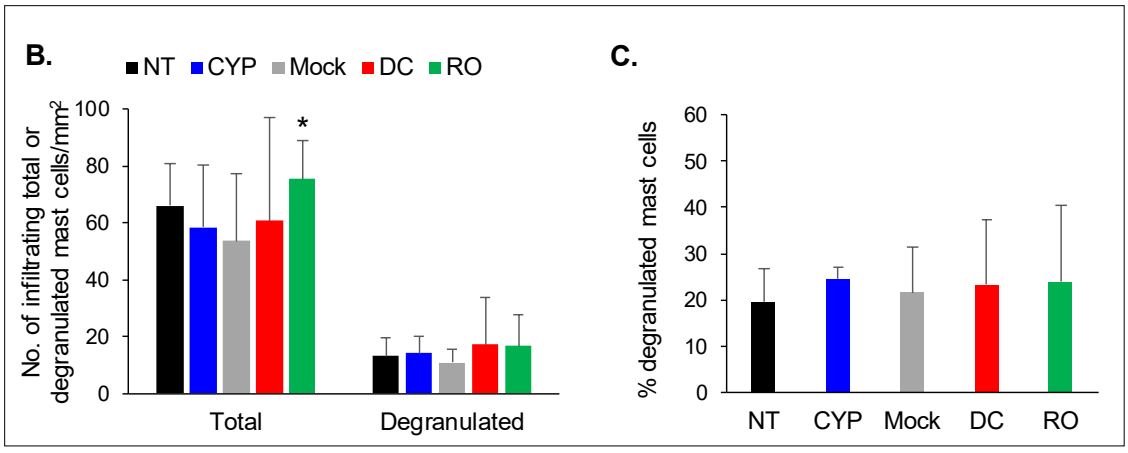
Figure 13. Mast cell infiltration and degranulation at the anagen-telogen transition (Exp-1, Timepoint-1). A. Representative images of toluidine blue-stained longitudinal sections of mouse dorsal skin. Red arrows, mast cells (MCs). B. Average number of infiltrating and degranulating MCs per mm2. C. % degranulation. D. Average number of degranulating perifollicular and follicular MCs. E. % degranulation of perifollicular and follicular MCs. The graph represents the mean ± SD. n = 4-7 mice per cohort *, $p \leq 0.05$; **, $p \leq 0.0001$. Refer to Table 20 for the adjusted p values for all data points. NT, nontreated; CYP, cyclophosphamide; DC, DevaCurl, RO, Rogaine. Scale bar = 200 µm.

Table 20. Mast cell infiltration and degranulation at Day 21 (Exp-1, Timepoint-1).

		Total No. MC/ mm²	No. Degranulated MC/ mm²	% Degranulation	No. Perifollicular MC/ mm²	No. Follicular MC/ mm²	% Perifollicular	% Follicular
NT	**mean ± SD**	20.1 ± 5.91	3.8 ± 2.55	18.41 ± 10.34	0.48 ± 0.35	0.55 ± 0.93	2.44 ± 2.31	2.88 ± 5.28
	adj. p-value							
CYP	**mean ± SD**	26.18 ± 6.54	9.85 ± 5.21	35.88 ± 9.37	0.95 ± 0.62	3.4 ± 1.8	3.41 ± 1.63	12.64 ± 5.22
	adj. p-value (vs. NT)	0.6287	0.6467	**<0.0001**	>0.9999	>0.9999	>0.9999	**0.0309**
MOCK	**mean ± SD**	29.44 ± 7.67	7.75 ± 2.51	26.71 ± 6.42	2.21 ± 1.09	0.05 ± 0.12	7.47 ± 2.73	0.18 ± 0.43
	adj. p-value (vs. NT)	**0.0300**	>0.9999	0.0818	>0.9999	>0.9999	>0.9999	>0.9999
DC	**mean ± SD**	23.73 ± 4.52	8.77 ± 3.6	36.33 ± 10.42	2.23 ± 1.55	0.04 ± 0.11	8.71 ± 4.9	0.15 ± 0.37
	adj. p-value (vs. Mock)	0.6736	>0.9999	**0.0224**	>0.9999	>0.9999	>0.9999	>0.9999
RO	**mean ± SD**	32.2 ± 9.03	10.34 ± 7.3	29.18 ± 15.24	2.29 ± 1.53	0 ± 0	6.69 ± 3.94	0 ± 0
	adj. p-value (vs. Mock)	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999

Two-way ANOVA with Bonferroni multiple comparison test was used for statistical analysis. Adjusted (adj.) *p* values are included. Bold type: $p \leq 0.05$. % degranulated = (No. degranulated MC ÷ Total No. of MC) x 100. MC, mast cells.


A. Mast cell infiltration at Day 85 (Exp-1, Timepoint-2)
Nontreated
CYP
Mock
DevaCurl
Rogaine


B.
NT
CYP
Mock
DC
RO
No. of infiltrating total or degranulated mast cells/mm2
100
80
60
40
20
0
*
Total
Degranulated
C.
% degranulated mast cells
60
50
40
30
20
10
0
NT
CYP
Mock
DC
RO

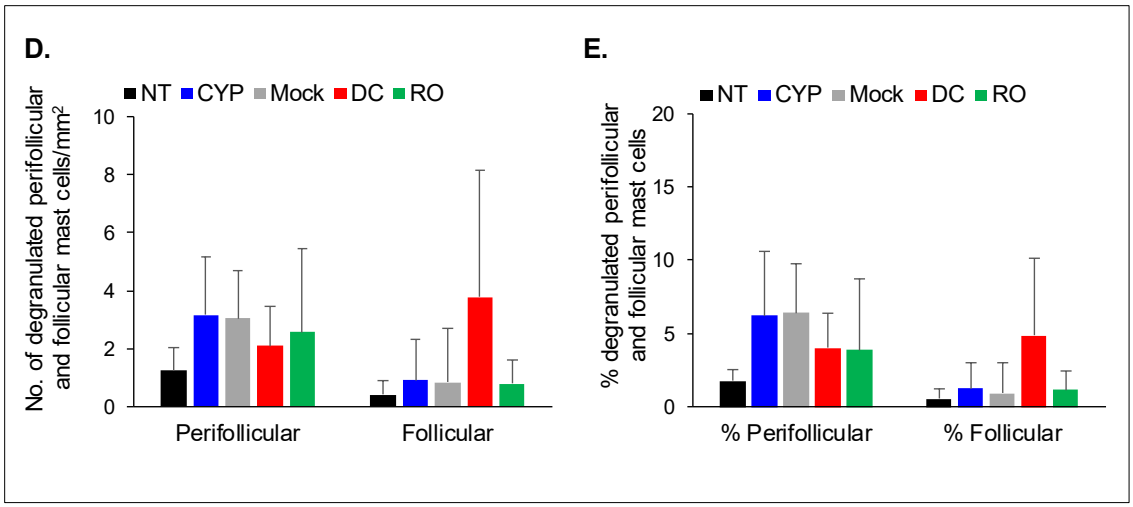


Figure 14. Mast cell infiltration and degranulation at the telogen-anagen transition (Exp-1, Timepoint-2). A. Representative images of toluidine blue-stained longitudinal sections of mouse dorsal skin. Red arrows, Mast cells (MCs). B. Average number of infiltrating and degranulating MCs per mm2. C. % degranulation. D. Average number of degranulating perifollicular and follicular MCs. E. % degranulation of perifollicular and follicular MCs. The graph represents the mean ± SD. n = 4-7 mice per cohort. *, $p \leq 0.05$. Refer to Table 21 for the adjusted (adj.) p values for all data points. NT, nontreated; CYP, cyclophosphamide; DC, DevaCurl, RO, Rogaine. Scale bar = 100 µm.

Table 21. Mast cell infiltration and degranulation at Day 85 (Exp-1, Timepoint-2).

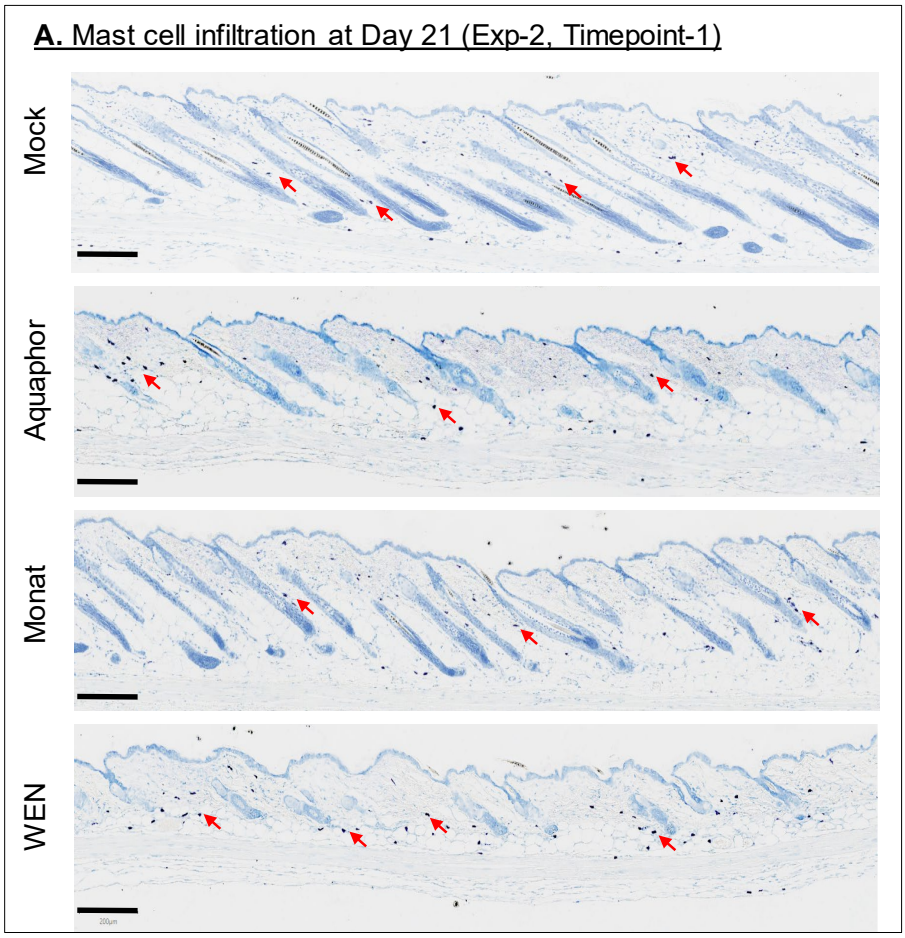
		Total No. MC/ mm²	No. Degranulated MC/ mm²	% Degranulation	No. Perifollicular MC/ mm²	No. Follicular MC/ mm²	% Perifollicular	% Follicular
NT	**mean ± SD**	66.13 ± 14.61	13.18 ± 6.68	19.5 ± 7.15	1.23 ± 0.82	0.42 ± 0.49	1.75 ± 0.75	0.57 ± 0.66
	adj. p-value							
CYP	**mean ± SD**	58.36 ± 22.16	14.32 ± 5.7	24.56 ± 2.44	3.15 ± 2	0.91 ± 1.43	6.22 ± 4.37	1.25 ± 1.75
	adj. p-value (vs. NT)	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
MOCK	**mean ± SD**	53.6 ± 23.69	10.86 ± 4.66	21.61 ± 9.96	3.04 ± 1.64	0.83 ± 1.86	6.41 ± 3.36	0.93 ± 2.07
	adj. p-value (vs. NT)	0.8859	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
DC	**mean ± SD**	60.82 ± 36.16	17.19 ± 16.44	23.29 ± 14.19	2.09 ± 1.37	3.77 ± 4.37	4.02 ± 2.39	4.87 ± 5.22
	adj. p-value (vs. Mock)	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
RO	**mean ± SD**	75.28 ± 13.56	16.86 ± 10.95	23.81 ± 16.74	2.56 ± 2.88	0.78 ± 0.83	3.89 ± 4.8	1.15 ± 1.28
	adj. p-value (vs. Mock)	**0.0204**	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999

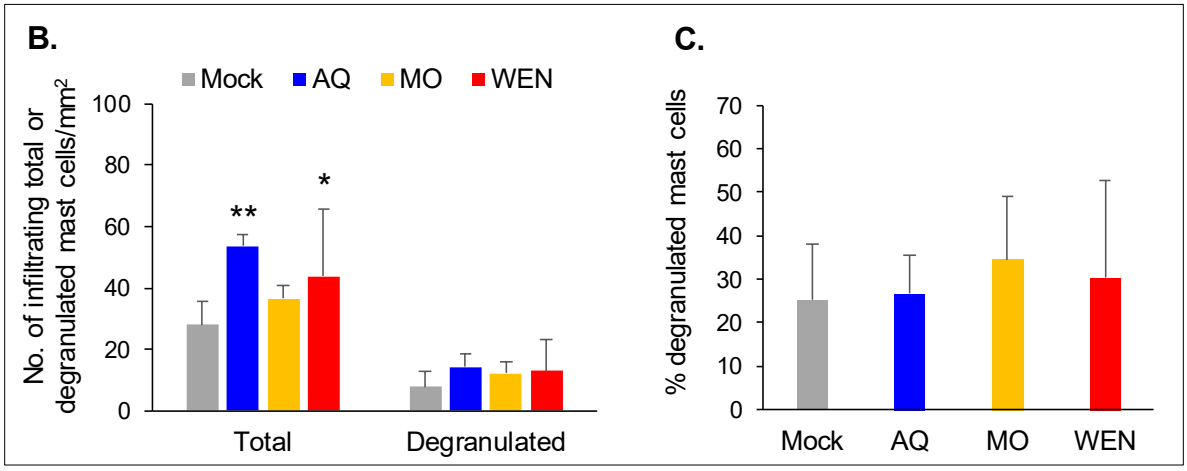
Two-way ANOVA with Bonferroni multiple comparison test was used for statistical analysis. Adjusted (adj.) *p* values are included. Bold type: $p \leq 0.05$. % degranulated = (No. degranulated MC ÷ Total No. of MC) x 100. MC, mast cells.

Table 22. Mast cell infiltration and degranulation at Day 21 (Exp-2, Timepoint-1).

		Total No. MC/ mm²	No. Degranulated MC/ mm²	% Degranulation	No. Perifollicular MC/ mm²	No. Follicular MC/ mm²	% Perifollicular	% Follicular
MOCK	**mean ± SD**	28.06 ± 7.3	7.7 ± 5.33	25.28 ± 12.67	1.07 ± 1.03	0 ± 0	3.32 ± 3.14	0 ± 0
	adj. p-value							
AQ	**mean ± SD**	53.69 ± 3.9	14.23 ± 4.47	26.62 ± 8.94	2.76 ± 1.29	0.66 ± 0.65	5.14 ± 2.41	1.26 ± 1.25
	adj. p-value (vs. Mock)	**<0.0001**	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
MO	**mean ± SD**	36.43 ± 4.47	12.16 ± 3.91	34.58 ± 14.4	2.56 ± 1.77	0.13 ± 0.2	7.31 ± 5.44	0.33 ± 0.47
	adj. p-value (vs. Mock)	0.4883	>0.9999	0.3191	>0.9999	>0.9999	>0.9999	>0.9999
WEN	**mean ± SD**	43.84 ± 22.02	13.05 ± 10.29	30.4 ± 22.35	2.88 ± 1.27	0 ± 0	7.27 ± 3.9	0 ± 0
	adj. p-value (vs. Mock)	**0.0140**	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999

Two-way ANOVA with Bonferroni multiple comparison test was used for statistical analysis. Adjusted (adj.) *p* values are included. Bold type: $p \leq 0.05$. % degranulated = (No. degranulated MC ÷ Total No. of MC) x 100. MC, mast cells.


A. Mast cell infiltration at Day 21 (Exp-2, Timepoint-1)
Mock
Aquaphor
Monat
WEN


B.
Mock
AQ
MO
WEN
No. of infiltrating total or degranulated mast cells/mm2
100
80
60
40
20
0
**
*
Total
Degranulated
C.
% degranulated mast cells
70
60
50
40
30
20
10
0
Mock
AQ
MO
WEN

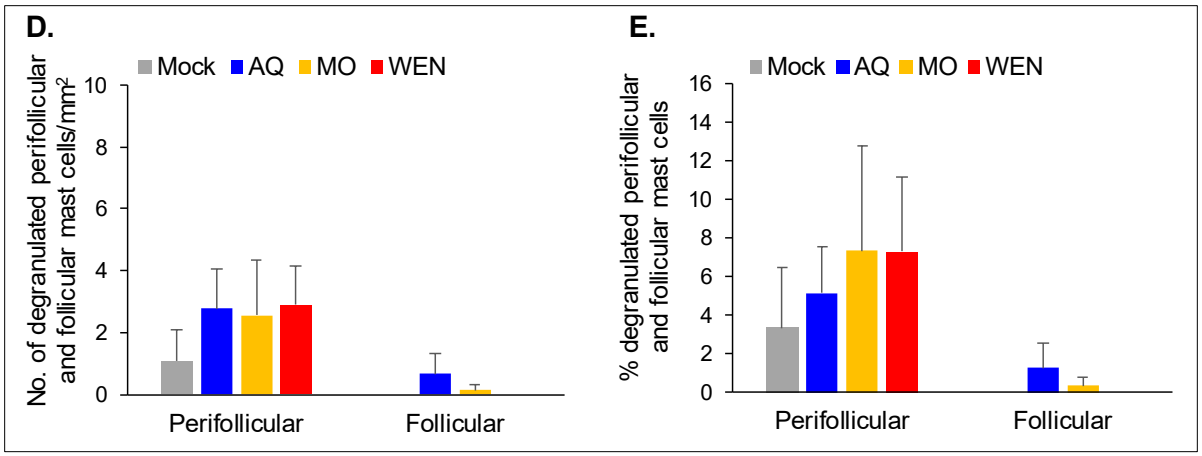
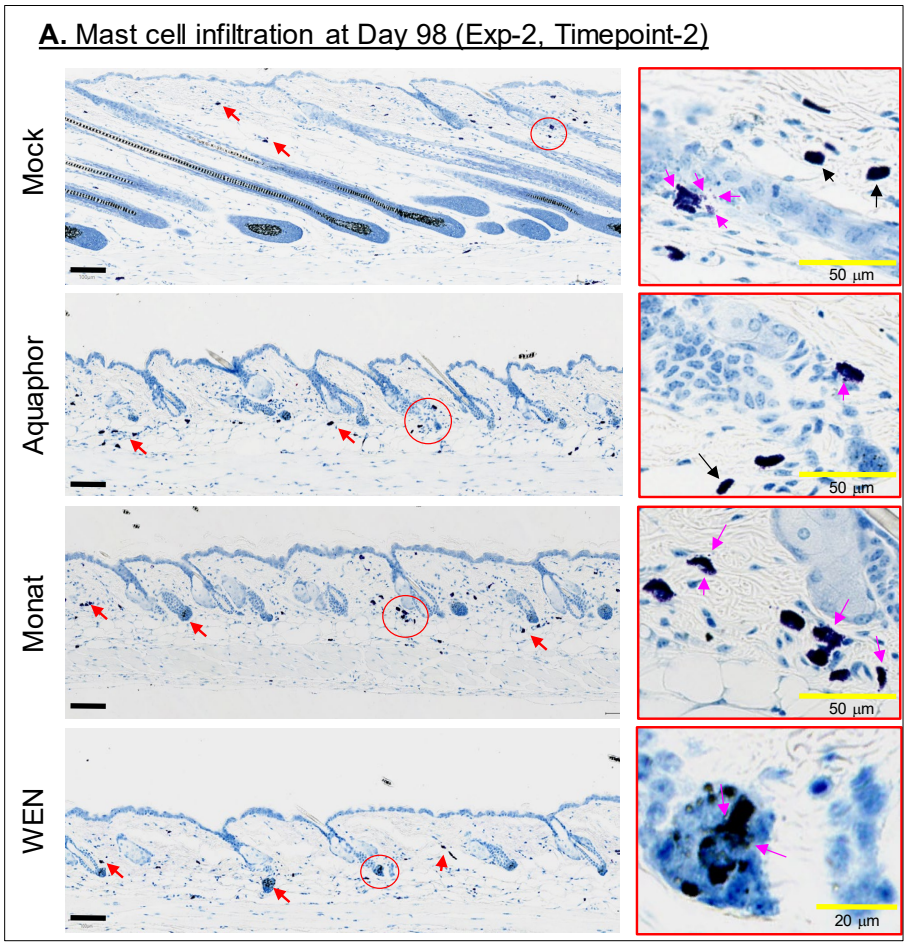


Figure 15. Mast cell infiltration and degranulation at the anagen-telogen transition (Exp-2, Timepoint-1). A. Representative images of toluidine blue-stained longitudinal sections of mouse dorsal skin. Red arrows, mast cells (MCs). B. The average number of infiltrating and degranulating MCs per mm2. C. % degranulation. D. Average number of degranulating perifollicular and follicular MCs. E. % degranulation of perifollicular and follicular MCs. The graph represents the mean ± SD. n = 4-7 mice per cohort. *, $p \leq 0.05$; **. $p \leq 0.0001$. Refer to Table 22 for the adjusted (adj.) p values for all data points. AQ, Aquaphor; MO, Monat. Scale bar = 200 µm.



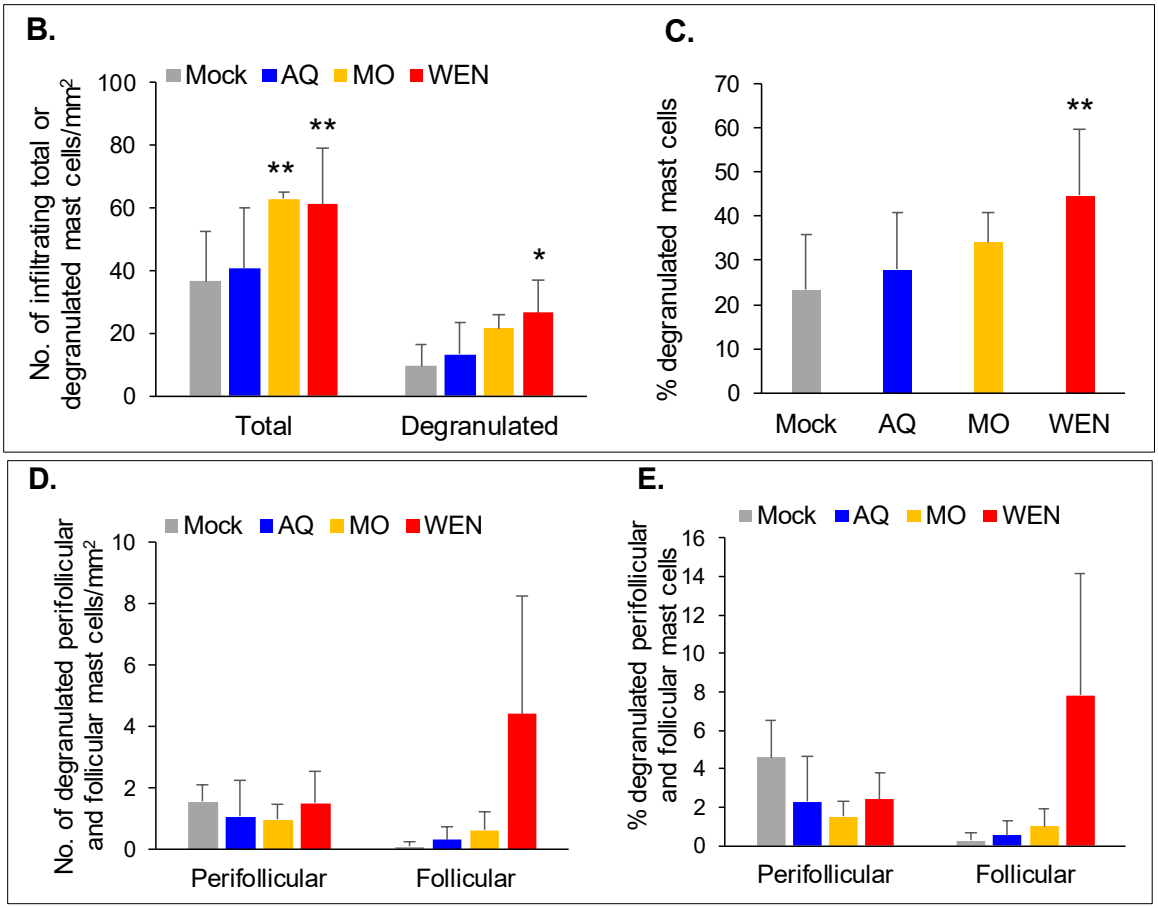


Figure 16. Mast cell infiltration and degranulation at the telogen-anagen transition (Exp-2, Timepoint-2). A. Representative images of toluidine blue-stained longitudinal sections of mouse dorsal skin. Red arrows, red circles, mast cells (MCs). Red box, higher magnification of red circle. Black arrows, regular MCs; magenta arrow, degranulating MCs. B. The average number of infiltrating and degranulating MCs per mm2. C. % degranulation. D. Average number of degranulating perifollicular and follicular MCs per mm2. E. % degranulation of perifollicular and follicular MCs. The graph represents the mean ± SD. n = 4-7 mice per cohort. *, $p \leq 0.05$; **. $p \leq 0.0001$. Refer to Table 23 for the adjusted (adj.) p values for all data points. AQ, Aquaphor; MO, Monat. Black scale bar = 100 µm.

Table 23. Mast cell infiltration and degranulation at Day 98 (Exp-2, Timepoint-2).

		Total No. MC/ mm²	No. Degranulated MC/ mm²	% Degranulation	No. Perifollicular MC/ mm²	No. Follicular MC/ mm²	% Perifollicular	% Follicular
Mock	**mean ± SD**	36.58 ± 15.87	9.36 ± 7.24	23.36 ± 12.59	1.56 ± 0.52	0.09 ± 0.16	4.59 ± 1.89	0.25 ± 0.45
	adj. p-value							
AQ	**mean ± SD**	40.6 ± 19.37	13.15 ± 10.36	27.9 ± 12.85	1.06 ± 1.2	0.32 ± 0.44	2.28 ± 2.39	0.54 ± 0.75
	adj. p-value (vs. Mock)	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999
MO	**mean ± SD**	63.02 ± 1.82	21.55 ± 4.27	34.18 ± 6.55	0.96 ± 0.5	0.62 ± 0.59	1.51 ± 0.78	0.99 ± 0.92
	adj. p-value (vs. Mock)	**<0.0001**	0.0850	0.1748	>0.9999	>0.9999	>0.9999	>0.9999
WEN	**mean ± SD**	61.13 ± 17.76	26.58 ± 10.13	44.66 ± 14.95	1.51 ± 1.01	4.4 ± 3.83	2.42 ± 1.35	7.8 ± 6.34
	adj. p-value (vs. Mock)	**<0.0001**	**0.0019**	**<0.0001**	>0.9999	>0.9999	>0.9999	0.6478

Two-way ANOVA with Bonferroni multiple comparison test was used for statistical analysis. Adjusted (adj.) *p* values are included. Bold type: $p \leq 0.05$. % degranulated = (No. degranulated MC ÷ Total No. of MC) x 100. MC, mast cells.

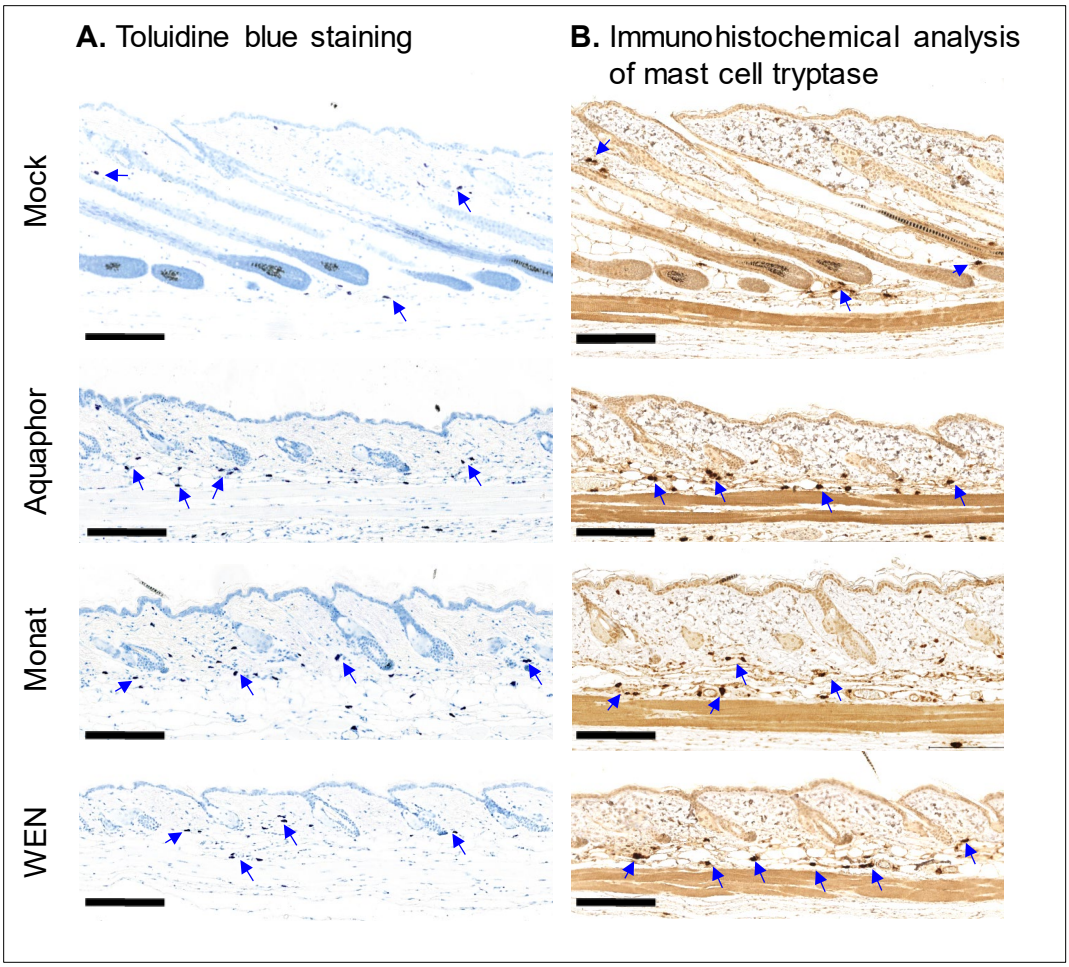


Figure 17. Detection of mast cells by toluidine blue staining (A) and immunohistochemical staining using mast cell tryptase antibody (B). Representative images are shown. n=5 tissue sections per assay. Blue arrows, mast cells. Scale bar = 200 μm.

4.8.3 Macrophage Evaluation

4.8.3.1 Evaluation of DevaCurl Low-Poo Delight Cleanser (Exp-1), and Aquaphor Baby Wash & Shampoo, Monat Renew Shampoo, and WEN Sweet Almond Mint Cleansing Conditioner (Exp-2)

Skin-resident macrophages have been implicated in the regulation of HF cycling, particularly during the transition from the anagen phase to the catagen phase. Decreases in macrophages occur before the onset of the anagen phase, and a selective reduction in the number of macrophages has been shown to induce premature entry into the anagen phase [46]. Immunohistochemical staining of skin sections using murine macrophage-specific anti-F4/80 antibody demonstrated higher numbers of F4/80+ macrophages in telogen skins (nontreated, CYP, DevaCurl, and Rogaine at D85 vs. D21, Exp-1) whereas the number of F4/80+ macrophages in the mock was significantly decreased compared to the nontreated cohort at Day 85 **(Figs. 18 &19, Table 24)**. While these data corroborate those from prior studies and suggest hair cycle-dependent fluctuations in macrophages, no significant changes were detectable in the numbers of F4/80+ cells or in their distribution in mice treated with the test products (Mock vs. DC, MO, WEN) **(Figs. 18-21; Tables 24 & 25)**.

4.8.3.2 Conclusion

All cohorts showed telogen-associated increases in the number of F4/80+ macrophages. No significant differences were observed among the test products in the number and distribution of F4/80+ macrophages. Recent studies have shown that TREM2+ macrophages promote HF stem cell quiescence during telogen and inhibit hair growth, suggesting the role of a distinct subset of dermal macrophages in hair loss associated with prolonged or arrested telogen [47]. Further studies using markers of specialized macrophages may help identify subpopulations relevant to alopecia.

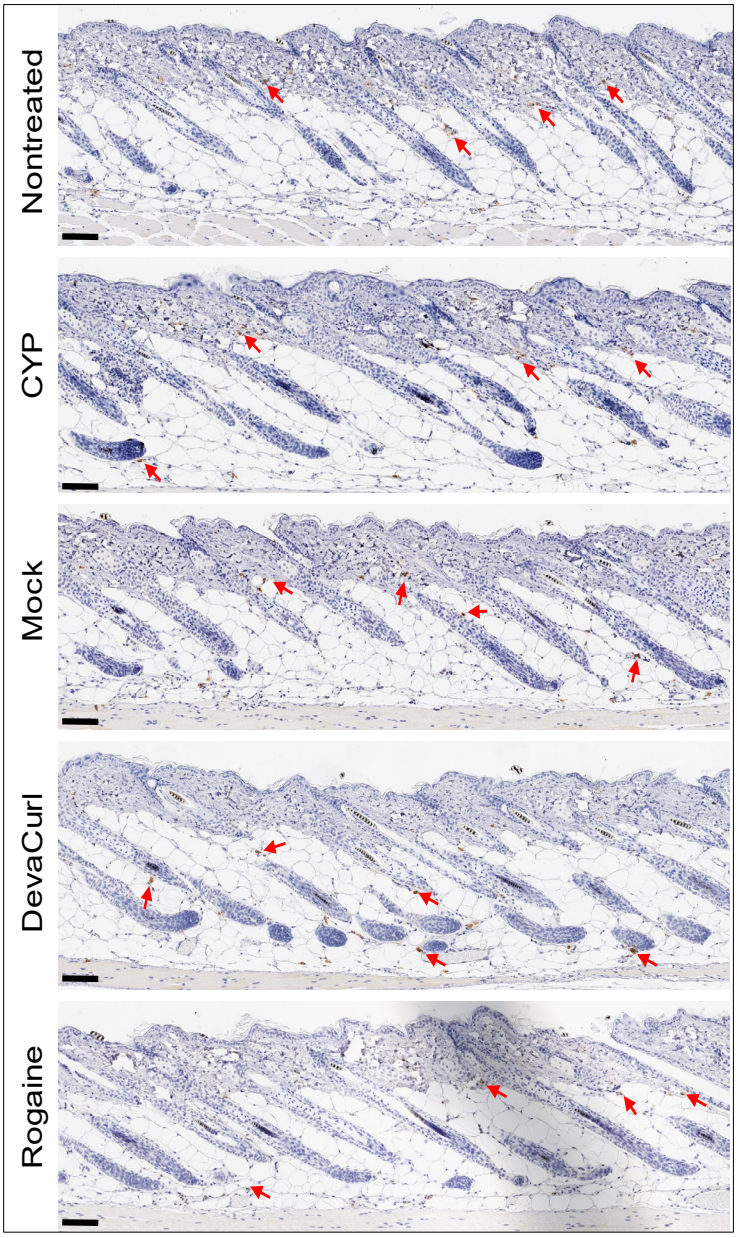


Figure 18. Immunohistochemical detection of macrophage (Exp-1, Timepoint-1). Representative images of longitudinal sections of mouse dorsal skin stained for F4/80. Red arrows, F4/80+ cells. Black scale bar = 100 µm.

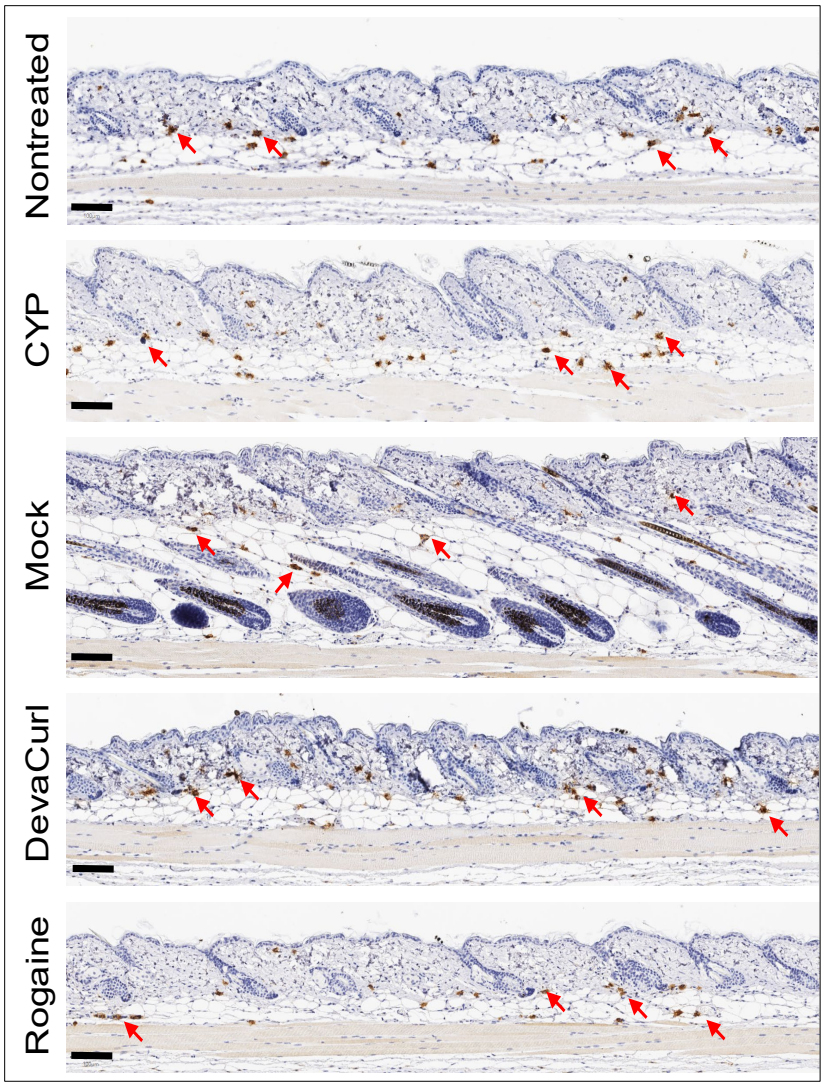


Figure 19. Immunohistochemical detection of macrophage (Exp-1, Timepoint-2). Representative images of longitudinal sections of mouse dorsal skin stained for F4/80. Red arrows, F4/80+ cells. Black scale bar = 100 μm.

Table 24. Macrophage analysis (Exp-1, Timepoints-1 and 2).

		NT	CYP	Mock	DC	Rogaine
D21	**mean ± SD**	13.86 ± .32	24.75 ± 4.63	22.02 ± 5.49	12.39 ± 4.6	17.96 ± 3.57
	adj. p-value (vs. NT or Mock)		>0.9999	>0.9999	>0.9999	>0.9999
D85	**mean ± SD**	64.69 ± 16.21	60.96 ± 6.31	35.94 ± 16.37	55.26 ± 25.16	66.73 ± 7.64
	adj. p-value (vs. NT or Mock)		>0.9999	**0.011**	0.22	**0.0054**

Two-way ANOVA with Bonferroni multiple comparison test was used for statistical analysis. Adjusted (adj.) p values are included. Bold type: $p \leq 0.05$. Data represent no. of F4/80+ Macrophages/mm^2

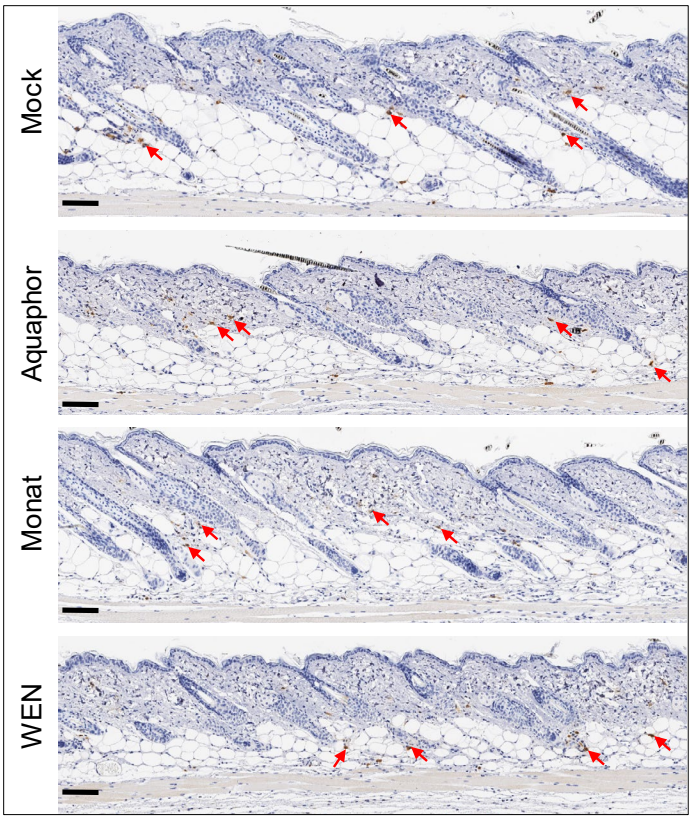


Figure 20. Immunohistochemical detection of macrophage (Exp-2, Timepoint-1). Representative images of longitudinal sections of mouse dorsal skin stained for F4/80. Red arrows, F4/80+ cells. Black scale bar = 100 µm.

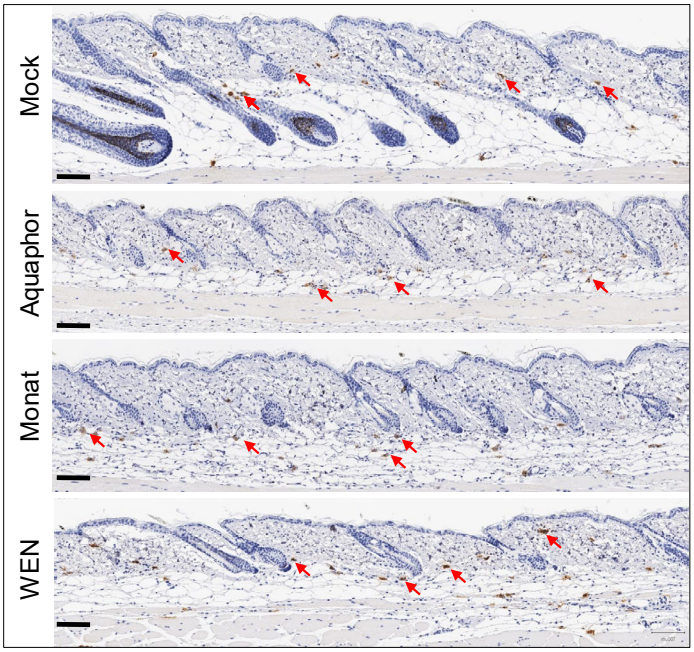


Figure 21. Immunohistochemical detection of macrophage (Exp-2, Timepoint-2). Representative images of longitudinal sections of mouse dorsal skin stained for F4/80. Red arrows, F4/80+ cells. Black scale bar = 100 µm.

Table 25. Macrophage analysis (Exp-2, Timepoints 1 and 2).

		Mock	AQ	MO	WEN
D21	**mean ± SD**	17.98 ± 3.03	32.08 ± 3.97	24.79 ± 6.04	30.92 ± 7.67
	adj. p-value (vs. Mock)		0.6171	>0.9999	0.7330
D98	**mean ± SD**	44.93 ± 31.03	32.57 ± 9.67	43.89 ± 4.77	39.92 ± 23.43
	adj. p-value (vs. Mock)		0.7956	>0.9999	>0.9999

Two-way ANOVA with Bonferroni multiple comparison test was used for statistical analysis. Adjusted (adj.) p values are included. Data represent no. of F4/80+ Macrophages/mm^2

4.8.4 Assessment of hair damage and structural abnormality

Hair care products with high detergent properties can remove the outer cuticle, leave hair frizzy and dull, and cause structural damage. To determine whether the test products cause structural abnormalities of the hair shafts, dorsal hairs were evaluated for structural weaknesses, hair breakage, focal bulge, and any abnormal features that represent defective hair [48]. In nontreated mice, the hair shafts showed well-maintained structures with <2% of the hairs showing anomalies **(Fig. 22, Table 26).** Pigmentation anomalies, including pigment clumping and depigmentation, were present in 10.00% of hairs in the CYP cohort at Day 21 (*p <0.0001*) **(Fig. 22, Table 26)**. While various defects in the hair shafts were observed at Day 85 in all cohorts, the severity of the damages was not statistically significant **(Fig. 23, Table 27)**. Similarly, overall hair damage in Exp-1 was comparable to that in Exp-2 at both HF transitions **(Figs. 24 & 25, Tables 28 & 29)**. Rather, structural weakness including hair breakage was reduced in the WEN cohort at Day 98 (0.50% WEN vs. 4.50% mock, *p=0.0112*) **(Fig. 25, Table 29).**

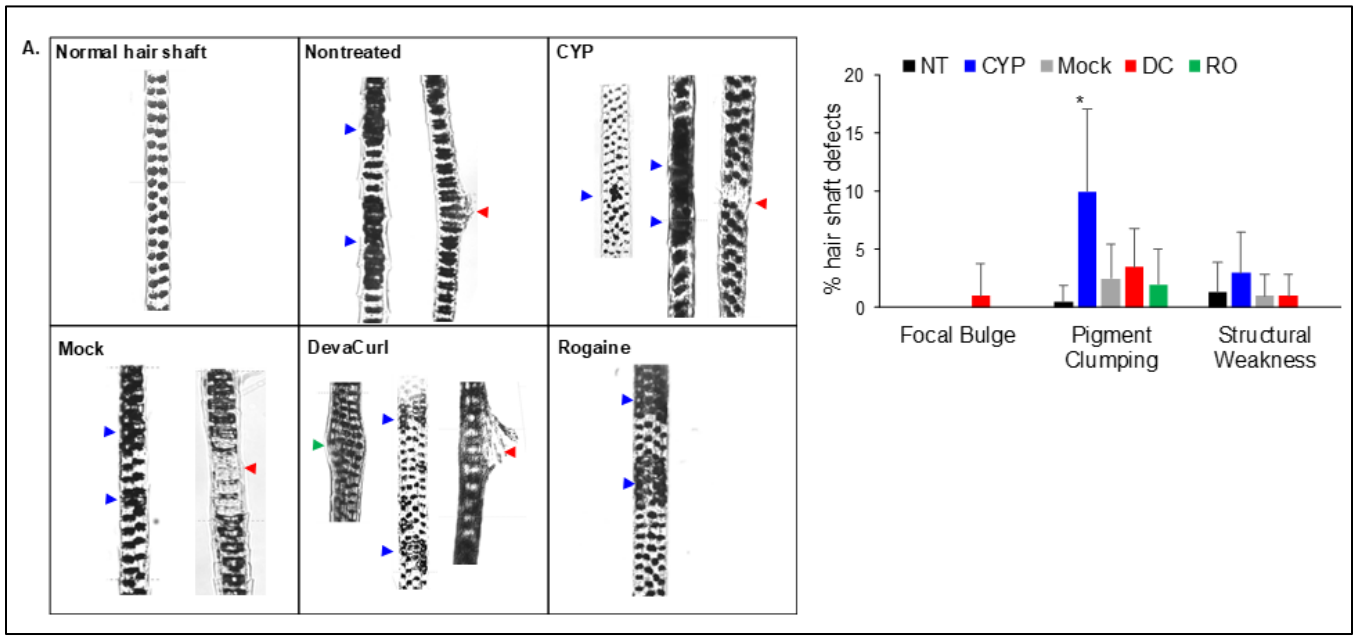


Figure 22. Hair shaft defects at the anagen-telogen transition (Exp-1, Timepoint-1). A. Representative images of various hair shaft defects. Green arrow, focal bulge; blue arrow, pigment clumping, depigmentation; red arrow, structural weakness, breakage. B. % damaged hairs. % damaged hairs = (No. of damaged hairs ÷ Total No. of hairs) x 100. The graph represents the mean ± SD. n =7-8 mice per cohort (25-35 hair shafts were assessed per mouse.) *, $p \leq 0.05$. Refer to Table 26 for the adjusted (adj.) p values for all data points. NT, nontreated; CYP, cyclophosphamide; DC, DevaCurl, RO, Rogaine.

Table 26. Hair shaft analysis at Day 21 (Exp-1, Timepoint-1).

		Focal Bulge (%)	Pigment Clumping (%)	Structural Weakness (%)
NT	**mean ± SD**	0 ± 0	0.50 ± 1.41	1.33 ± 2.57
	adj. p-value			
CYP	**mean ± SD**	0 ± 0	10.00 ± 7.09	3.00 ± 3.55
	adj. p-value (vs. NT)	>0.9999	**<0.0001**	>0.9999
Mock	**mean ± SD**	0 ± 0	2.50 ± 2.98	1.00 ± 1.85
	adj. p-value (vs. NT)	>0.9999	>0.9999	>0.9999
DC	**mean ± SD**	1.00 ± 2.83	3.50 ± 3.34	1.00 ± 1.85
	adj. p-value (vs. Mock)	>0.9999	>0.9999	>0.9999
RO	**mean ± SD**	0 ± 0	2.00 ± 3.02	0 ± 0
	adj. p-value (vs. Mock)	>0.9999	>0.9999	>0.9999

Two-way ANOVA with Bonferroni multiple comparison test was used for statistical analysis. Adjusted (adj.) p values are included. Bold type: $p \leq 0.05$.

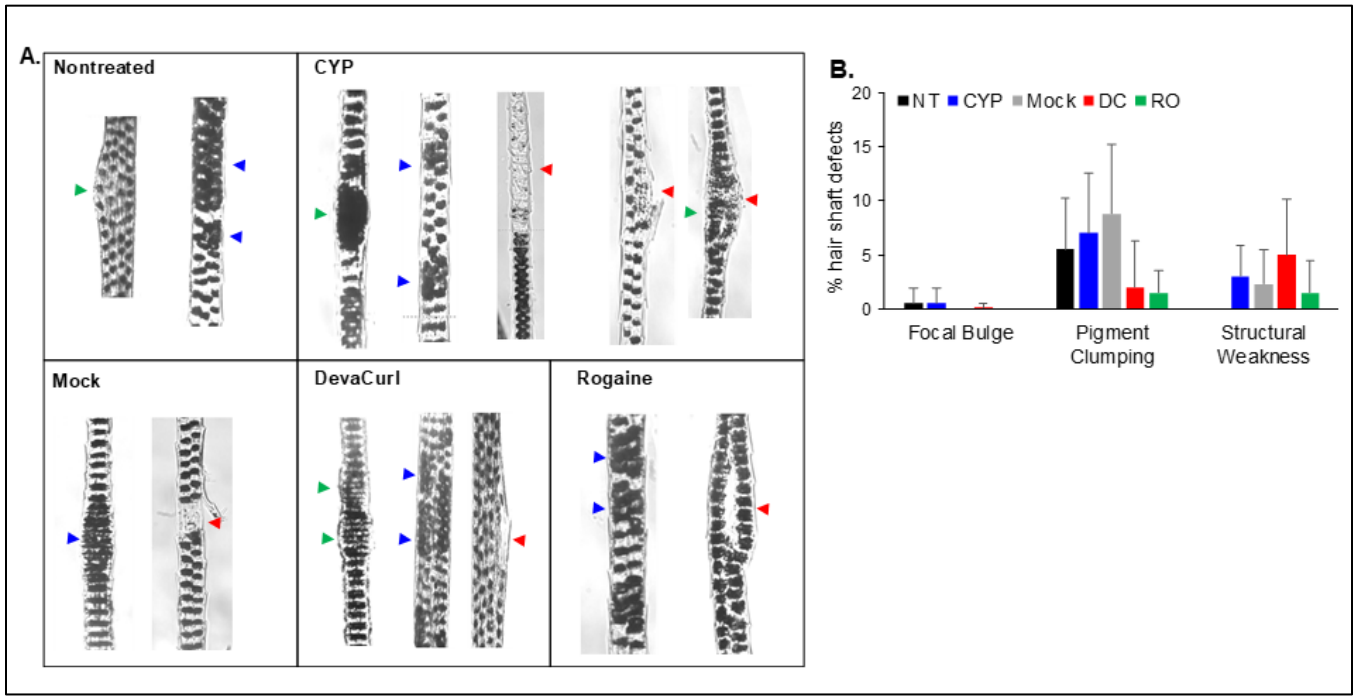


Figure 23. Hair shaft defects at the telogen-anagen transition (Exp-1, Timepoint-2). A. Representative images of various hair shaft defects. Green arrow, focal bulge; blue arrow, pigment clumping, depigmentation; red arrow, structural weakness, breakage. B. % damaged hairs. % damaged hairs = (No. of damaged hairs ÷ Total No. of hairs) x 100. The graph represents the mean ± SD. n =7-8 mice per cohort (25-35 hair shafts were assessed per mouse.) *, $p \leq 0.05$. Refer to Table 27 for the adjusted (adj.) p values for all data points. NT, nontreated; CYP, cyclophosphamide; DC, DevaCurl, RO, Rogaine.

Table 27. Hair shaft analysis at Day 85 (Exp-1, Timepoint-2).

		Focal Bulge (%)	Pigment Clumping (%)	Structural Weakness (%)
NT	**mean ± SD**	0.50 ± 1.41	5.50 ± 4.75	0 ± 0
	adj. p-value			
CYP	**mean ± SD**	0.50 ± 1.41	7.00 ± 5.55	3.00 ± 2.83
	adj. p-value (vs. NT)	>0.9999	>0.9999	0.7992
Mock	**mean ± SD**	0 ± 0	6.29 ± 6.47	2.29 ± 3.15
	adj. p-value (vs. NT)	>0.9999	>0.9999	>0.9999
DC	**mean ± SD**	0.13 ± 0.35	2.00 ± 4.28	5.00 ± 5.13
	adj. p-value (vs. Mock)	>0.9999	0.1636	>0.9999
RO	**mean ± SD**	0 ± 0	1.50 ± 2.07	1.50 ± 2.98
	adj. p-value (vs. Mock)	>0.9999	0.0755	>0.9999

Two-way ANOVA with Bonferroni multiple comparison test was used for statistical analysis. Adjusted (adj.) p values are included.

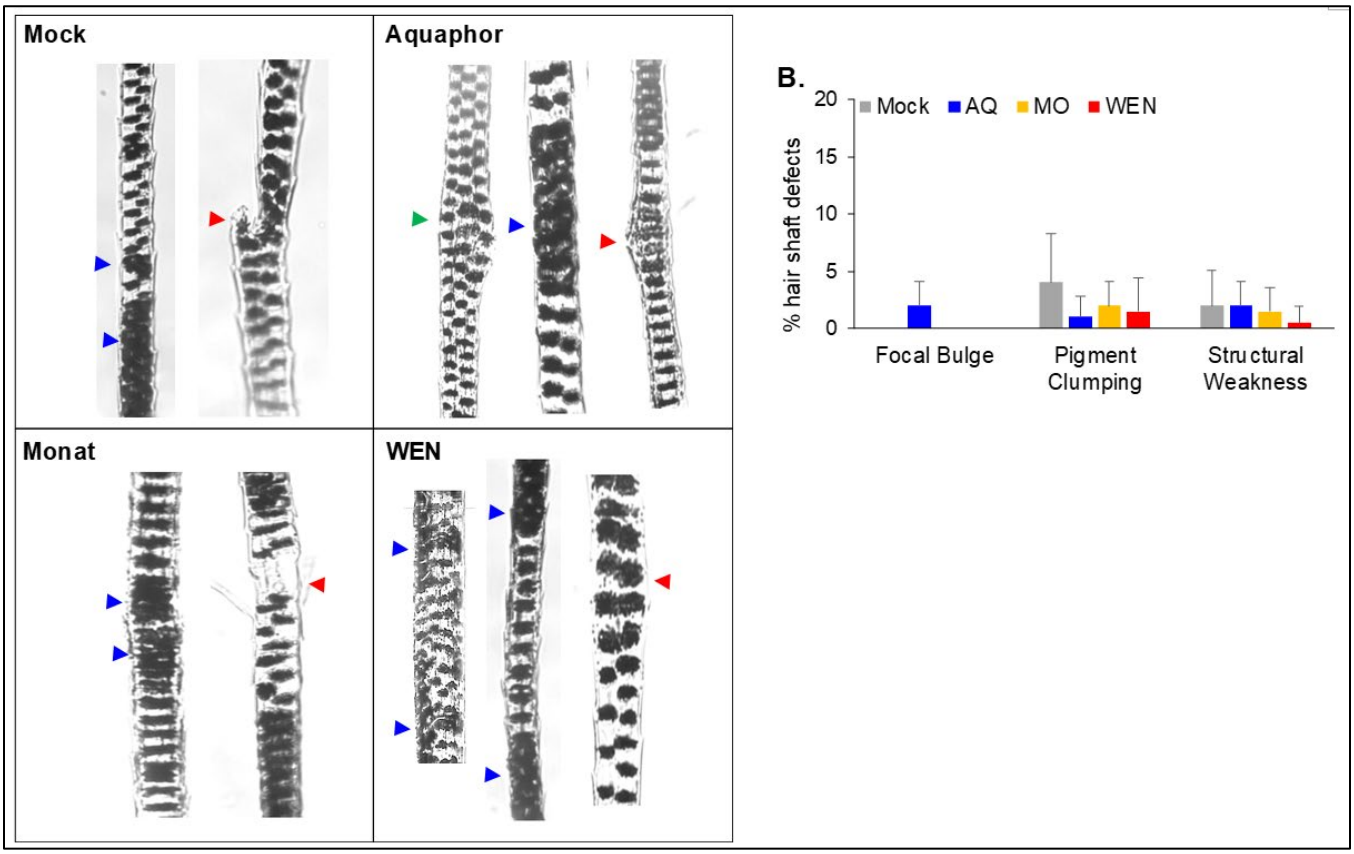


Figure 24. Hair shaft defects at the anagen-telogen transition (Exp-2, Timepoint-1). A. Representative images of various hair shaft defects. Green arrow, focal bulge; blue arrow, pigment clumping, depigmentation; red arrow, structural weakness, breakage. B. % damaged hairs. % damaged hairs = (No. of damaged hairs ÷ Total No. of hairs) x 100. The graph represents the mean ± SD. n =7-8 mice per cohort (25-35 hair shafts were assessed per mouse.) *, $p \leq 0.05$. Refer to Table 28 for the adjusted (adj.) p values for all data points. AQ, Aquaphor; MO, Monat.

Table 28. Hair shaft analysis at Day 21 (Exp-2, Timepoint-1).

		Focal Bulge (%)	Pigment Clumping (%)	Structural Weakness (%)
Mock	**mean ± SD**	0 ± 0	4.00 ± 4.28	2.00 ± 3.02
	adj. p-value			
AQ	**mean ± SD**	2.00 ± 2.14	1.00 ± 1.85	2.00 ± 2.14
	adj. p-value (vs. Mock)	0.4595	0.0518	>0.9999
MO	**mean ± SD**	0 ± 0	2.00 ± 2.14	1.50 ± 2.07
	adj. p-value (vs. Mock)	>0.9999	0.4595	>0.9999
WEN	**mean ± SD**	0 ± 0	1.50 ± 2.98	0.50 ± 1.41
	adj. p-value (vs. Mock)	>0.9999	0.1659	>0.9999

Two-way ANOVA with Bonferroni multiple comparison test was used for statistical analysis. Adjusted (adj.) p values are included.

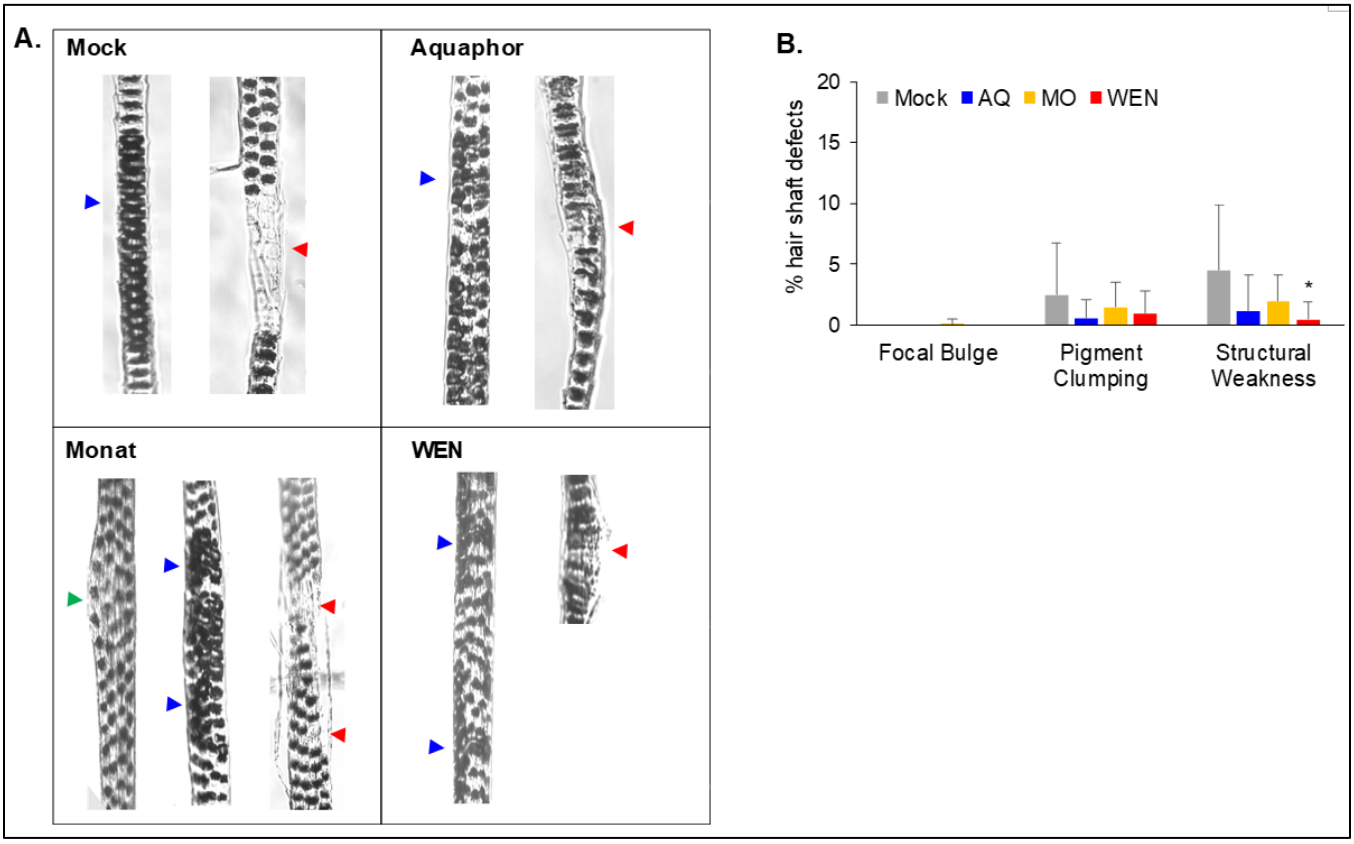


Figure 25. Hair shaft defects at the telogen-anagen transition (Exp-2, Timepoint-2). A. Representative images of various hair shaft defects. Green arrow, focal bulge; blue arrow, pigment clumping, depigmentation; red arrow, structural weakness, breakage. B. % damaged hairs. % damaged hairs = (No. of damaged hairs ÷ Total No. of hairs) x 100. The graph represents the mean ± SD. n =7-8 mice per cohort (25-35 hair shafts were assessed per mouse.) *, $p \leq 0.05$. Refer to Table 29 for the p values for all data points. AQ, Aquaphor; MO, Monat.

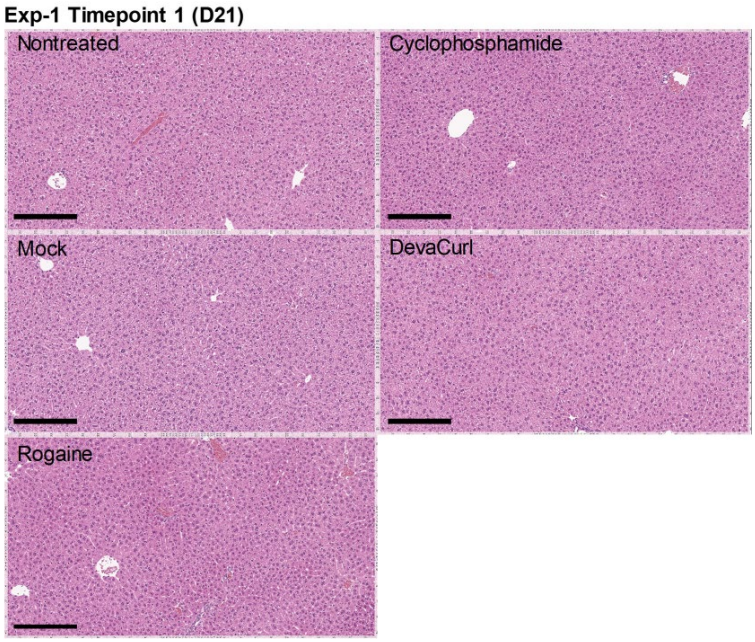
Table 29. Hair shaft analysis at Day 98 (Exp-2, Timepoint-2).

		Focal Bulge (%)	Pigment Clumping (%)	Structural Weakness (%)
Mock	**mean ± SD**	0 ± 0	2.50 ± 4.24	4.50 ± 5.42
	adj. p-value			
AQ	**mean ± SD**	0 ± 0	0.57 ± 1.51	1.14 ± 3.02
	adj. p-value (vs. Mock)	>0.9999	0.8283	0.0651
MO	**mean ± SD**	0.13 ± 0.35	1.50 ± 2.07	2.00 ± 2.14
	adj. p-value (vs. Mock)	>0.9999	>0.9999	0.2866
WEN	**mean ± SD**	0 ± 0	1.00 ± 1.85	0.50 ± 1.41
	adj. p-value (vs. Mock)	>0.9999	>0.9999	**0.0112**

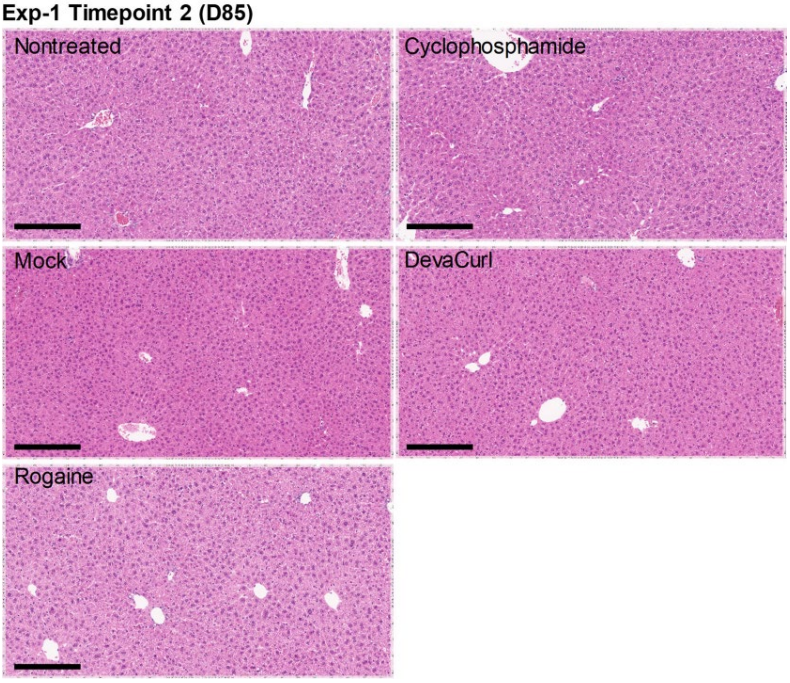
Two-way ANOVA with Bonferroni multiple comparison test was used for statistical analysis. Adjusted (adj.) p values are included. Bold type: $p \leq 0.05$.

4.8.5 Histopathological evaluation of liver

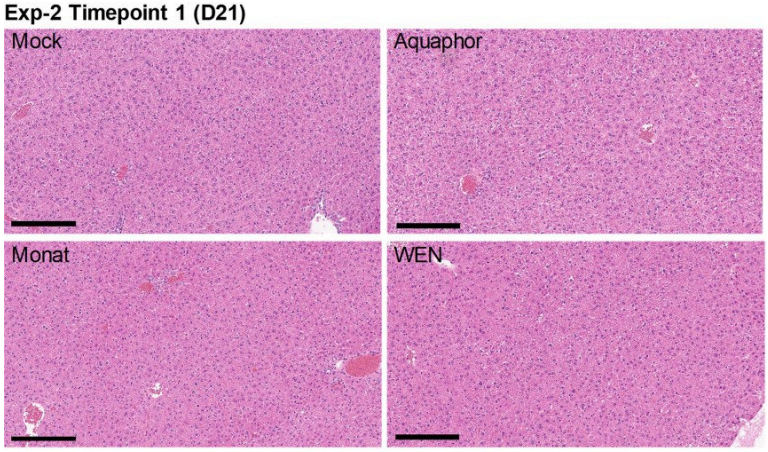
For both Exp-1 and Exp-2, no substantial histopathological changes were observed in the liver tissues collected at Timepoint 1 and Timepoint 2 in all cohorts. **Fig. 26** shows the representative images of 7-8 mice per cohort.



Exp-1 Timepoint 2 (D85)



Exp-2 Timepoint 1 (D21)



Exp-2 Timepoint 2 (D98)

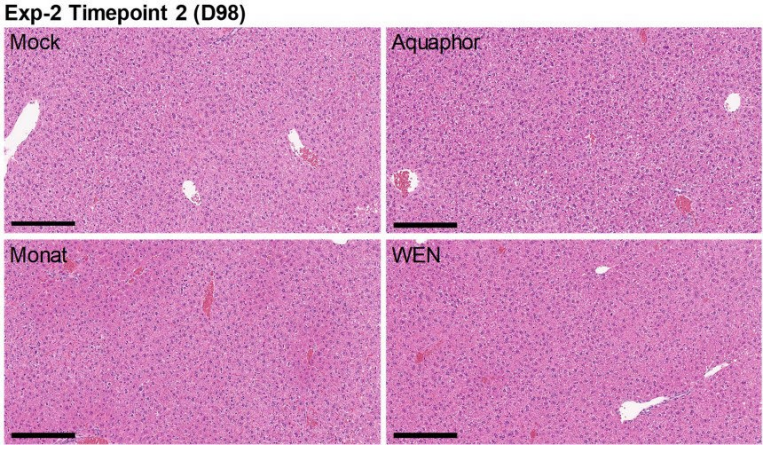


Figure 26. Histopathological evaluation of liver. Representative images of H&E-stained sections of mouse livers harvested at Day 21 (D21) and Day 85 (D85) for Exp-1, and at Day 21 (D21) and Day 98 (D98) for Exp-2. Scale bar = 200 μm.

4.8.6 Body weight measurement

In Exp-1, while no substantial differences were observed in average body weights in all cohorts, the mock and DevaCurl cohorts showed less body weight gain compared to the nontreated mice **(Table 30).** In Exp-2, one mouse in the WEN cohort **(Fig. 9D, red box)** lost 20% of its body weight during the second week of treatment. One mouse in the Aquaphor cohort died around Day 26. At Day 24, mice in this cohort weighed between 16.8g and 20g, and the weight of this mouse was 18.7g. Therefore, the cause of death is not likely due to weight loss. We also did not observe skin irritation, as well as any other conditions interfering with eating or drinking in experimental animals. The total body weight gain from Day 0 appeared to be higher in the mock and WEN cohorts. However, the average body weight at Day 98 did not differ substantially in all cohorts (21.53g mock, 20.74g Aquaphor, 20.54g Monat, and 21.88g WEN) **(Table 31)**.

Table 30. Body weight measurement (g) (Exp-1).

		Nontreated	CYP	Mock	Dev aCurl	Rogaine
D0	**mean ± SD**	18.38 ± 1.05	18.26 ± 0.86	17.68 ± 1.19	17.93 ± 0.87	18.12 ± 0.62
	adj. p-value (vs. D0)					
	adj. p-value (vs. nontreated or mock)		>0.9999	0.1747	>0.9999	>0.9999
D7	**mean ± SD**	19.04 ± 0.93	18.89 ± 0.95	18.73 ± 0.86	18.56 ± 0.89	19.09 ± 0.72
	adj. p-value (vs. D0)	0.6644	0.6644	**0.0112**	0.2795	**0.0112**
	adj. p-value (vs. nontreated or mock)		>0.9999	>0.9999	>0.9999	>0.9999
D14	**mean ± SD**	19.96 ± 0.93	19.46 ± 1.07	19.18 ± 0.98	18.95 ± 0.8	19.56 ± 0.84
	adj. p-value (vs. D0)	**<0.0001**	**0.0008**	**<0.0001**	**0.0031**	**<0.0001**
	adj. p-value (vs. nontreated or mock)		0.8928	0.0663	>0.9999	>0.9999
D20	**mean ± SD**	20.02 ± 1.1	19.98 ± 1.07	19.51 ± 0.99	19.47 ± 1	19.99 ± 0.72
	adj. p-value (vs. D0)	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
	adj. p-value (vs. nontreated or mock)		>0.9999	0.8928	>0.9999	0.8928
D28	**mean ± SD**	19.98 ± 0.53	20.33 ± 0.69	18.55 ± 0.57	18.34 ± 0.75	19.26 ± 0.45
	adj. p-value (vs. D0)	**0.0002**	**<0.0001**	0.2018	>0.9999	**0.0144**
	adj. p-value (vs. nontreated or mock)		>0.9999	**0.0079**	>0.9999	0.9253
D35	**mean ± SD**	20.08 ± 0.62	20.61 ± 0.65	19.2 ± 0.73	18.83 ± 0.86	19.55 ± 0.59
	adj. p-value (vs. D0)	**<0.0001**	**<0.0001**	**0.0005**	0.2018	**0.0005**
	adj. p-value (vs. nontreated or mock)		>0.9999	0.3066	>0.9999	>0.9999
D42	**mean ± SD**	20.21 ± 0.51	20.51 ± 0.34	18.99 ± 0.58	19.31 ± 0.87	20.13 ± 0.74
	adj. p-value (vs. D0)	**<0.0001**	**<0.0001**	**0.0052**	**0.0017**	**<0.0001**
	adj. p-value (vs. nontreated or mock)		>0.9999	**0.0400**	>0.9999	0.0830
D49	**mean ± SD**	20.53 ± 0.75	21.1 ± 0.78	19.01 ± 0.75	19.58 ± 0.76	20.43 ± 0.7
	adj. p-value (vs. D0)	**<0.0001**	**<0.0001**	**0.0052**	**<0.0001**	**<0.0001**
	adj. p-value (vs. nontreated or mock)		>0.9999	**0.0033**	>0.9999	**0.0079**
D57	**mean ± SD**	21.16 ± 0.47	21.25 ± 0.69	19.6 ± 0.75	19.54 ± 0.99	21.46 ± 0.71
	adj. p-value (vs. D0)	**<0.0001**	**<0.0001**	**<0.0001**	**0.0002**	**<0.0001**
	adj. p-value (vs. nontreated or mock)		>0.9999	**0.0013**	>0.9999	**<0.0001**

		Nontreated	CYP	Mock	Dev aCurl	Rogaine
D60	**mean ± SD**	21.48 ± 0.39	21.76 ± 0.82	19.71 ± 0.8	19.6 ± 1.02	21.45 ± 0.92
	adj. p-value (vs. D0)	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
	adj. p-value (vs. nontreated or mock)		>0.9999	**0.0002**	>0.9999	**0.0002**
D63	**mean ± SD**	21.79 ± 0.33	21.8 ± 0.84	19.83 ± 0.88	20.01 ± 0.93	21.46 ± 0.79
	adj. p-value (vs. D0)	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
	adj. p-value (vs. nontreated or mock)		>0.9999	**<0.0001**	>0.9999	**0.0005**
D66	**mean ± SD**	21.93 ± 0.64	21.66 ± 0.5	19.79 ± 0.8	20.06 ± 0.91	21.58 ± 0.81
	adj. p-value (vs. D0)	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
	adj. p-value (vs. nontreated or mock)		>0.9999	**<0.0001**	>0.9999	**0.0002**
D70	**mean ± SD**	21.89 ± 0.86	21.65 ± 0.54	19.88 ± 0.61	20.13 ± 0.9	21.83 ± 0.74
	adj. p-value (vs. D0)	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
	adj. p-value (vs. nontreated or mock)		>0.9999	**<0.0001**	>0.9999	**<0.0001**
D73	**mean ± SD**	22.16 ± 0.53	21.65 ± 0.63	20.19 ± 0.66	20.06 ± 0.79	22.14 ± 0.6
	adj. p-value (vs. D0)	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
	adj. p-value (vs. nontreated or mock)		>0.9999	**<0.0001**	>0.9999	**<0.0001**
D77	**mean ± SD**	21.99 ± 0.45	20.99 ± 1.46	20.41 ± 0.87	20.03 ± 0.84	21.81 ± 0.69
	adj. p-value (vs. D0)	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
	adj. p-value (vs. nontreated or mock)		0.1636	**0.0013**	>0.9999	**0.0079**
D80	**mean ± SD**	22.01 ± 0.67	21.53 ± 0.91	20.66 ± 1.04	20.51 ± 1.03	22.1 ± 0.73
	adj. p-value (vs. D0)	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
	adj. p-value (vs. nontreated or mock)		>0.9999	**0.0183**	>0.9999	**0.0079**
D84	**mean ± SD**	22.48 ± 0.63	22.2 ± 0.81	20.69 ± 0.93	20.96 ± 0.86	22.28 ± 0.49
	adj. p-value (vs. D0)	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
	adj. p-value (vs. nontreated or mock)		>0.9999	**0.0002**	>0.9999	**0.0013**
	Total weight gain from D0 (g)	4.10 ± 0.63	3.94 ±0.81	3.01 ± 0.93	3.03 ± 0.86	4.16 ± 0.49

Data are represented as mean (g) ± SD. Two-way ANOVA with Bonferroni multiple comparison test was used for statistical analysis. Adjusted (adj.) *p* values are included. Bold type: $p \leq 0.05$. CYP, cyclophosphamide.

Table 31. Body weight measurement (g) (Exp-2).

		Mock	Aquaphor	Monat	W EN
D0	**mean ± SD**	18.58 ± 0.61	18.8 ± 1.01	18.34 ± 0.54	18.63 ± 0.84
	adj. p-value (vs. Mock)		>0.9999	>0.9999	>0.9999
D7	**mean ± SD**	18.94 ± 0.6	19.21 ± 0.94	18.86 ± 0.74	19.4 ± 0.78
	adj. p-value (vs. D0)	>0.9999	>0.9999	0.8249	0.0736
	adj. p-value (vs. Mock)		0.9739	>0.9999	0.2839
D14	**mean ± SD**	19.58 ± 0.59	19.62 ± 0.92	19.48 ± 0.72	19.71 ± 1.55*
	adj. p-value (vs. D0)	**0.0042**	**0.0416**	**0.0005**	**0.0013**
	adj. p-value (vs. Mock)		>0.9999	>0.9999	>0.9999
D24	**mean ± SD**	18.56 ± 0.55	18.65 ± 1.15	18.51 ± 0.48	19.36 ± 0.59
	adj. p-value (vs. D0)	>0.9999	>0.9999	>0.9999	0.4267
	adj. p-value (vs. Mock)		>0.9999	>0.9999	0.1199

		Mock	Aquaphor	Monat	W EN
D28**	**mean ± SD**	18.73 ± 0.56	19.03 ± 0.82	18.66 ± 0.31	19.3 ± 0.4
	adj. p-value (vs. D0)	>0.9999	>0.9999	>0.9999	0.6574
	adj. p-value (vs. Mock)		>0.9999	>0.9999	0.4287
D35	**mean ± SD**	18.93 ± 0.61	19.09 ± 0.88	18.76 ± 0.54	19.71 ± 0.4
	adj. p-value (vs. D0)	>0.9999	>0.9999	>0.9999	**0.0197**
	adj. p-value (vs. Mock)		>0.9999	>0.9999	0.1356
D42	**mean ± SD**	19.15 ± 0.63	19.13 ± 0.76	19.03 ± 0.33	19.8 ± 0.57
	adj. p-value (vs. D0)	>0.9999	>0.9999	0.5710	**0.0077**
	adj. p-value (vs. Mock)		>0.9999	>0.9999	0.2847
D49	**mean ± SD**	19.69 ± 0.7	19.74 ± 0.84	19.11 ± 1.16	20.06 ± 0.61
	adj. p-value (vs. D0)	**0.0145**	0.1097	0.3150	**0.0004**
	adj. p-value (vs. Mock)		>0.9999	0.4081	>0.9999
D56	**mean ± SD**	19.54 ± 0.64	19.61 ± 0.88	19.69 ± 0.5	20.31 ± 0.57
	adj. p-value (vs. D0)	0.0630	0.3055	**0.0010**	**<0.0001**
	adj. p-value (vs. Mock)		>0.9999	>0.9999	0.1440
D63	**mean ± SD**	19.68 ± 0.67	19.99 ± 0.97	19.94 ± 0.4	20.46 ± 0.77
	adj. p-value (vs. D0)	**0.0161**	**0.0109**	**<0.0001**	**<0.0001**
	adj. p-value (vs. Mock)		>0.9999	>0.9999	0.1356
D70	**mean ± SD**	19.61 ± 0.73	19.87 ± 1.2	20.15 ± 0.54	20.44 ± 0.65
	adj. p-value (vs. D0)	**0.0325**	**0.0349**	**<0.0001**	**<0.0001**
	adj. p-value (vs. Mock)		>0.9999	0.4953	0.0993
D77	**mean ± SD**	20.13 ± 0.58	20.23 ± 1.2	20.19 ± 0.68	21.14 ± 0.61
	adj. p-value (vs. D0)	**<0.0001**	**0.0008**	**<0.0001**	**<0.0001**
	adj. p-value (vs. Mock)		>0.9999	>0.9999	**0.0288**
D84	**mean ± SD**	20.64 ± 0.89	20.76 ± 1.01	20.45 ± 0.48	21.33 ± 0.72
	adj. p-value (vs. D0)	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
	adj. p-value (vs. Mock)		>0.9999	>0.9999	0.2289
D91	**mean ± SD**	21.01 ± 0.71	20.93 ± 1.24	20.26 ± 0.38	21.59 ± 0.34
	adj. p-value (vs. D0)	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
	adj. p-value (vs. Mock)		>0.9999	0.1623	0.4087
D98	**mean ± SD**	21.53 ± 0.91	20.74 ± 1.13	20.54 ± 0.6	21.88 ± 0.41
	adj. p-value (vs. D0)	**<0.0001**	**<0.0001**	**<0.0001**	**<0.0001**
	adj. p-value (vs. Mock)		0.1500	**0.0333**	>0.9999
	Total weight gain from D0 (g)	2.93 ± 0.91	1.94 ± 1.13	2.24 ± 0.6	3.28 ± 0.41

*, The body weight calculation included the mouse that lost 20% of its body weight during the second week. **, n=7 for the Aquaphor cohort from D28. Data are represented as mean (g) ± SD. Two-way ANOVA with Bonferroni multiple comparison test was used for statistical analysis. Adjusted (adj.) *p* values are included. Bold type: $p \leq 0.05$.

4.9 Summary and Conclusion

In all test cohorts (i.e., DevaCurl, Aquaphor, Monat, WEN), the onset and duration of the 1st anagen phase and the subsequent entry into the 1st catagen phase were normal and indicated relatively synchronous HF cycling.

Anagen–telogen transition (TP-1): Compared to the mock cohort, mice treated with Aquaphor, Monat, or WEN showed a higher proportion of HFs in the telogen phase at Day 21, suggesting accelerated entry into the 1st telogen phase. However, this difference was not statistically significant.

Telogen–anagen transition (TP-2): Compared to the mock cohorts, a delay in progression to the 2nd anagen phase was observed in mice treated with DevaCurl or WEN, with 46.39% of DevaCurl HFs and 71.2% of WEN HFs remaining in the 1st telogen phase at Day 85 and Day 98, respectively. The observed delay in the WEN cohort was statistically significant.

Monat, WEN, and Rogaine were associated with increases in total mast cell numbers at the telogen–anagen transition. In addition, WEN resulted in significant increases in mast cell activation.

Mast cell activity appeared to be preferentially localized within the HFs in WEN-treated mice. However, this observation was not statistically significant and will require further investigation.

Overall, hair damage in the test cohorts was not statistically significantly higher than in the mock cohorts.

4.10 Potential limitations and Recommendations

Differences in the viscosity of the product formulations
The dermal penetration of a number of substances (e.g., benzoic acid) has been shown to be enhanced by increasing the water content of the vehicles, and viscous formulations are generally considered to reduce the diffusion coefficient of molecules in the vehicle, thus retarding or eliminating its skin partitioning and absorption [49]. The undiluted WEN formulation is highly viscous compared to other undiluted test products, which may impact retention and absorption of the ingredients present in the test products by the skin.

Scents associated with the test products
Environmental factors, such as humidity, cage type, and diet, can cause stress in rodents, leading to various abnormal behaviors and physiological responses, including alopecia [37, 38, 50]. In addition, mice are sensitive to various scents, and certain chemosignals or pheromones have been shown to invoke stress responses in mice [51]. All test products contain fragrance mixtures and a number of individual scent ingredients. Peppermint oil and red clover can act as effective mouse deterrents, as can menthol, which can be synthesized or obtained from the mint family. Menthol and red clover extract were present in WEN and Monat products, respectively.

Accelerated hair growth in the depilation model
Depilation-mediated HF cycling progresses rapidly, reaching a fully mature anagen HF on Day 9. Although the depilation method is widely used for hair growth studies, this accelerated model might mask the effects of the test products during the 1st anagen-telogen HF transition, or fail to provide a sufficient dose regimen or time to observe the effects of the test products.

Variation from animal to animal
Hair growth during the first 14 weeks after birth (P90) is well defined in female C57BL/6 mice, and HFs are well synchronized during each stage of this period. In this study, the P90 period corresponded to Day 28, after which the rates of hair cycling and subsequent hair recovery varied among the animals. This variation and the small size of the sample limited the study's statistical power.

5 PROJECT 2: In vitro cytotoxicity assessments of ingredients found in hair care products.

5.1 Project Rationale and Objectives

The objective of this study was to identify ingredients of hair care products that affect the viability and growth of DPCs and NHEKs using in vitro methodologies. A list of priority ingredients was provided by the FDA. Sources and suppliers were identified by Columbia University. One ingredient (Capixyl) could not be obtained. This report summarizes the results for the four test products **(Table 32)**, and 20 of the 21 ingredients **(Table 33)**. Those ingredients that are present in the test products are shown in **Table 34**.

5.2 Test Products and Ingredients

Table 32. Test Products

Name	Lot No	Supplier
Aquaphor Baby Wash & Shampoo- Fragrance free *	1065957	Amazon
DevaCurl Low-Poo Delight Cleanser	0006019A	Amazon
Monat Renew Shampoo	19J0813144	Monat
Wen Sweet Almond Mint Cleansing Conditioner	07022-006	WEN

*, Included as a product control for in vivo studies.

Table 33. Test ingredients and controls.

	Name	CAS No	Lot No	Supplier
1	Acetyl tetrapeptide-3	827306-88-7	0552357-1	Cayman Chemical
2	Calendula Officinalis Extract	84776-23-8	1836350-11798	Lotioncrafter
3	Capixyl*	N/A	N/A	Lucasmeyer Cosmetics
4	Caryocar Brasiliense Fruit Oil (Pequi oil)	394238-03-0	26515	Amazon
5	Cinnamidopropyltrimonium chloride (CRODASORB UV-283), CATC	177190-98-6	1515298	Croda
6	Citrus Limon (Lemon) Peel Oil	8008-56-8	FSS191127-36	Formulator Sample Shop
7	Cocamidopropyl Betaine (SurfProTM CAPB)	61789-40-0	V053019-78837-11760	Lotioncrafter
8	Cocos Nucifera (Coconut) Oil	8001-31-8	181592	Making Cosmetics
9	Dextran 40	9004-54-0	F3KHE-BO	Fischer Scientific
10	Dextran 70	9004-54-0	8SVZN-TQ	Fischer Scientific
11	Guar Hydroxypropyltrimonium Chloride	65497-29-2	P19074A-11234	Lotioncrafter
12	Helianthus Annuus (Sunflower seed) Oil	8001-21-6	180703	Making Cosmetics
13	Lavandula Angustifolia (Lavender) Oil	8000-28-0	N/A	Making Cosmetics
14	Methylchloroisothiazolinone (5-Chloro-2-methyl-4-isothiazolin-3-one, MCI)	26172-55-4	LC33138	AK Scientific Inc.
15	Methylisothiazolinone (MI)	2682-20-4	TC42338PU1	AK Scientific Inc.
16	Pisum Sativum (Pea) Peptide	90082-41-0	FSS191127-35	Formulator Sample Shop
17	Polysorbate 60	9005-67-8	U29C007	Fischer Scientific
18	Rosmarinus Officinalis Leaf Extract (Rosemary Oleoresin, ROE)	84604-14-8	EB25211-11712	Lotioncrafter
19	Solanum Lycopersicum (Tomato) Seed Oil	90131-63-8	FSS191009-677	Formulator Sample Shop
20	Trifolium Pratense (Red Clover) Blossom Extract	85085-25-2	B21B11652	Making Cosmetics

	Name	CAS No	Lot No	Supplier
21	Vegetable oil (Olus oil)	68956-68-3	160117371A	Naturallythinking
22	Minoxidil**	38304-91-5	R050S0	Sigma-Aldrich
23	Cisplatin**	15663-27-1	S116613	Selleckchem

*, Unable to obtain.
**, Included as controls.
N/A, Not available.

Table 34. The presence of the test ingredients in the four selected products.

	Name	AQ	DC	MO	WEN	Function
1	Acetyl tetrapeptide-3			✓		Skin conditioning
2	Calendula Officinalis Extract		✓		✓	Emollient, anti-inflammatory
3	Capixyl *			✓		Anti-inflammatory
4	Caryocar Brasiliense Fruit Oil (Pequi oil)			✓		Skin conditioning
5	Cinnamidopropyltrimonium chloride (CRODASORB UV-283), CATC			✓		Antistatic
6	Citrus Limon (Lemon) Peel Oil			✓		Hair/skin conditioning, fragrance
7	Cocamidopropyl Betaine (SurfProTM CAPB)	✓	✓	✓		Surfactant, viscosity control, antistatic
8	Cocos Nucifera (Coconut) Oil			✓		Skin conditioning
9	Dextran 40			✓**		Viscosity control
10	Dextran 70			✓**		Viscosity control
11	Guar Hydroxypropyltrimonium Chloride		✓	✓		Antistatic, viscosity control
12	Helianthus Annuus (Sunflower seed) Oil			✓		Emollient, fragrance, antioxidant
13	Lavandula Angustifolia (Lavender) Oil					fragrance
14	Methylchloroisothiazolinone (5-Chloro-2-methyl-4-isothiazolin-3-one, MCI)				✓	Preservative
15	Methylisothiazolinone (MI)				✓	Preservative
16	Pisum Sativum (Pea) Peptide			✓		Skin conditioning
17	Polysorbate 60					Surfactant, emulsifier
18	Rosmarinus Officinalis Leaf Extract (Rosemary Oleoresin, ROE)		✓		✓	Skin conditioning, anti-microbial, fragrance
19	Solanum Lycopersicum (Tomato) Seed Oil			✓		Emollient, skin conditioning, fragrance
20	Trifolium Pratense (Red Clover) Blossom Extract			✓		Skin conditioning
21	Vegetable oil (Olus oil)					Emollient

AQ, Aquaphor Baby Wash & Shampoo; DC, DevaCurl Low-Poo Delight; MO, Monat Renew; WEN, WEN Sweet Almond Mint Cleansing Conditioner
✓, Representing the presence of ingredients in test products.
*, Unable to obtain.
**, listed as Dextran on the product.

5.3 Cell Viability Assessment

5.3.1 Assay Protocols

Experiments were carried out using two-dimensional cultures of (i) primary DPCs isolated from normal human scalps (602-05A, Sigma-Aldrich) and (ii) NHEKs isolated from adult skin (00192627, Lonza). Cells were maintained at 37 °C in 5% CO_2 at 95% relative humidity. A serial dilution of the test products/ingredients was prepared in tissue culture medium and filter-sterilized before use. Cell viability

was assessed using CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (G5430, Promega), a quantitative assay that provides a readout of cell viability through the measurement of metabolic activity.

Specifically, it measures the reducing potential of MTS [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium] by metabolically active cells. In this assay system, MTS is used in combination with an intermediate electron acceptor reagent (phenazine methyl sulfate) to facilitate cell permeability. In healthy cells, MTS is converted by mitochondrial enzymes into soluble purple formazan products. After two hours of incubation at 37°C, the number of viable cells was determined by measuring absorbance at 490 nm in a microplate reader. The MTS assay was performed following the manufacturer's protocol.[1]

Day 0: For each test product and ingredient, four 96-well plates (one plate each for four time points) were seeded at 1,500 cells/well in 100 µl. The assay was performed over 6 days without replacing the medium. To prevent evaporation and the risks associated with edge effects during the 6-day treatment, we used 96-well plates with a built-in moat divided into four sectional reservoirs that can be filled with sterile water (Thermo Scientific™ Nunc™ Edge 2.0). The number of cells seeded initially was optimized to avoid confluent cultures, and no morphological changes were observed in nontreated control cells during the treatment duration.

Day 1: Cells were treated with the test products/ingredients across a wide range of concentrations from 2.5E-06 to 10% **(Table 35)**. The test concentrations for products/ingredients were determined based on information from literature as well as the results, including solubility and cell viability, from the pilot study (data not shown), to ensure the validity of the data. Five to six wells of cells were treated per concentration (i.e., n=5-6 replicates per concentration). Each plate included nontreated cells, as well as cells treated with minoxidil or cisplatin as controls. For test ingredients that have limited water solubility, ethanol or dimethyl sulfoxide (DMSO) was used as solvents.

Days 2 – 7: MTS was added (20 µl/well) at 24, 48, and 72 hours (h), and 6 days (D) after treatment. After two hours of incubation at 37°C in a humidified, 5% CO_2 atmosphere, absorbance at 490 nm was obtained using a microplate reader (Bio-Rad).

Table 35. Experimental design for MTS assay.

Test products/Ingredients	Solvent	Concentrations Tested (%)	Treatment Frequency	Treatment Duration (days)
Aquaphor Baby Wash & Shampoo- Fragrance free	Medium	0.0001 – 0.1	1	1,2,3,6
DevaCurl Low-Poo Delight Cleanser	Medium	0.0001 – 0.1	1	1,2,3,6
Monat Renew Shampoo	Medium	0.0001 – 0.1	1	1,2,3,6
WEN Sweet Almond Mint Cleansing Conditioner	Medium	0.0001 – 0.1	1	1,2,3,6
Acetyl tetrapeptide-3	Medium	0.0001 – 1	1	1,2,3,6
Calendula Officinalis Extract	Medium	2.5E-06 – 0.25	1	1,2,3,6
Capixyl*	N/A	N/A	N/A	N/A
Caryocar Brasiliense Fruit Oil (Pequi oil)	1% Ethanol	2.5E-05 – 0.25	1	1,2,3,6
Cinnamidopropyltrimonium chloride (CRODASORB UV-283), CATC	Medium	0.0001 – 5	1	1,2,3,6
Citrus Limon (Lemon) Peel Oil	1% Ethanol	0.0001 – 1	1	1,2,3,6
Cocamidopropyl Betaine (SurfProTM CAPB)	Medium	0.00001 – 0.1	1	1,2,3,6
Cocos Nucifera (Coconut) Oil	Medium	0.0001 – 1	1	1,2,3,6
Dextran 40	Medium	0.0001 – 5	1	1,2,3,6
Dextran 70	Medium	0.0001 – 5	1	1,2,3,6
Guar Hydroxypropyltrimonium Chloride	Medium	0.0001 – 0.1	1	1,2,3,6
Helianthus Annuus (Sunflower seed) Oil	1% Ethanol	0.0001 – 1	1	1,2,3,6

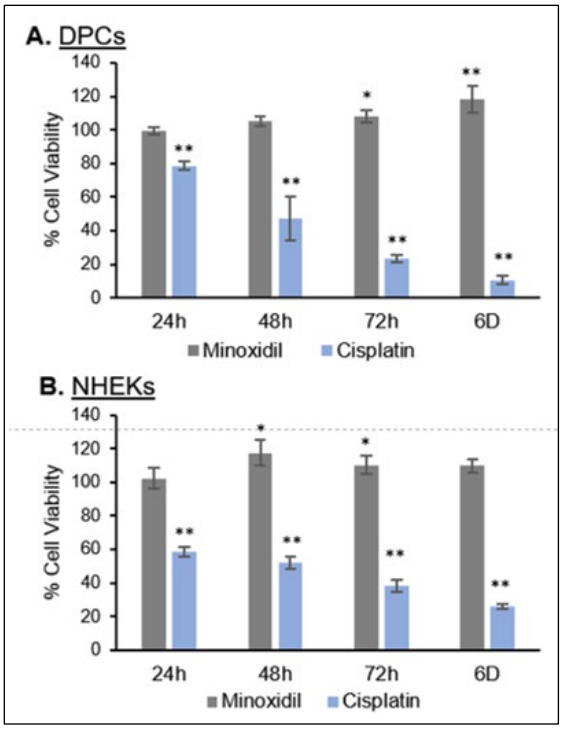
[1] *CellTiter 96® Aqueous Non-Radioactive Cell Proliferation Assay,* Technical Bulletin Part# TB169, Promega Corporation

Test products/Ingredients	Solvent	Concentrations Tested (%)	Treatment Frequency	Treatment Duration (days)
Lavandula Angustifolia (Lavender) Oil	1% Ethanol	0.0001 – 1	1	1,2,3,6
Methylchloroisothiazolinone (5-Chloro-2-methyl-4-isothiazolin-3-one, MCI)	Medium	0.00001 – 0.1	1	1,2,3,6
Methylisothiazolinone (MI)	Medium	0.00001 – 0.1	1	1,2,3,6
Pisum Sativum (Pea) Peptide	Medium	0.0001 – 5	1	1,2,3,6
Polysorbate 60	20% Ethanol***	0.001 – 2.5	1	1,2,3,6
Rosmarinus Officinalis Leaf Extract (Rosemary Oleoresin, ROE)	2.5% DMSO	0.00001 – 0.01	1	1,2,3,6
Solanum Lycopersicum (Tomato) Seed Oil	Medium	0.0001 – 1	1	1,2,3,6
Trifolium Pratense (Red Clover) Blossom Extract	Medium	0.001 – 10	1	1,2,3,6
Vegetable oil (Olus oil)	Medium	0.0001 – 0.5	1	1,2,3,6
Minoxidil**	100% Ethanol†	3 µm	1	1,2,3,6
Cisplatin**	DMSO	100 µm	1	1,2,3,6

*, Not tested because the ingredient was not obtainable.
**, Included as controls.
***, Polysorbate 60 was prepared by 1:1 dilution (v/v) in 20% ethanol, yielding a stock solution of 50% of Polysorbate 60 in 10% ethanol. The stock solution was further diluted in the medium. The ethanol concentration at the highest concentration of Polysorbate 60 tested was 0.5%, which is well-tolerated by various cell lines [52].
†, Stock solution of 30 mM of Minoxidil was prepared in 100% ethanol and was further diluted in the medium.
N/A, not available.

5.3.2 Assay Controls

Each assay included cisplatin and minoxidil as controls. Cisplatin, an alkylating agent, has been shown to inhibit proliferation and induce apoptosis in several hair follicle compartments, including dermal papilla and matrix keratinocytes [53, 54]. Consistent with its cytotoxic effect on dividing cells, cisplatin (100 µm) substantially reduced the viability of DPCs and NHEKs. Minoxidil is implicated in hair growth and has been shown to shorten the telogen stage and prolong the anagen stage through both proliferative and anti-apoptotic effects on the dermal papilla cells [23]. Overall increase in cell viability was observed in DPCs and NHEKs treated with minoxidil (3 µm). **Fig. 27** shows representative datasets.



Viability (%) of DPCs treated with Minoxidil or Cisplatin.

Treatment Duration		Nontreated (0 μM)	Minoxidil (3 μM)	Cisplatin (100 μM)
24 h	**mean ± SD**	100 ± 2.46	99.48 ± 1.86	78.62 ± 2.53
	adj. p-value		>0.9999	<0.0001
48 h	**mean ± SD**	100 ± 4.65	105 ± 2.96	**47.44 ± 12.9**
	adj. p-value		0.3400	<0.0001
72 h	**mean ± SD**	100 ± 4.62	108.17 ± 3.92	**23.44 ± 2.29**
	adj. p-value		0.0263	<0.0001
6 D	**mean ± SD**	100 ± 4.27	118.06 ± 7.96	**10.66 ± 2.56**
	adj. p-value		<0.0001	<0.0001

Viability (%) of NHEKs treated with Minoxidil or Cisplatin.

Treatment Duration		Nontreated (0 μM)	Minoxidil (3 μM)	Cisplatin (100 μM)
24 h	**mean ± SD**	100 ± 2.49	102.34 ± 6.14	58.3 ± 2.87
	adj. p-value		>0.9999	<0.0001
48 h	**mean ± SD**	100 ± 3.97	117.39 ± 7.57	52.1 ± 3.33
	adj. p-value		0.0002	<0.0001
72 h	**mean ± SD**	100 ± 10.53	110.37 ± 5.49	**38.32 ± 3.48**
	adj. p-value		0.0356	<0.0001
6 D	**mean ± SD**	100 ± 8.33	109.76 ± 3.74	**25.85 ± 1.57**
	adj. p-value		0.0536	<0.0001

Figure 27. Cytotoxicity assessment of minoxidil and cisplatin in DPCs (A) and NHEKs (B). Cells cultured in the presence of the assay controls for 24, 48, 72 hours (h), and 6 days (D) were assessed by MTS assay. Cell viability (%) was obtained relative to nontreated control. Data in the tables are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) p values were generated using Bonferroni multiple comparison test. Highlighted values correspond to cell viability < 50%. *, $p \leq 0.05$; **, $p \leq 0.0001$.

5.3.3 Statistical Methods & Data Presentation

Statistical analysis was performed using GraphPad (Version 9.4.1.681, GraphPad Software, Inc.). Data were analyzed using two-way ANOVA with Bonferroni multiple comparison test. Dose-response curve, R^2 (the coefficient of determination or goodness of fit), and IC50 (the half maximal inhibitory concentrations) were obtained using GraphPad. Adjusted *p* values generated from Bonferroni tests, IC50 values, and R^2 values of $\geq$0.9 are included in the corresponding tables. Data are presented as mean ± SD. $p \leq 0.05$ was considered statistically significant. IC50 values that were unobtainable due to no detectable level of cytotoxicity within the tested dose range and an R^2 value of <0.9 were marked with "N/A" (not available) in the tables.

5.3.4 Results

Test Products

Of the four hair care products **(Table 32)**, we found Monat Renew shampoo to be the most cytotoxic to DPCs, killing 80.94% of the cells in 24h at 0.04% **(Fig. 28A, Table 36)**. On the other hand, WEN Sweet Almond Mint cleansing conditioner had the least effect on DPC viability. WEN-induced cytotoxicity was apparent only at 0.1% after 48h **(Fig. 29A, Table 38)**, and the average IC50 of WEN was 7-fold higher than that of MO (0.22% WEN vs. 0.03% MO) in DPCs. Similar cytotoxicity profiles were observed for DevaCurl Low-Poo Delight Cleanser **(Fig. 30A, Table 40)** and Aquaphor Baby Wash & Shampoo **(Fig. 31A, Table 42)** in DPCs with an average IC50 of 0.05% for both. Although Aquaphor Baby Wash & Shampoo appeared to be slightly more toxic than DevaCurl Low-Poo Delight Cleanser at the early time points, the difference was unsubstantial.

NHEKs were strikingly more sensitive to all the test products except for WEN. Although their effects were less substantial at 24h, the IC50s of Monat Renew shampoo **(Fig. 28B, Table 37)**, DevaCurl Low-Poo Delight Cleanser **(Fig. 30B, Table 41)**, and Aquaphor Baby Wash & Shampoo **(Fig. 31B, Table 43)** were >25-fold lower than those observed in DPCs. NHEKs were less sensitive to WEN, with the maximum decrease occurring at 0.1% 6 days after treatment **(Fig. 29B, Table 39)**.

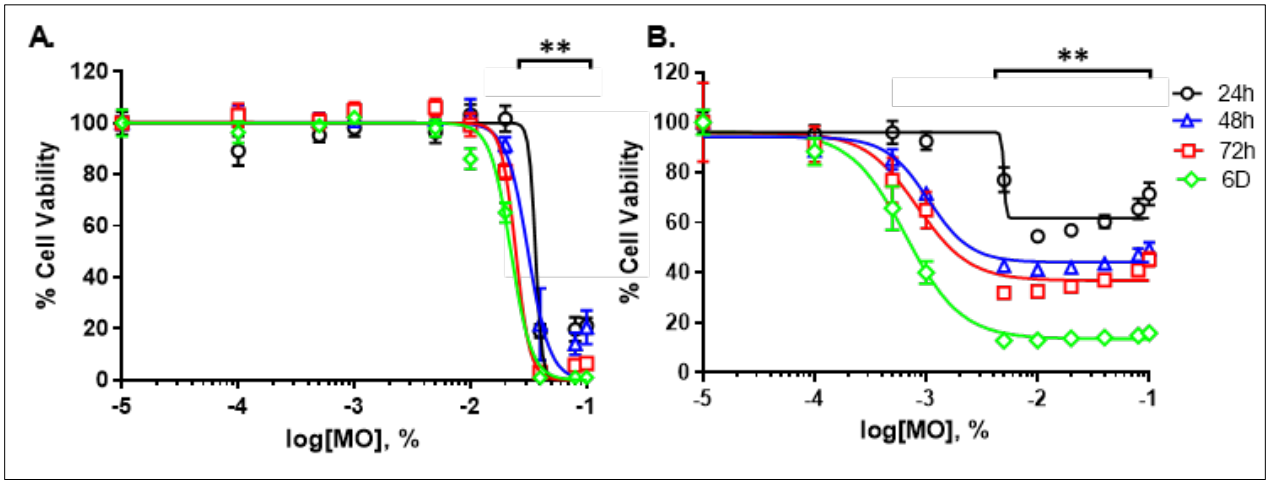


Figure 28. Cytotoxicity assessment of Monat Renew Shampoo (MO) in DPCs (A) and NHEKs (B). Cells cultured in the presence of the test product for 24, 48, 72 hours (h), and 6 days (D) were assessed by MTS assay. Dose response curves were obtained using GraphPad. **, $p \leq 0.0001$ (vs. nontreated control). n=5-6 replicates per concentration.

Table 36. Viability (%) of DPCs treated with Monat Renew Shampoo.

		0%	0.0001%	0.0005%	0.001%	0.005%	0.01%	0.02%	0.04%	0.08%	0.1%	IC50	R^2
24h	mean ± SD	100 ± 4.47	89.14 ± 5.67	95.3 ± 2.77	98.1 ± 3.39	96.42 ± 4.23	103.25 ± 3.88	101.68 ± 5.02	**19.06 ± 2.6**	**19.73 ± 4.74**	**21.19 ± 3.06**	0.035	0.9787
	adj. p-value		0.0001	0.4961	>0.9999	>0.9999	>0.9999	>0.9999	<0.0001	<0.0001	<0.0001		
48h	mean ± SD	100 ± 2.51	102.66 ± 3.99	100.84 ± 3.05	102.66 ± 4.17	101.4 ± 3.12	103.16 ± 6.16	91.44 ± 2.98	**21.67 ± 13.97**	**14.17 ± 4.31**	**20.55 ± 6.47**	0.031	0.9364
	adj. p-value		>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	0.005	<0.0001	<0.0001	<0.0001		
72h	mean ± SD	100 ± 2.08	103.05 ± 4.47	100.61 ± 3.11	104.73 ± 3.36	105.95 ± 3.27	99.2 ± 4.46	81.01 ± 2.95	**2.96 ± 0.33**	**5.73 ± 1.83**	**6.48 ± 0.97**	0.025	0.9887
	adj. p-value		>0.9999	>0.9999	0.4812	0.139	>0.9999	<0.0001	<0.0001	<0.0001	<0.0001		
6 D	mean ± SD	100 ± 5.25	96.36 ± 4.16	98.92 ± 1.88	102.18 ± 1.96	97.97 ± 3.36	86.08 ± 4.06	65.18 ± 3.82	**0.96 ± 0.13**	**1.01 ± 0.23**	**1.03 ± 0.31**	0.022	0.9854
	adj. p-value		>0.9999	>0.9999	>0.9999	>0.9999	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. R^2 > 0.9 is indicated. Highlighted values correspond to cell viability< 50%.

Table 37. Viability (%) of NHEKs treated with Monat Renew Shampoo.

		0%	0.0001%	0.0005%	0.001%	0.005%	0.01%	0.02%	0.04%	0.08%	0.1%	IC50	R^2
24h	mean ± SD	100 ± 3.97	95.5 ± 3.47	96.01 ± 4.66	92.53 ± 3.55	77.07 ± 4.96	54.55 ± 1.8	56.91 ± 1.85	60.39 ± 2.51	65.4 ± 4.05	71.44 ± 4.47	N/A	N/A
	adj. p-value		0.743	>0.9999	0.0379	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
48h	mean ± SD	100 ± 2.44	88.71 ± 2.35	85.2 ± 4.09	71.65 ± 1.81	**42.6 ± 1.23**	**41.2 ± 1.16**	**42.06 ± 1.94**	**43.69 ± 1.64**	**47.2 ± 2.59**	**49.22 ± 2.78**	0.0010	0.9644
	adj. p-value		0.0002	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
72h	mean ± SD	100 ± 15.74	91.23 ± 7.16	76.85 ± 8.96	64.99 ± 7.25	**31.86 ± 1.14**	**32.49 ± 1.28**	**34.5 ± 1.76**	**37.08 ± 2.21**	**40.97 ± 2.21**	**45.21 ± 2.81**	0.0008	0.9065
	adj. p-value		0.0074	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
6 D	mean ± SD	100 ± 4.95	88.32 ± 5.29	65.79 ± 8.77	**40.06 ± 4.48**	**12.89 ± 0.31**	**12.97 ± 0.3**	**13.71 ± 0.59**	**14.02 ± 0.65**	**14.89 ± 0.86**	**15.84 ± 1.2**	0.0007	0.9828
	adj. p-value		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. R^2 > 0.9 is indicated. Highlighted values correspond to cell viability< 50%. N/A, not available.

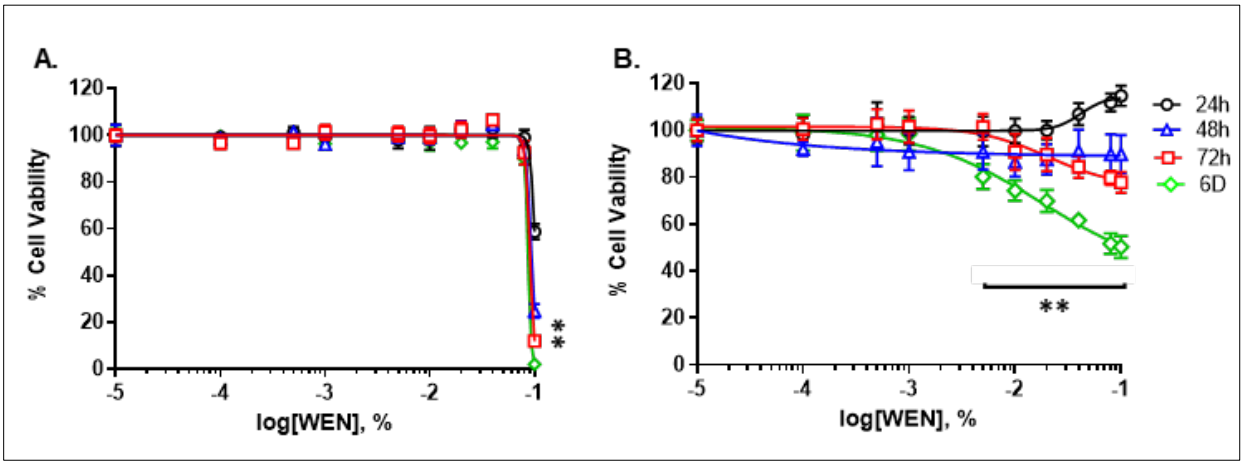


Figure 29. Cytotoxicity assessment of WEN Sweet Almond Mint Cleansing Conditioner (WEN) in DPCs (A) and NHEKs (B). Cells cultured in the presence of the test product for 24, 48, 72 hours (h), and 6 days (D) were assessed by MTS assay. Dose response curves were obtained using GraphPad. **, $p \leq 0.0001$ (vs. nontreated control). n=5-6 replicates per concentration.

Table 38. Viability (%) of DPCs treated with WEN Sweet Almond Mint Cleansing Conditioner.

		0%	0.0001%	0.0005%	0.001%	0.005%	0.01%	0.02%	0.04%	0.08%	0.1%	IC50	R^2
24h	mean ± SD	100 ± 4.58	99.27 ± 1.67	101.1 ± 2.54	99.27 ± 4.28	98.07 ± 3.71	98.62 ± 4.89	102.48 ± 3.35	102.93 ± 3.94	98.99 ± 3.42	58.74 ± 3.31	0.408	N/A
	adj. p-value		>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	<0.0001		
48h	mean ± SD	100 ± 4.47	98.52 ± 2.16	101.36 ± 1.9	96.11 ± 1.79	99.14 ± 2.03	99.14 ± 2.97	102.78 ± 3.3	104.69 ± 2.08	93.82 ± 2.35	**24.89 ± 2.93**	0.192	N/A
	adj. p-value		>0.9999	>0.9999	0.3645	>0.9999	>0.9999	>0.9999	0.1236	0.0115	<0.0001		
72h	mean ± SD	100 ± 2.78	96.62 ± 2.56	96.57 ± 2.19	101.37 ± 3.22	100.64 ± 3.17	99.85 ± 3.38	102.45 ± 3.37	106.51 ± 1.22	92.51 ± 5.18	**11.94 ± 0.96**	0.160	N/A
	adj. p-value		0.6729	0.6363	>0.9999	>0.9999	>0.9999	>0.9999	0.0063	0.0009	<0.0001		
6 D	mean ± SD	100 ± 2.86	99.57 ± 2	99.83 ± 2.28	96.36 ± 2.18	100.23 ± 1.82	96.76 ± 3.32	96.78 ± 1.63	97.18 ± 2.84	90.1 ± 2.59	**2.05 ± 0.44**	0.121	N/A
	adj. p-value		>0.9999	>0.9999	0.4948	>0.9999	0.7834	0.8084	>0.9999	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. Highlighted values correspond to cell viability< 50%. N/A, not available.

Table 39. Viability (%) of NHEKs treated with WEN Sweet Almond Mint Cleansing Conditioner.

		0%	0.0001%	0.0005%	0.001%	0.005%	0.01%	0.02%	0.04%	0.08%	0.1%	IC50	R^2
24h	mean ± SD	100 ± 5.35	98.07 ± 7.29	102.03 ± 9.8	99.89 ± 5.8	99.46 ± 6.33	99.79 ± 5.21	100 ± 3.93	106.85 ± 4.73	111.78 ± 3.79	114.67 ± 4.33	N/A	N/A
	adj. p-value		>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	0.5219	0.0111	0.0006		
48h	mean ± SD	100 ± 6.77	91.93 ± 2.89	94.98 ± 10.32	90.75 ± 7.83	90.75 ± 7.6	86.44 ± 6.33	87.54 ± 6.54	91.14 ± 9.09	89.73 ± 8.53	89.73 ± 8.25	N/A	N/A
	adj. p-value		0.2321	>0.9999	0.0972	0.0972	0.0019	0.0058	0.1312	0.0426	0.0426		
72h	mean ± SD	100 ± 4.54	100.49 ± 4.64	102.5 ± 6.46	101.57 ± 6.8	101.32 ± 5.48	90.73 ± 7.69	89.55 ± 7.11	84.36 ± 4.66	79.7 ± 3.09	77.73 ± 4.56	N/A	N/A
	adj. p-value		>0.9999	>0.9999	>0.9999	>0.9999	0.0956	0.0366	0.0002	<0.0001	<0.0001		
6 D	mean ± SD	100 ± 4.31	98.98 ± 7.64	98.01 ± 6.91	98.36 ± 6.88	80.1 ± 5.37	74.23 ± 4.34	69.85 ± 4.74	61.68 ± 2.93	51.64 ± 4.41	50.22 ± 4.7	0.018	0.9252
	adj. p-value		>0.9999	>0.9999	>0.9999	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. N/A, not available.

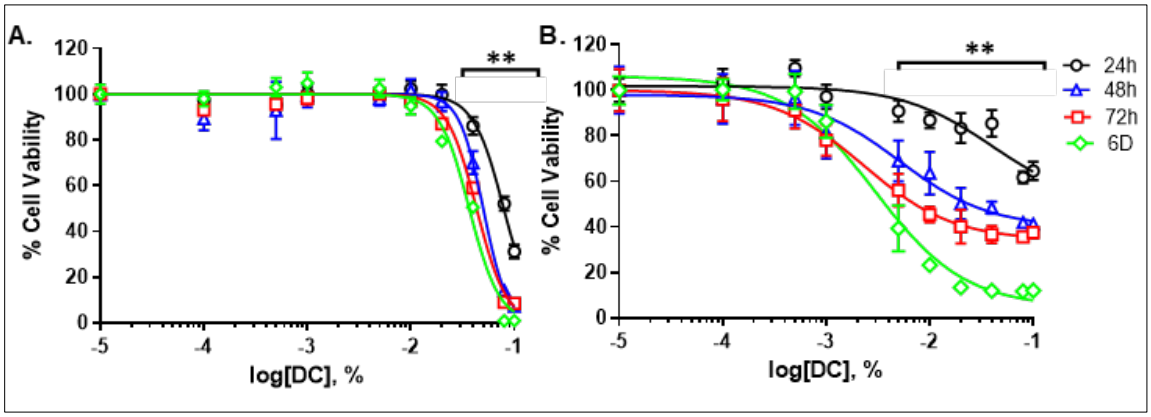


Figure 30. Cytotoxicity assessment of DevaCurl Low-Poo Delight Cleanser (DC) in DPCs (A) and NHEKs (B). Cells cultured in the presence of the test product for 24, 48, 72 hours (h), and 6 days (D) were assessed by MTS assay. Dose response curves were obtained using GraphPad. **, $p \leq 0.0001$ (vs. nontreated control). n=5-6 replicates per concentration.

Table 40. Viability (%) of DPCs treated with DevaCurl Low-Poo Delight Cleanser.

		0%	0.0001%	0.0005%	0.001%	0.005%	0.01%	0.02%	0.04%	0.08%	0.1%	IC50	R^2
24h	mean ± SD	100 ± 1.8	97.25 ± 3	95.31 ± 3.46	100.31 ± 2.93	98.47 ± 2.55	102.34 ± 3.39	99.9 ± 4.33	86.15 ± 3.79	52.13 ± 3.16	**31.25 ± 3.03**	0.079	0.9736
	adj. p-value		>0.9999	0.2563	>0.9999	>0.9999	>0.9999	>0.9999	<0.0001	<0.0001	<0.0001		
48h	mean ± SD	100 ± 1.71	89.34 ± 5.08	92.85 ± 12.54	99.36 ± 5.04	98 ± 3.02	101.72 ± 4.82	96.71 ± 4.27	70.09 ± 5.18	**14.22 ± 1.69**	**7.35 ± 0.8**	0.050	0.9651
	adj. p-value		<0.0001	0.0081	>0.9999	>0.9999	>0.9999	>0.9999	<0.0001	<0.0001	<0.0001		
72h	mean ± SD	100 ± 2.27	93.13 ± 1.65	95.6 ± 2.38	98.25 ± 2.7	100.13 ± 1.96	97.22 ± 1.62	87.06 ± 2.03	59.01 ± 2.39	**9.05 ± 1.76**	**8.67 ± 1.49**	0.043	0.9873
	adj. p-value		0.0127	0.3566	>0.9999	>0.9999	>0.9999	<0.0001	<0.0001	<0.0001	<0.0001		
6 D	mean ± SD	100 ± 4.2	98.01 ± 3.31	103 ± 4.05	104.82 ± 4.63	102.55 ± 3.78	94.88 ± 3.77	79.49 ± 2.33	50.65 ± 1.66	**0.94 ± 0.12**	**1.07 ± 0.21**	0.037	0.9803
	adj. p-value		>0.9999	>0.9999	0.2187	>0.9999	0.1508	<0.0001	<0.0001	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were

generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. Highlighted values correspond to cell viability< 50%.

Table 41. Viability (%) of NHEKs treated with DevaCurl Low-Poo Delight Cleanser.

		0%	0.0001%	0.0005%	0.001%	0.005%	0.01%	0.02%	0.04%	0.08%	0.1%	IC50	R^2
24h	mean ± SD	100 ± 5.21	103.61 ± 5.7	109.74 ± 3.65	97.16 ± 5.38	90.81 ± 4.73	86.87 ± 3.42	83.37 ± 6.49	85.45 ± 5.86	61.71 ± 2.66	64.55 ± 4.04	N/A	N/A
	adj. p-value		>0.9999	0.0878	>0.9999	0.1317	0.0048	0.0001	0.0012	<0.0001	<0.0001		
48h	mean ± SD	100 ± 10.33	96.26 ± 10.93	96.66 ± 11.79	81.45 ± 11.54	68.92 ± 8.9	63.55 ± 9.39	50.2 ± 6.81	**48.41 ± 2.63**	**41.99 ± 1.66**	**41.33 ± 1.14**	0.005	N/A
	adj. p-value		>0.9999	>0.9999	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
72h	mean ± SD	100 ± 9.25	95.95 ± 9.5	91.08 ± 7.94	78.01 ± 6.82	56.17 ± 7.15	**45.44 ± 3.37**	**40.09 ± 7.49**	**36.62 ± 3.84**	**35.68 ± 2.02**	**37.45 ± 2.46**	0.002	0.9441
	adj. p-value		>0.9999	0.1597	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
6 D	mean ± SD	100 ± 6.24	100.25 ± 4.71	99.48 ± 7.65	86.37 ± 7.26	**39.35 ± 10.04**	**23.2 ± 2.57**	**13.45 ± 1.77**	**12 ± 0.26**	**11.77 ± 0.42**	**12.1 ± 0.38**	0.003	0.9704
	adj. p-value		>0.9999	>0.9999	0.003	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. Highlighted values correspond to cell viability< 50%. N/A, not available.

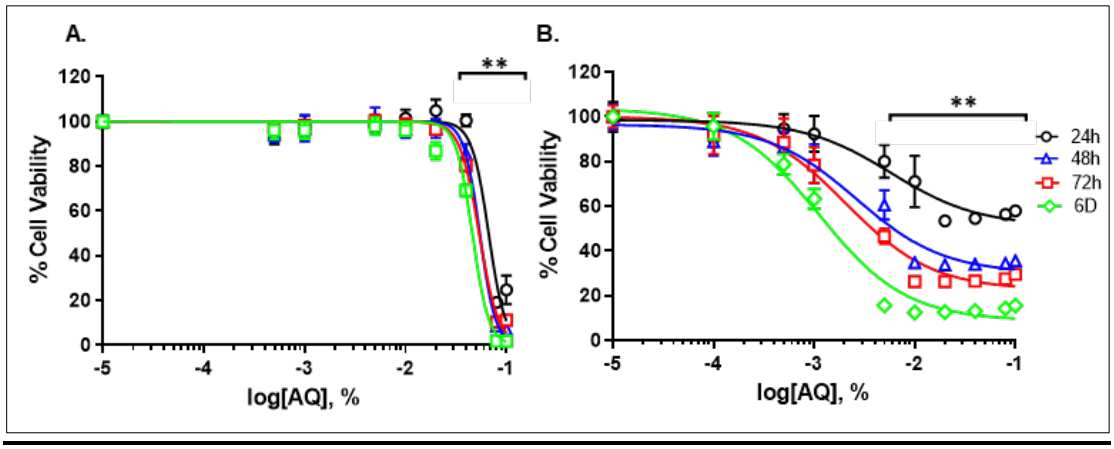


Figure 31. Cytotoxicity assessment of Aquaphor Baby Wash & Shampoo (AQ) in DPCs (A) and NHEKs (B). Cells cultured in the presence of the test product for 24, 48, 72 hours (h), and 6 days (D) were assessed by MTS assay. Dose response curves were obtained using GraphPad. **, $p \leq 0.0001$ (vs. nontreated control). n=5-6 replicates per concentration.

Table 42. Viability (%) of DPCs treated with Aquaphor Baby Wash & Shampoo.

		0%	0.0001%	0.0005%	0.001%	0.005%	0.01%	0.02%	0.04%	0.08%	0.1%	IC50	R^2
24h	mean ± SD	100 ± 2.85	84.99 ± 5.7	92.86 ± 3.02	97.72 ± 5.05	97.83 ± 2.69	101.55 ± 3.75	104.86 ± 5.01	100.41 ± 2.76	**19.17 ± 2.44**	**24.76 ± 6.4**	0.067	0.9446
	adj. p-value		<0.0001	0.1649	>0.9999	>0.9999	>0.9999	0.9609	>0.9999	<0.0001	<0.0001		
48h	mean ± SD	100 ± 2.58	96.91 ± 3.33	94.29 ± 3.5	96.97 ± 5.91	101.31 ± 4.97	96.7 ± 4.06	96.84 ± 4.26	85.35 ± 4.6	**8.68 ± 1.91**	**6.96 ± 2.91**	0.054	0.9837
	adj. p-value		>0.9999	0.5286	>0.9999	>0.9999	>0.9999	>0.9999	<0.0001	<0.0001	<0.0001		
72h	mean ± SD	100 ± 1.22	94.99 ± 2.76	97.12 ± 2.56	98.27 ± 1.99	100.27 ± 2.81	98.67 ± 2.78	96.54 ± 2.51	80.38 ± 2.54	**10.35 ± 0.62**	**11.15 ± 1.75**	0.054	0.9913
	adj. p-value		0.8676	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	<0.0001	<0.0001	<0.0001		
6 D	mean ± SD	100 ± 2.55	84.45 ± 4.84	80.33 ± 24.64	96.05 ± 3.82	97.9 ± 4.05	96 ± 2.46	86.84 ± 3.78	69.2 ± 2.81	**1.81 ± 0.28**	**1.76 ± 0.55**	0.046	0.9799
	adj. p-value		<0.0001	<0.0001	>0.9999	>0.9999	>0.9999	0.0002	<0.0001	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. Highlighted values correspond to cell viability< 50%.

Table 43. Viability (%) of NHEKs treated with Aquaphor Baby Wash & Shampoo.

		0%	0.0001%	0.0005%	0.001%	0.005%	0.01%	0.02%	0.04%	0.08%	0.1%	IC50	R^2
24h	mean ± SD	100 ± 6.72	94.68 ± 5.95	94.68 ± 6.48	92.4 ± 8.02	80.02 ± 7.31	71.01 ± 11.57	53.42 ± 0.82	54.51 ± 1.41	56.35 ± 1.78	57.98 ± 2.1	N/A	N/A
	adj. p-value		0.6035	0.6035	0.0827	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
48h	mean ± SD	100 ± 5.65	88.8 ± 6.32	86.37 ± 6.85	79.07 ± 8.63	60.5 ± 6.59	**34.7 ± 0.55**	**33.73 ± 0.72**	**34.01 ± 0.69**	**34.42 ± 0.35**	**35.54 ± 0.55**	0.003	0.9396
	adj. p-value		0.0013	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
72h	mean ± SD	100 ± 5.05	91.9 ± 8.58	88.59 ± 10.72	78.36 ± 8.05	**46.59 ± 3.44**	**26.24 ± 0.72**	**26.28 ± 0.85**	**26.58 ± 0.85**	**27.47 ± 0.83**	**29.55 ± 1.64**	0.002	0.9526
	adj. p-value		0.0498	0.001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
6 D	mean ± SD	100 ± 1.28	95.77 ± 5.9	78.79 ± 4.64	63.24 ± 4.43	**15.67 ± 2.01**	**12.46 ± 0.45**	**12.61 ± 0.35**	**13.18 ± 0.35**	**14.3 ± 0.56**	**15.52 ± 0.78**	0.001	0.9755
	adj. p-value		>0.9999	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. Highlighted values correspond to cell viability< 50%.

Test Ingredients

Acetyl tetrapeptide-3

Acetyl tetrapeptide-3 is a four amino acid peptide, typically used at 0.5–5% in cosmetics. Herbal extract combinations containing acetyl tetrapeptide-3 have been implicated in hair growth [55, 56], and a mixture of acetyl tetrapeptide-3 (326 ppm, 0.0326%) and red clover extract is marketed as Follicle Booster (MakingCosmetics). The effects of acetyl tetrapeptide-3 alone on hair growth are unknown. In DPCs, acetyl tetrapeptide-3 resulted in an overall 60% decrease in cell viability, but only in cells exposed to the highest concentration (1%) for 6 days **(Fig. 32A, Table 44)**. The overall cytotoxicity profiles of acetyl tetrapeptide-3 in NHEKs were similar to those observed in DPCs. However, NHEKs were somewhat more sensitive at later time points. At day 6, the viability of NHEKs treated with 0.5% acetyl tetrapeptide-3 was reduced to 25.3% (vs. 64.3% in DPCs) **(Fig. 32B, Table 45).**

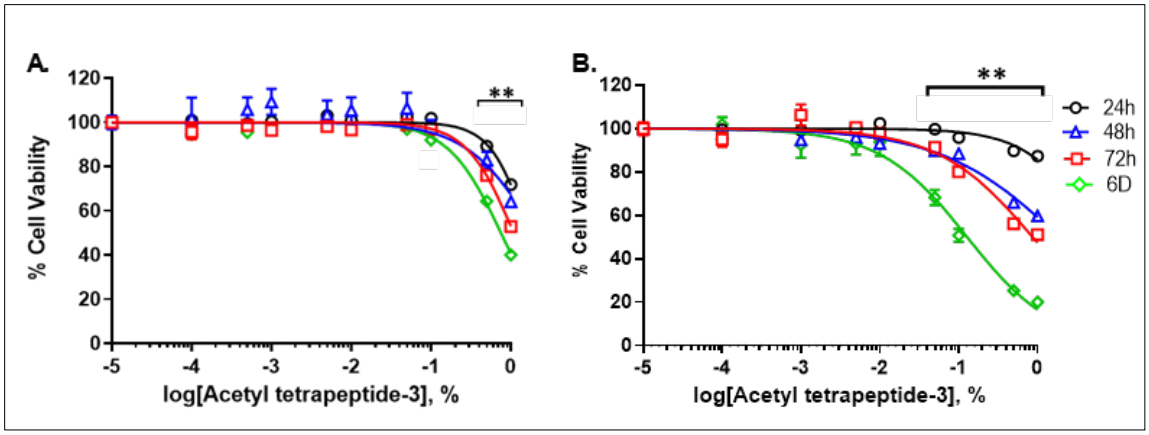


Figure 32. Cytotoxicity assessment of Acetyl tetrapeptide-3 in DPCs (A) and NHEKs (B). Cells cultured in the presence of the test ingredient for 24, 48, 72 hours (h), and 6 days (D) were assessed by MTS assay. Dose response curves were obtained using GraphPad. **, $p \leq 0.0001$ (vs. nontreated control). n=5-6 replicates per concentration.

Table 44. Viability (%) of DPCs treated with Acetyl tetrapeptide-3.

		0%	0.0001%	0.0005%	0.001%	0.005%	0.01%	0.05%	0.1%	0.5%	1%	IC50	R^2
24h	mean ± SD	100 ± 4.89	100.92 ± 4.83	99.46 ± 5.64	100.49 ± 3.87	103.08 ± 3.43	100.92 ± 4.03	102.49 ± 3.43	101.84 ± 4.77	89.29 ± 4.55	71.88 ± 5.72	N/A	N/A
	adj. p-value		>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	0.1616	<0.0001		
48h	mean ± SD	100 ± 7.69	101.74 ± 22.97	105.75 ± 13.44	109.3 ± 14.63	103.58 ± 15.45	105.47 ± 14.38	106.34 ± 17.2	98.17 ± 7.13	82.83 ± 9.41	64.11 ± 5.77	N/A	N/A
	adj. p-value		>0.9999	>0.9999	0.3556	>0.9999	>0.9999	>0.9999	>0.9999	0.0016	<0.0001		
72h	mean ± SD	100 ± 5.01	95.61 ± 8.13	98.7 ± 2.96	96.42 ± 2.05	98.12 ± 3.67	96.62 ± 2.03	99 ± 4.46	96.84 ± 4.23	76.05 ± 3.76	52.84 ± 2.61	0.610	0.9246
	adj. p-value		>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	<0.0001	<0.0001		
6 D	mean ± SD	100 ± 3.68	94.55 ± 2.98	95.49 ± 4.62	99.05 ± 2.72	99.57 ± 3.37	100.73 ± 4.09	96.81 ± 3.58	92.03 ± 4.72	64.3 ± 3.04	**40 ± 2.52**	0.864	0.9614
	adj. p-value		>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	0.6963	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. Highlighted values correspond to cell viability< 50%. N/A, not available.

Table 45. Viability (%) of NHEKs treated with Acetyl tetrapeptide-3.

		0%	0.0001%	0.001%	0.005%	0.01%	0.05%	0.1%	0.5%	1%	IC50	R^2
24h	mean ± SD	100 ± 3.65	99.53 ± 3.69	99.18 ± 3.74	100.47 ± 3.72	102.46 ± 2.55	99.77 ± 3.32	95.89 ± 3.63	89.79 ± 3.41	87.32 ± 3.65	N/A	N/A
	adj. p-value		>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	0.0171	0.0012		
48h	mean ± SD	100 ± 3.67	97.7 ± 3.55	94.77 ± 2.58	95.96 ± 3.33	93.11 ± 4.69	89.72 ± 1.72	88.43 ± 1.29	65.93 ± 1.77	59.78 ± 1.47	0.386	0.9461
	adj. p-value		>0.9999	0.8953	>0.9999	0.2958	0.0159	0.0042	<0.0001	<0.0001		
72h	mean ± SD	100 ± 7.5	94.95 ± 8.21	106.25 ± 11.7	100.5 ± 4.73	97.73 ± 5.2	91.33 ± 5.14	80.03 ± 4.23	56.15 ± 2.91	51.17 ± 3	N/A	N/A
	adj. p-value		>0.9999	0.4631	>0.9999	>0.9999	0.071	<0.0001	<0.0001	<0.0001		
6 D	mean ± SD	100 ± 2.97	101.9 ± 7.3	92.55 ± 13.31	93 ± 11.36	93.7 ± 14.13	68.2 ± 7.98	50.7 ± 6.8	**25.3 ± 0.91**	**20 ± 0.4**	0.086	0.9323
	adj. p-value		>0.9999	0.315	0.4218	0.6475	<0.0001	<0.0001	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. Highlighted values correspond to cell viability< 50%. N/A, not available.

Calendula Officinalis Extract

Calendula extract, prepared from *Calendula officinalis* Linn (Asteraceae), has long been used in traditional medicine for treating skin disorders (e.g., skin ulcers, minor burns, rashes, inflammation) and promoting wound healing. In this context, it has been shown to enhance PI3K-mediated proliferation and migration of lung fibroblasts [57], as well as proliferation of mouse embryonic fibroblasts via TGFβ1 and βFGF [58]. It has also been shown to inhibit the growth of various cancer cells in vitro and in vivo [59]. Calendula extract is typically used at 0.5–5% in cosmetics. However, the formulation used in this study contained 1.5–3.5% calendula extract, thereby limiting the feasibility of assessing concentrations >0.25%. Despite this limitation, clear dose- and time-dependent decreases in cell viability were observed at concentrations >0.125%. DPCs treated with 0.25% calendula extract showed a 58.68% and 84.34% reduction in cell viability at 72h and at 6 days after treatment, respectively **(Fig. 33A, Table 46)**. It is important to note that the calendula formulation used in this study contains additives and preservatives (i.e., potassium sorbate and sodium benzoate, 0.2–0.3%). Given that sodium benzoate has been shown to reduce the viability of human gingival fibroblasts, albeit at higher concentrations [60], its possible influence on DPC viability cannot be completely excluded.

In contrast, calendula extract increased NHEK viability at 24h, followed by gradual decreases at later time points **(Fig. 33B, Table 47)**. At concentrations <0.05%, calendula extract appeared to promote cell growth, while concentrations >0.2% were cytotoxic, leading to a reduction in cell viability of >50%. However, this effect was apparent only at day 6. Although uncertain, this decrease at later time points could be due to nutrition deprivation and/or pH changes in the medium.

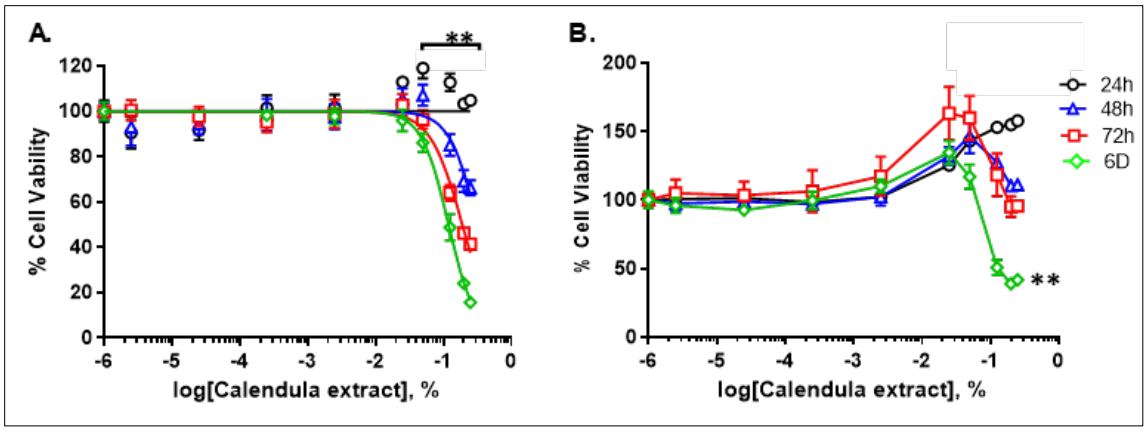


Figure 33. Cytotoxicity assessment of Calendula extract in DPCs (A) and NHEKs (B). Cells cultured in the presence of the test ingredient for 24, 48, 72 hours (h), and 6 days (D) were assessed by MTS assay. Dose response curves were obtained using GraphPad. **, *p ≤ 0.0001* (vs. nontreated control). n=5-6 replicates per concentration.

Table 46. Viability (%) of DPCs treated with Calendula extract.

		0%	0.0000025%	0.000025%	0.00025%	0.0025%	0.025%	0.05%	0.125%	0.2%	0.25%	IC50	R^2
24h	mean ± SD	100 ± 4.74	90.55 ± 6.86	91.79 ± 4.47	101.46 ± 5.68	101.69 ± 5.69	112.93 ± 2.45	119 ± 4.5	112.71 ± 3.83	103.37 ± 1.68	104.84 ± 1.65	N/A	N/A
	adj. p-value		0.0194	0.0675	>0.9999	>0.9999	0.0003	<0.0001	0.0004	>0.9999	>0.9999		
48h	mean ± SD	100 ± 3.64	93.08 ± 8.21	94.6 ± 4.81	98.42 ± 7.08	97.69 ± 5.56	105.55 ± 4.46	107.13 ± 4.61	85.16 ± 4.76	69.24 ± 4.77	66.22 ± 3.37	N/A	N/A
	adj. p-value		0.2155	0.6929	>0.9999	>0.9999	0.6255	0.1794	<0.0001	<0.0001	<0.0001		
72h	mean ± SD	100 ± 2.45	100.4 ± 4.52	97.77 ± 4.21	95.23 ± 4.47	98.84 ± 6.29	102.76 ± 4.77	96.34 ± 4.04	64.01 ± 3.49	**46.27 ± 1.99**	**41.32 ± 0.78**	0.190	0.9550
	adj. p-value		>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	<0.0001	<0.0001	<0.0001		
6 D	mean ± SD	100 ± 3.94	nd	nd	98.27 ± 1.64	97.79 ± 3.68	95.94 ± 4.77	86.19 ± 4.11	**48.73 ± 5.86**	**24.04 ± 0.98**	**15.66 ± 0.79**	0.120	0.9888
	adj. p-value				>0.9999	>0.9999	>0.9999	<0.0001	<0.0001	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. Highlighted values correspond to cell viability< 50%. N/A, not available.

Table 47. Viability (%) of NHEKs treated with Calendula extract.

		0%	0.0000025%	0.000025%	0.00025%	0.0025%	0.025%	0.05%	0.125%	0.2%	0.25%	IC50	R^2
24h	mean ± SD	100 ± 4.11	100.5 ± 5.77	101.25 ± 3.62	98.49 ± 5.46	102.26 ± 2.63	125.47 ± 1.94	143.16 ± 4.13	152.95 ± 3.61	154.96 ± 1.3	157.72 ± 1.56	N/A	N/A
	adj. p-value		>0.9999	>0.9999	>0.9999	>0.9999	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
48h	mean ± SD	100 ± 3.35	97.34 ± 5.39	98.67 ± 4.66	97.16 ± 3.11	102.22 ± 6.54	132 ± 6.52	146.45 ± 12.53	127.48 ± 3.79	110.46 ± 2.81	111.08 ± 1.73	N/A	N/A
	adj. p-value		>0.9999	>0.9999	>0.9999	>0.9999	<0.0001	<0.0001	<0.0001	0.1847	0.1278		
72h	mean ± SD	100 ± 5.35	104.87 ± 10.11	103.47 ± 9.92	106.28 ± 15.38	117.21 ± 14.26	163.15 ± 19.42	159.75 ± 16.03	118.32 ± 15.5	95.05 ± 7.57	95.64 ± 1.2	N/A	N/A
	adj. p-value		>0.9999	>0.9999	>0.9999	0.0015	<0.0001	<0.0001	0.0006	>0.9999	>0.9999		
6 D	mean ± SD	100 ± 6.29	95.89 ± 5.48	92.53 ± 3.52	99.55 ± 6.45	110.08 ± 4.82	134.5 ± 8.59	116.87 ± 8.83	50.77 ± 5.48	**38.84 ± 0.34**	**41.68 ± 0.29**	0.237	N/A
	adj. p-value		>0.9999	0.8709	>0.9999	0.2298	<0.0001	0.002	<0.0001	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. Highlighted values correspond to cell viability< 50%. N/A, not available.

Caryocar Brasiliense Fruit Oil (Pequi oil)

The oil extracted from the pulp and seeds of Pequi fruit has been widely used in folk medicine for wound healing and to treat joint and muscular pains. Pequi oil at 12% was shown to inhibit xylene-induced inflammation and accelerated cutaneous wound repair in mice [61]. While its effects on hair growth and HF cycling are unknown, Pequi oil increased the viability of both DPCs and NHEKs. DPCs

showed significant dose-dependent increases in cell viability, with the maximum increase occurring 24h after treatment **(Fig. 34A, Table 48)**. In NHEKs, substantial increases in cell viability were observed at concentrations >0.125%, which peaked at 24h and then gradually decreased at later time points. The maximum increase in cell viability, 111.18%, was observed at 24h in cells treated with 0.25% **(Fig. 34B, Table 49)**.

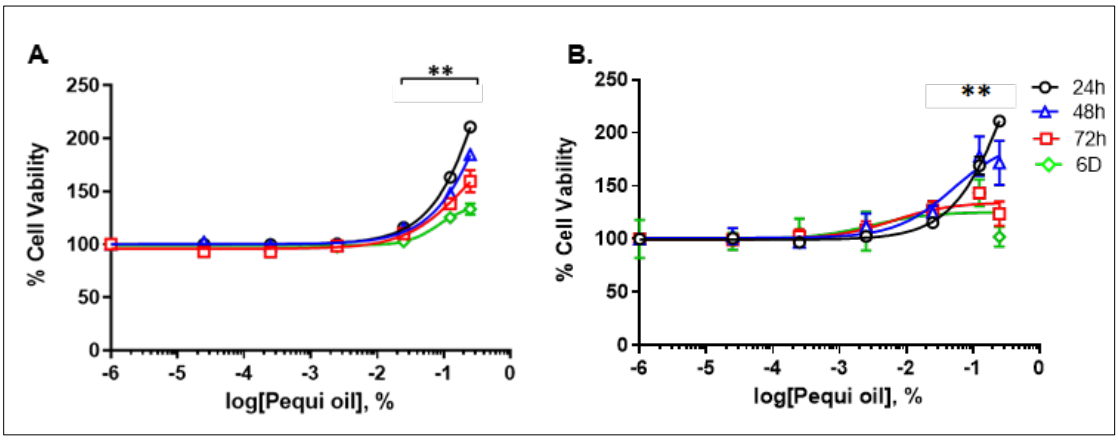


Figure 34. Cytotoxicity assessment of Pequi oil in DPCs (A) and NHEKs (B). Cells cultured in the presence of the test ingredient for 24, 48, 72 hours (h), and 6 days (D) were assessed by MTS assay. Dose response curves were obtained using GraphPad. **, $p \leq 0.0001$ (vs. nontreated control). n=5-6 replicates per concentration.

Table 48. Viability (%) of DPCs treated with Pequi oil.

		0%	0.000025%	0.00025%	0.0025%	0.025%	0.125%	0.25	IC50	R^2
24h	mean ± SD	100 ± 1.81	100.07 ± 3.86	99.93 ± 4.1	100.58 ± 2.66	115.84 ± 2.65	163.16 ± 3.41	210.77 ± 4.05	N/A	0.9942
	adj. p-value		>0.9999	>0.9999	>0.9999	<0.0001	<0.0001	<0.0001		
48h	mean ± SD	100 ± 3.85	102.2 ± 2.78	98.55 ± 1.26	99 ± 3.06	115.08 ± 4.63	147.88 ± 2.44	184.65 ± 3.75	N/A	0.9876
	adj. p-value		>0.9999	>0.9999	>0.9999	<0.0001	<0.0001	<0.0001		
72h	mean ± SD	100 ± 4.21	92.97 ± 3.18	92.85 ± 2.71	98.46 ± 4.41	110.19 ± 3.52	138.34 ± 4.1	159.38 ± 10.51	N/A	0.9522
	adj. p-value		0.0143	0.012	>0.9999	<0.0001	<0.0001	<0.0001		
6 D	mean ± SD	100 ± 2.99	98.82 ± 2.55	99.34 ± 3.2	97.69 ± 3.89	102.71 ± 3.31	125.29 ± 2.74	133.23 ± 5.24	N/A	0.9448
	adj. p-value		>0.9999	>0.9999	>0.9999	>0.9999	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. N/A, not available.

Table 49. Viability (%) of NHEKs treated with Pequi oil.

		0%	0.000025%	0.00025%	0.0025%	0.025%	0.125%	0.25	IC50	R^2
24h	mean ± SD	100 ± 2.1	101.03 ± 2.2	96.92 ± 2.08	102.52 ± 3.64	115.47 ± 2.76	169.25 ± 8.31	211.18 ± 2.42	N/A	N/A
	adj. p-value		>0.9999	>0.9999	>0.9999	0.0436	<0.0001	<0.0001		
48h	mean ± SD	100 ± 2.46	102.78 ± 7.69	96.93 ± 5.13	111.91 ± 11.87	124.89 ± 6.22	178.1 ± 18.55	171.68 ± 21.02	N/A	N/A
	adj. p-value		>0.9999	>0.9999	0.2266	0.0001	<0.0001	<0.0001		
72h	mean ± SD	100 ± 3.79	99.45 ± 5.1	102.58 ± 6.57	108.44 ± 8.42	127.49 ± 8.64	143.5 ± 4.72	123.8 ± 11.47	N/A	N/A
	adj. p-value		>0.9999	>0.9999	0.8368	<0.0001	<0.0001	0.0003		
6 D	mean ± SD	100 ± 18.03	97.8 ± 7.98	106.06 ± 13.11	107.44 ± 18.1	129.68 ± 5.66	143.47 ± 12.44	102.2 ± 9.26	N/A	N/A
	adj. p-value		>0.9999	>0.9999	>0.9999	<0.0001	<0.0001	>0.9999		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. N/A, not available.

Cinnamidopropyltrimonium chloride (CATC)

CATC is a quaternary ammonium compound with an antistatic property. It has also been shown to absorb UV radiation, thereby protecting hair from UV-induced damage [62]. The safety data related to

CATC is not available, although similar quaternary ammonium compounds have been shown to cause reproductive toxicity in mice [63]. A typical concentration range of quaternary ammonium compounds in cosmetic products is between 150 – 400 ppm (0.015 – 0.04%). At concentrations ≥ 0.5%, we observed a clear dose- and time-dependent cytotoxicity in DPCs **(Fig. 35A, Table 50)**. DPCs treated with 0.5% CATC showed a 75.26% reduction in cell viability 48 hours after treatment, which was further reduced to 2.42% at day 6 **(Table 50)**. Concentrations ≥ 2% were toxic, killing most cells in 24 hours. In stark contrast to the acute cytotoxicity observed in DPCs, CATC caused only modest decreases in NHEK viability. For example, at 24h, >60% of NHEKs remained viable when exposed to >2.5% of CATC **(Fig. 35B, Table 51)**.

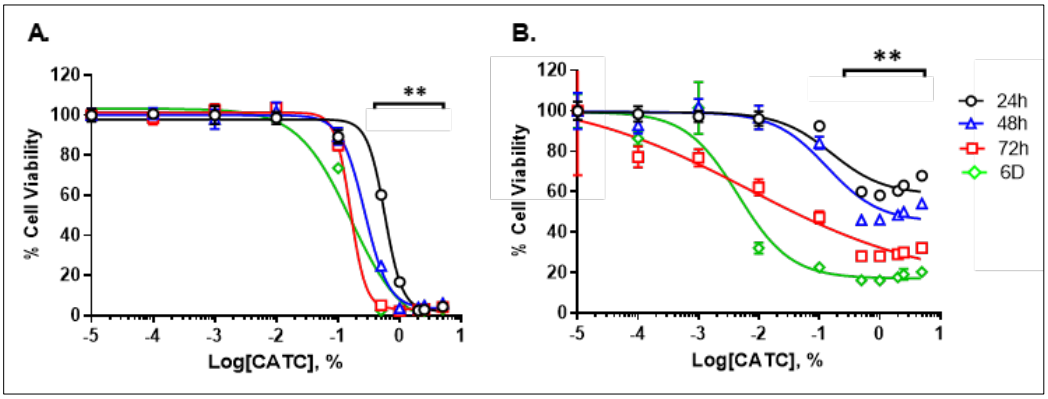


Figure 35. Cytotoxicity assessment of CATC in DPCs (A) and NHEKs (B). Cells cultured in the presence of the test ingredient for 24, 48, 72 hours (h), and 6 days (D) were assessed by MTS assay. Dose response curves were obtained using GraphPad. **, *p ≤ 0.0001* (vs. nontreated control). n=5-6 replicates per concentration.

Table 50. Viability (%) of DPCs treated with CATC.

		0%	0.0001%	0.001%	0.01%	0.1%	0.5%	1%	2%	2.5%	5%	IC50	R^2
24h	mean ± SD	100 ± 3.24	100.7 ± 2.12	100.1 ± 4.62	98.65 ± 3.06	89.24 ± 3.35	60.28 ± 1.65	**16.78 ± 0.65**	**2.58 ± 0.5**	**2.93 ± 0.29**	**4.42 ± 0.61**	0.573	0.9924
	adj. p-value		>0.9999	>0.9999	>0.9999	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
48h	mean ± SD	100 ± 3.15	100.7 ± 2.82	98.04 ± 4.9	102.01 ± 4.29	89.48 ± 4.2	**24.74 ± 1.38**	**3.6 ± 0.68**	**4.02 ± 0.56**	**4.91 ± 1.47**	**6.17 ± 0.66**	0.270	0.9945
	adj. p-value		>0.9999	>0.9999	>0.9999	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
72h	mean ± SD	100 ± 3.26	98.64 ± 3.35	102.66 ± 2.89	103.79 ± 2.48	84.94 ± 2.64	**5.07 ± 0.49**	**2.23 ± 0.36**	**2.81 ± 0.28**	**3.24 ± 0.45**	**4.23 ± 0.44**	0.159	0.9975
	adj. p-value		>0.9999	0.4477	0.0489	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
6 D	mean ± SD	100 ± 2.64	100.25 ± 1.04	101.68 ± 2.53	100.35 ± 3.55	73.58 ± 1.53	**2.42 ± 0.65**	**2.24 ± 0.32**	**2.51 ± 0.39**	**2.37 ± 0.33**	**3.16 ± 0.37**	0.162	0.9724
	adj. p-value		>0.9999	>0.9999	>0.9999	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. Highlighted values correspond to cell viability< 50%.

Table 51. Viability (%) of NHEKs treated with CATC.

		0%	0.0001%	0.001%	0.01%	0.1%	0.5%	1%	2%	2.5%	5%	IC50	R^2
24h	mean ± SD	100 ± 4.51	98.48 ± 3.87	97.2 ± 2.87	96.15 ± 3.59	92.54 ± 1.3	60.02 ± 1.03	58.28 ± 1.91	60.49 ± 1.92	63.17 ± 1.91	67.95 ± 2.4	N/A	N/A
	adj. p-value		>0.9999	>0.9999	>0.9999	0.3772	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
48h	mean ± SD	100 ± 8.98	92.88 ± 4.04	101.96 ± 3.88	96.8 ± 5.94	84.16 ± 2.99	**46.09 ± 0.44**	**46.17 ± 0.88**	**48.58 ± 1.17**	50.09 ± 0.79	54.27 ± 0.99	0.134	0.9204
	adj. p-value		0.4692	>0.9999	>0.9999	0.0002	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
72h	mean ± SD	100 ± 31.71	77.3 ± 5.28	76.77 ± 4.3	62.16 ± 3.94	**47.39 ± 3.11**	**28.18 ± 0.44**	**28.18 ± 0.84**	**29.18 ± 0.61**	**29.91 ± 1.59**	**32.25 ± 1.75**	N/A	N/A
	adj. p-value		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
6 D	mean ± SD	100 ± 8.47	86.36 ± 3.43	101.45 ± 12.8	**32.18 ± 2.73**	**22.67 ± 1.8**	**16.29 ± 1.22**	**16.2 ± 0.58**	**17.71 ± 2.08**	**19.17 ± 2.74**	**20.33 ± 1.06**	0.005	0.9377
	adj. p-value		0.0021	>0.9999	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. Highlighted values correspond to cell viability< 50%. N/A, not available.

Citrus Limon (Lemon) Peel Oil

C. Limon oil is widely used in cosmetic formulations as fragrances and/or skin conditioning agents. It is an established contact allergen and may cause cross-allergy with balsam of Peru. It is also categorized as a phototoxic fragrance ingredient. The International Fragrance Association (IFRA) has restricted its use to 2% in leave-on products that are applied to skin areas exposed to direct sunlight [64]. According to Cosmetic Ingredient Review (CIR), there are no restrictions on rinse-off products and products that are not applied to the skin [65]. In our study, treating DPCs with Lemon peel oil led to significant dose-dependent increases in cell viability, with the maximum increase occurring 24h after treatment **(Fig. 36A, Table 52)**. This increase attenuated at 72h, returning to the basal level at day 6. Similar dose- and time-dependent increases were also observed in NHEKs until 72h, but this increase was not sustained at day 6, as cell viability diminished from 139.77% (24h) to 69.78% **(Fig. 36B, Table 53)**.

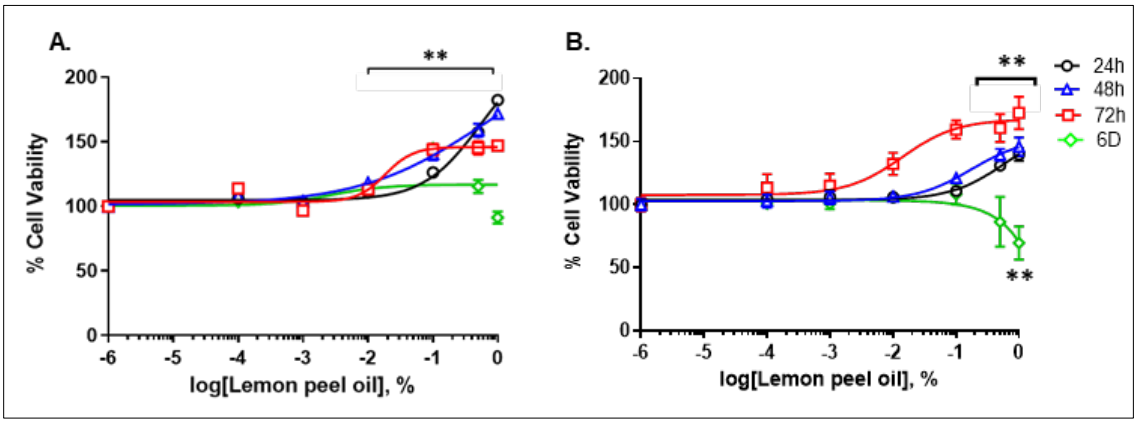


Figure 36. Cytotoxicity assessment of Lemon peel oil in DPCs (A) and NHEKs (B). Cells cultured in the presence of the test ingredient for 24, 48, 72 hours (h), and 6 days (D) were assessed by MTS assay. Dose response curves were obtained using GraphPad. **, $p \leq 0.0001$ (vs. nontreated control). n=5-6 replicates per concentration.

Table 52. Viability (%) of DPCs treated with Lemon peel oil.

		0%	0.0001%	0.001%	0.01%	0.1%	0.5%	1%	IC50	R^2
24h	mean ± SD	100 ± 3.74	106.02 ± 4.1	103.05 ± 2.12	110.73 ± 3.39	126.25 ± 3.63	157.41 ± 3.6	182.2 ± 2.16	N/A	0.9862
	adj. p-value		0.0302	0.9098	<0.0001	<0.0001	<0.0001	<0.0001		
48h	mean ± SD	100 ± 3.17	108.1 ± 2.63	103.85 ± 3.38	118.23 ± 3.7	140.65 ± 5.06	158.88 ± 5.05	171.82 ± 4.04	N/A	0.9728
	adj. p-value		0.0011	0.4228	<0.0001	<0.0001	<0.0001	<0.0001		
72h	mean ± SD	100 ± 3.09	113.82 ± 3.72	96.53 ± 3.14	113.09 ± 2.45	143.95 ± 4.5	145.25 ± 5.16	146.94 ± 2.67	N/A	0.9171
	adj. p-value		<0.0001	0.6148	<0.0001	<0.0001	<0.0001	<0.0001		
6 D	mean ± SD	100 ± 3.39	103.46 ± 3.19	101.33 ± 3.05	113.44 ± 2.55	144.88 ± 2.88	115.5 ± 5.23	91.18 ± 4.68	N/A	N/A
	adj. p-value		0.6212	>0.9999	<0.0001	<0.0001	<0.0001	0.0003		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. N/A, not available.

Table 53. Viability (%) of NHEKs treated with Lemon peel oil.

		0%	0.0001%	0.001%	0.01%	0.1%	0.5%	1%	IC50	R^2
24h	mean ± SD	100 ± 3.84	104.56 ± 3.2	106.08 ± 5.11	106.08 ± 1.9	110.88 ± 3.89	131.11 ± 2.24	139.77 ± 4.79	N/A	0.9277
	adj. p-value		>0.9999	>0.9999	>0.9999	0.086	<0.0001	<0.0001		
48h	mean ± SD	100 ± 4.7	102.92 ± 5.11	105.36 ± 4.9	106.62 ± 3.37	121.21 ± 3.42	139.51 ± 4.78	146.06 ± 7.36	N/A	0.9294
	adj. p-value		>0.9999	>0.9999	0.7993	<0.0001	<0.0001	<0.0001		
72h	mean ± SD	100 ± 5.27	113.47 ± 10.93	115.02 ± 9.79	132.39 ± 8.82	159.6 ± 7.19	160.81 ± 11.02	172.71 ± 12.89	N/A	N/A
	adj. p-value		0.0154	0.0048	<0.0001	<0.0001	<0.0001	<0.0001		
6 D	mean ± SD	100 ± 4.31	100.82 ± 1.81	100.7 ± 4.29	105.86 ± 3.87	108.2 ± 8.25	86.47 ± 19.71	69.78 ± 13.2	N/A	N/A
	adj. p-value		>0.9999	>0.9999	>0.9999	0.3811	0.0148	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. N/A, not available.

Cocamidopropyl Betaine (CAPB)

CAPB is an amphoteric synthetic surfactant used in cosmetics and personal hygiene products including cleansers, liquid soaps, shampoos, and conditioners. It is a known contact allergen [66]. In addition, despite being the allergen that had the eighth-most frequent incidence of reactions in a recent ten-year retrospective review of pediatric patients' medical records, CAPB is a common ingredient in products recommended for children who have allergic contact dermatitis [67]. It is typically used at concentrations of up to 30%. CAPB was cytotoxic to DPCs; however, a substantial decrease in cell viability was apparent only in cells treated with the highest concentration (0.1%), at which the maximum reduction of 74.62% was observed within 24h **(Fig. 37A, Table 54)**. In NHEKs, CAPB caused dose- and time-dependent decreases in cell viability, with IC50 doses of 0.0009% and 0.0006% at 72h and day 6, respectively **(Fig. 37B, Table 55)**.

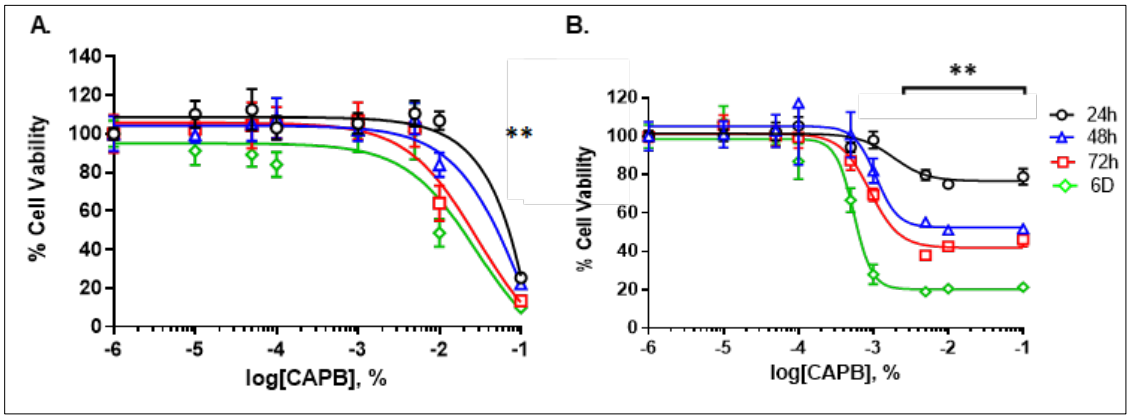


Figure 37. Cytotoxicity assessment of CAPB in DPCs (A) and NHEKs (B). Cells cultured in the presence of the test ingredient for 24, 48, 72 hours (h), and 6 days (D) were assessed by MTS assay. Dose response curves were obtained using GraphPad. **, $p \leq 0.0001$ (vs. nontreated control). n=5-6 replicates per concentration.

Table 54. Viability (%) of DPCs treated with CAPB.

		0%	0.00001%	0.00005%	0.0001%	0.001%	0.005%	0.01%	0.1%	IC50	R^2
24h	mean ± SD	100 ± 3.05	110.18 ± 6.88	112.52 ± 10.62	103.07 ± 6.1	105.41 ± 5.3	110.58 ± 6.73	106.77 ± 4.84	**25.38 ± 1.49**	0.067	N/A
	adj. p-value		0.1526	0.0349	>0.9999	>0.9999	0.1205	0.8788	<0.0001		
48h	mean ± SD	100 ± 9.03	99.33 ± 3.87	105.21 ± 8.91	108.02 ± 10.56	101.89 ± 5.7	107.87 ± 8.17	83.99 ± 6.35	**22.26 ± 2.23**	0.046	N/A
	adj. p-value		>0.9999	>0.9999	0.4908	>0.9999	0.528	0.0026	<0.0001		
72h	mean ± SD	100 ± 9.86	102.64 ± 3.47	104.42 ± 11.91	105.77 ± 8.11	106.69 ± 9.42	102.89 ± 9.57	63.97 ± 9.08	**13.33 ± 0.51**	0.026	N/A
	adj. p-value		>0.9999	>0.9999	>0.9999	0.9098	>0.9999	<0.0001	<0.0001		
6 D	mean ± SD	100 ± 6.77	91.13 ± 7.54	89.2 ± 6.21	84.08 ± 6.42	99.83 ± 9.25	101.28 ± 14.73	**48.55 ± 7.22**	**9.83 ± 1.2**	0.018	N/A
	adj. p-value		0.317	0.1059	0.0028	>0.9999	>0.9999	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. Highlighted values correspond to cell viability< 50%. N/A, not available.

Table 55. Viability (%) of NHEKs treated with CAPB.

		0%	0.00001%	0.00005%	0.0001%	0.0005%	0.001%	0.005%	0.01%	0.1%	IC50	R^2
24h	mean ± SD	100 ± 2.81	100.5 ± 3.13	102.58 ± 4.59	105.47 ± 4.7	94.33 ± 2.49	98.01 ± 4.46	79.82 ± 2.83	75.05 ± 1.7	78.93 ± 4.07	N/A	N/A
	adj. p-value		>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	<0.0001	<0.0001	<0.0001		
48h	mean ± SD	100 ± 7.52	100.93 ± 6.9	102.72 ± 8.53	117.28 ± 32.12	100.72 ± 11.88	82.01 ± 6.4	55.34 ± 1.18	51.11 ± 1.73	51.83 ± 2.03	N/A	N/A
	adj. p-value		>0.9999	>0.9999	0.0005	>0.9999	0.0003	<0.0001	<0.0001	<0.0001		

		0%	0.00001%	0.00005%	0.0001%	0.0005%	0.001%	0.005%	0.01%	0.1%	IC50	R^2
72h	mean ± SD	100 ± 2.95	105.61 ± 5.42	100.11 ± 5.36	99.49 ± 5.64	87.25 ± 4.91	69.57 ± 3.3	**38.01 ± 1.47**	**42.67 ± 1.82**	**46.21 ± 3.6**	9.00E-04	0.9684
	adj. p-value		>0.9999	>0.9999	>0.9999	0.0237	<0.0001	<0.0001	<0.0001	<0.0001		
6 D	mean ± SD	100 ± 6.01	107.39 ± 8.45	98.45 ± 2.78	86.92 ± 9.3	66.75 ± 6.17	**28.09 ± 5.13**	**19.04 ± 0.36**	**20.64 ± 0.77**	**21.47 ± 1.69**	6.00E-04	0.9659
	adj. p-value		0.7788	>0.9999	0.0426	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. Highlighted values correspond to cell viability< 50%. N/A, not available.

Cocos Nucifera (Coconut) Oil

Coconut oil was described to have a high affinity for hair proteins which may help prevent hair damage due to protein loss during the grooming process and ultraviolet (UV) exposure [68, 69]. In both DPCs and NHEKs, coconut oil led to substantial increases in cell viability, with the maximum increases occurring 24h after treatment **(Fig. 38, Tables 56 & 57)**.

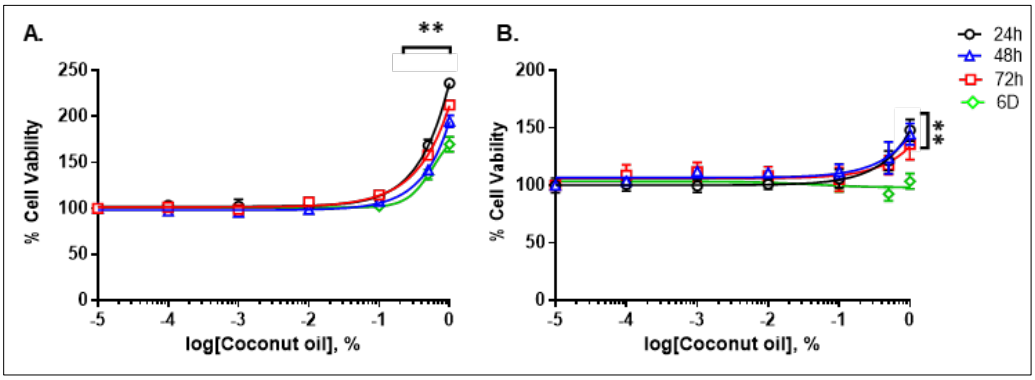


Figure 38. Cytotoxicity assessment of Coconut oil in DPCs (A) and NHEKs (B). Cells cultured in the presence of the test ingredient for 24, 48, 72 hours (h), and 6 days (D) were assessed by MTS assay. Dose response curves were obtained using GraphPad. **, $p \leq 0.0001$ (vs. nontreated control). n=5-6 replicates per concentration.

Table 56. Viability (%) of DPCs treated with Coconut oil

		0%	0.0001%	0.001%	0.01%	0.1%	0.5%	1%	IC50	R^2
24h	mean ± SD	100 ± 3.4	103.47 ± 3.89	102.32 ± 7.59	103.11 ± 2.33	113.47 ± 5.36	168.48 ± 6.54	236.25 ± 4.97	N/A	0.9901
	adj. p-value		>0.9999	>0.9999	>0.9999	<0.0001	<0.0001	<0.0001		
48h	mean ± SD	100 ± 2.08	97.14 ± 2.98	95.87 ± 3.93	98.91 ± 4.08	107.84 ± 3.26	141.74 ± 3.16	194.71 ± 6.42	N/A	0.9872
	adj. p-value		>0.9999	0.6192	>0.9999	0.0135	<0.0001	<0.0001		
72h	mean ± SD	100 ± 2.26	101.24 ± 4.04	98.76 ± 4.96	107.07 ± 4.45	114.77 ± 4.34	157.78 ± 3.23	212.3 ± 2.01	N/A	0.9898
	adj. p-value		>0.9999	>0.9999	0.0341	<0.0001	<0.0001	<0.0001		
6 D	mean ± SD	100 ± 3.03	101.27 ± 1.9	99.89 ± 3.5	102.58 ± 3.67	102.22 ± 2.31	136.8 ± 5.41	169.64 ± 8.22	N/A	0.9731
	adj. p-value		>0.9999	>0.9999	>0.9999	>0.9999	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. N/A, not available.

Table 57. Viability (%) of NHEKs treated with Coconut oil.

		0%	0.0001%	0.001%	0.01%	0.1%	0.5%	1%	IC50	R^2
24h	mean ± SD	100 ± 6.4	100.78 ± 5.68	99.51 ± 5.82	101.17 ± 4.66	105.92 ± 8.06	121.46 ± 8.46	148.16 ± 9.35	N/A	N/A
	adj. p-value		>0.9999	>0.9999	>0.9999	0.9303	<0.0001	<0.0001		
48h	mean ± SD	100 ± 3.21	103.7 ± 1.77	112.25 ± 3.79	111.04 ± 1.56	111.89 ± 6.6	123.93 ± 13.81	144.52 ± 9.48	N/A	N/A
	adj. p-value		>0.9999	0.0219	0.0516	0.0283	<0.0001	<0.0001		
72h	mean ± SD	100 ± 2.13	108.71 ± 9.18	111.8 ± 8.22	108.28 ± 7.97	105.19 ± 10.48	117.67 ± 8.31	135.7 ± 13.47	N/A	N/A
	adj. p-value		0.2239	0.0304	0.286	>0.9999	0.0002	<0.0001		
6 D	mean ± SD	100 ± 3.72	104.02 ± 4.44	105.92 ± 2.56	103.39 ± 2.69	99.84 ± 5.27	92.62 ± 5.96	103.58 ± 6.63	N/A	N/A
	adj. p-value		>0.9999	0.9333	>0.9999	>0.9999	0.4623	>0.9999		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. N/A, not available.

Dextran 40 and Dextran 70

Dextran 40 and Dextran 70 are polysaccharides used in skincare products to promote skin hydration. While no substantial effects were observed in DPCs **(Figs. 39A & 40A, Tables 58 & 60)**, both Dextran 40 **(Fig. 39B, Table 59)** and Dextran 70 **(Fig. 40B, Table 61)** caused >60% increases in NHEK viability at 24h when treated with the highest dose (5%).

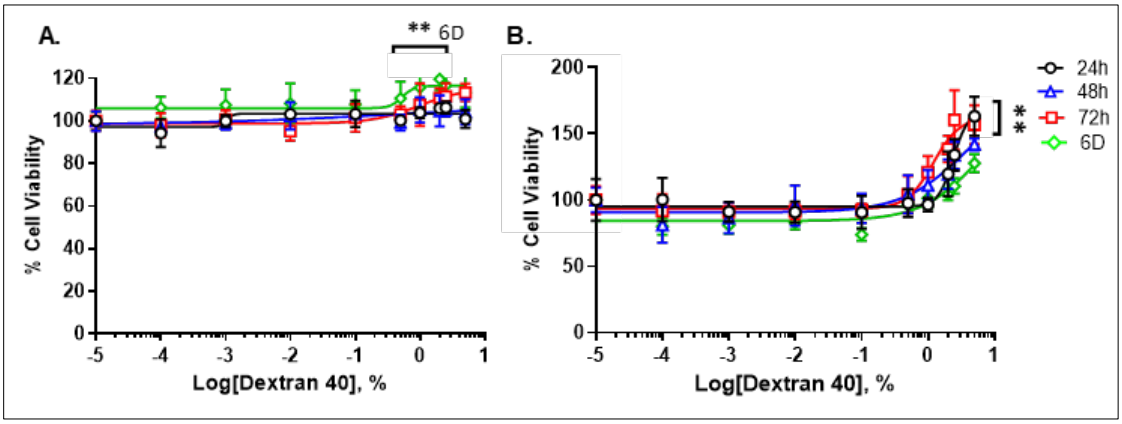


Figure 39. Cytotoxicity assessment of Dextran 40 in DPCs (A) and NHEKs (B). Cells cultured in the presence of the test ingredient for 24, 48, 72 hours (h), and 6 days (D) were assessed by MTS assay. Dose response curves were obtained using GraphPad. **, $p \leq 0.0001$ (vs. nontreated control). n=5-6 replicates per concentration.

Table 58. Viability (%) of DPCs treated with Dextran 40.

		0%	0.0001%	0.001%	0.01%	0.1%	0.5%	1%	2%	2.5%	5%	IC50	R^2
24h	**mean ± SD**	100 ± 2.46	94.24 ± 6.64	99.93 ± 2.8	103.14 ± 2.19	103.29 ± 6.24	100.3 ± 1.2	103.89 ± 2.53	105.98 ± 2.04	106.28 ± 2.96	100.9 ± 4.14	N/A	N/A
	adj. p-value		0.832	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	0.7249	0.5998	>0.9999		
48h	**mean ± SD**	100 ± 4.65	97.21 ± 1.93	98.93 ± 3.18	102.36 ± 6.37	103.36 ± 1.31	99.14 ± 3.66	105.14 ± 5.95	104.64 ± 7.31	105.36 ± 3.36	104.28 ± 5.96	N/A	N/A
	adj. p-value		>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999		
72h	**mean ± SD**	100 ± 4.62	98.07 ± 5.66	100.66 ± 4.32	95.15 ± 4.5	101.4 ± 6.51	103.06 ± 3.81	107.58 ± 10.02	110.77 ± 4.5	111.5 ± 6.22	113.23 ± 4.13	N/A	N/A
	adj. p-value		>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	0.2454	0.0166	0.0082	0.0013		
6 D	**mean ± SD**	100 ± 4.27	106.25 ± 5.07	107.47 ± 7.27	108.16 ± 9.45	107.76 ± 6.64	110.54 ± 7.93	115.69 ± 5.09	119.63 ± 5.49	116.56 ± 8.08	113.32 ± 7.06	N/A	N/A
	adj. p-value		0.61	0.2654	0.1576	0.2145	0.0207	<0.0001	<0.0001	<0.0001	0.0012		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. N/A, not available.

Table 59. Viability (%) of NHEKs treated with Dextran 40.

		0%	0.0001%	0.001%	0.01%	0.1%	0.5%	1%	2%	2.5%	5%	IC50	R^2
24h	**mean ± SD**	100 ± 15.72	100.47 ± 16.47	91.21 ± 7.39	90.89 ± 8.08	90.73 ± 12.34	97.8 ± 10.52	96.54 ± 5.25	119.79 ± 16.92	134.08 ± 11.9	163.29 ± 14.8	N/A	N/A
	adj. p-value		>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	0.022	<0.0001	<0.0001		
48h	**mean ± SD**	100 ± 9.17	81.26 ± 13.48	86.58 ± 11.48	95.86 ± 15.19	93.67 ± 11.07	104.47 ± 14.4	111.14 ± 11.63	119.59 ± 10.96	133.69 ± 10.2	141.87 ± 4.5	N/A	N/A
	adj. p-value		0.0365	0.3466	>0.9999	>0.9999	>0.9999	0.7673	0.0242	<0.0001	<0.0001		
72h	**mean ± SD**	100 ± 10.62	91.68 ± 12.95	91.25 ± 6.4	91.37 ± 5.82	92.36 ± 4.83	104.13 ± 14.28	120.9 ± 12.14	138.66 ± 10.08	160.42 ± 22.44	156.91 ± 14.62	N/A	N/A
	adj. p-value		>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	0.0125	<0.0001	<0.0001	<0.0001		
6 D	**mean ± SD**	100 ± 3.42	80.12 ± 6.44	80.93 ± 6.13	82.94 ± 5.06	73.9 ± 4.63	96.31 ± 6.26	101.5 ± 7.48	104.49 ± 4.59	110.66 ± 5.98	127.77 ± 6.78	N/A	N/A
	adj. p-value		0.0482	0.0675	0.1493	0.0025	>0.9999	>0.9999	>0.9999	>0.9999	0.001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. N/A, not available.

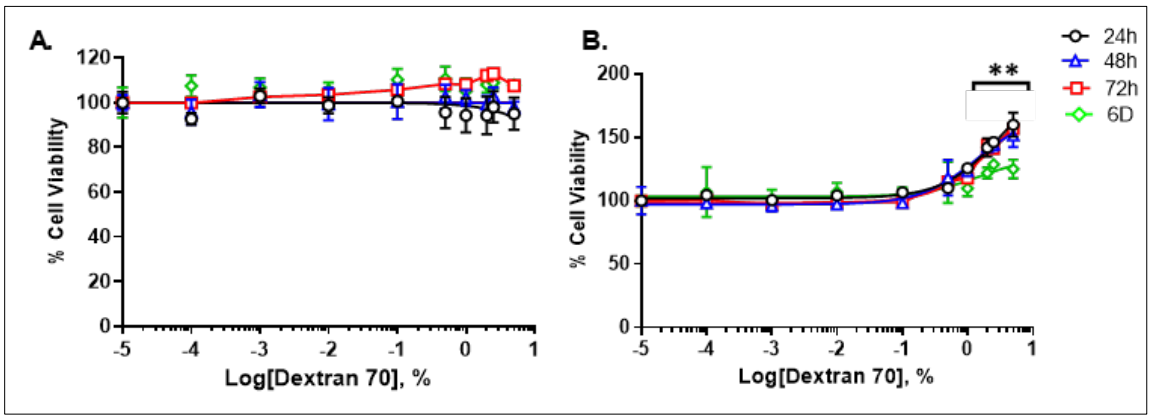


Figure 40. Cytotoxicity assessment of Dextran 70 in DPCs (A) and NHEKs (B). Cells cultured in the presence of the test ingredient for 24, 48, 72 hours (h), and 6 days (D) were assessed by MTS assay. Dose response curves were obtained using GraphPad. **, $p \leq 0.0001$ (vs. nontreated control). n=5-6 replicates per concentration.

Table 60. Viability (%) of DPCs treated with Dextran 70.

		0%	0.0001%	0.001%	0.01%	0.1%	0.5%	1%	2%	2.5%	5%	IC50	R^2
24h	mean ± SD	100 ± 4.88	92.78 ± 2.6	102.92 ± 3.34	98.77 ± 3.58	100.69 ± 2.21	95.62 ± 7.02	94.31 ± 7.59	94.39 ± 8.43	98 ± 7.07	95.01 ± 7.27	N/A	N/A
	adj. p-value		0.276	>0.9999	>0.9999	>0.9999	>0.9999	0.7917	0.8308	>0.9999	>0.9999		
48h	mean ± SD	100 ± 3.36	95.84 ± 5.55	103.55 ± 5.57	99.4 ± 7.22	100.45 ± 7.82	102.64 ± 5.27	102.19 ± 3.54	99.32 ± 3.85	102.87 ± 3.99	97.88 ± 2.68	N/A	N/A
	adj. p-value		>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999		
72h	mean ± SD	100 ± 6.36	99.86 ± 3.81	102.54 ± 2.59	103.52 ± 4	105.78 ± 5.93	108.38 ± 5.49	108.1 ± 5.31	112.19 ± 5.9	112.96 ± 4.66	107.54 ± 7.34	N/A	N/A
	adj. p-value		>0.9999	>0.9999	>0.9999	0.7472	0.1109	0.1396	0.0029	0.0012	0.2175		
6 D	mean ± SD	100 ± 6.78	107.48 ± 4.83	106.79 ± 4.18	105.54 ± 3.41	110.27 ± 4.84	110.62 ± 5.52	105.19 ± 5.7	108.05 ± 3.6	108.9 ± 2.76	107.88 ± 2.36	N/A	N/A
	adj. p-value		0.2281	0.3776	0.8694	0.0205	0.0147	>0.9999	0.146	0.0715	0.1674		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. N/A, not available.

Table 61. Viability (%) of NHEKs treated with Dextran 70.

		0%	0.0001%	0.001%	0.01%	0.1%	0.5%	1%	2%	2.5%	5%	IC50	R^2
24h	mean ± SD	100 ± 3.35	104.66 ± 3.26	100.54 ± 3.85	104.23 ± 2.78	106.72 ± 1.86	110.08 ± 3.5	125.68 ± 3.25	142.15 ± 7.16	146.48 ± 4.12	160.13 ± 9.56	N/A	N/A
	adj. p-value		>0.9999	>0.9999	>0.9999	>0.9999	0.2396	<0.0001	<0.0001	<0.0001	<0.0001		
48h	mean ± SD	100 ± 10.9	98.04 ± 2.75	96.16 ± 4.43	97.2 ± 3.49	98.32 ± 3.39	118.31 ± 14.14	123.34 ± 4.09	142.56 ± 3.79	144.44 ± 4.68	151.57 ± 9.14	N/A	N/A
	adj. p-value		>0.9999	>0.9999	>0.9999	>0.9999	0.0006	<0.0001	<0.0001	<0.0001	<0.0001		
72h	mean ± SD	100 ± 5.58	100.77 ± 3.42	96.98 ± 4.67	98.88 ± 3.68	98.74 ± 5.09	115.3 ± 18.76	117.68 ± 8.97	144.07 ± 4.12	141.12 ± 12.74	156.7 ± 5.76	N/A	N/A
	adj. p-value		>0.9999	>0.9999	>0.9999	>0.9999	0.0076	0.0011	<0.0001	<0.0001	<0.0001		
6 D	mean ± SD	100 ± 4.18	106.96 ± 19.73	100.58 ± 8.22	107 ± 7.35	103.17 ± 7.17	114.35 ± 16.2	109.67 ± 6.24	121.89 ± 4.4	128.81 ± 1.46	125.02 ± 7.34	N/A	N/A
	adj. p-value		>0.9999	>0.9999	>0.9999	>0.9999	0.0154	0.3	<0.0001	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. N/A, not available.

Guar Hydroxypropyltrimonium Chloride (Guar)

Guar is a water-soluble, quaternary ammonium derivative of guar gum. It is an antistatic agent and is typically used at 0.2 – 2% in hair care products. Guar also functions as a thickener, causing solution to gel at concentrations higher than 0.1%. Therefore, the highest concentration tested in in vitro studies was 0.1%. Despite this limitation, our data shows that DPCs were sensitive to Guar even at concentrations lower than 0.2%. We observed significant dose- and time-dependent decreases in cell viability at concentrations 0.08% and 0.1%. At day 6, cell viability was reduced to 49.26% and 21.24% in cells treated with 0.08% and 0.1% Guar, respectively **(Fig. 41A, Table 62)**. In contrast to the

substantial cytotoxicity observed in DPCs, Guar increased NHEK viability at 24h, followed by gradual decreases at later time points **(Fig. 41B, Table 63)**.

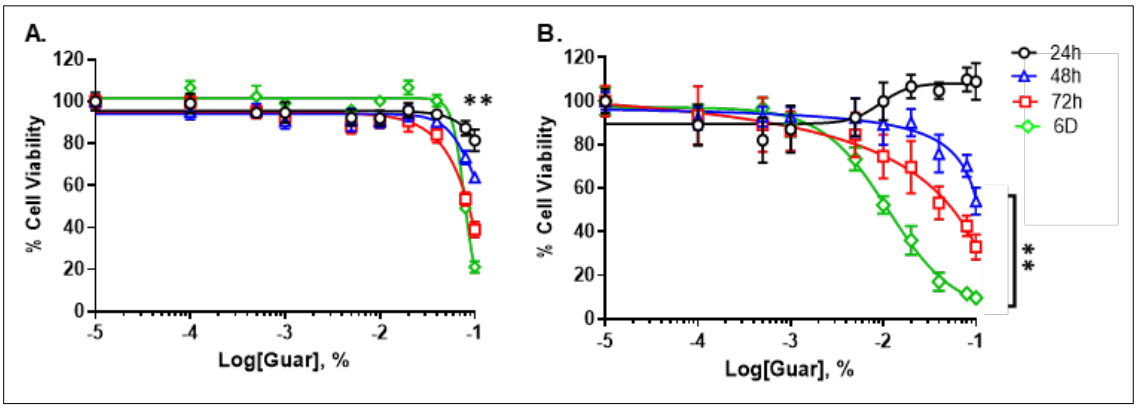


Figure 41. Cytotoxicity assessment of Guar in DPCs (A) and NHEKs (B). Cells cultured in the presence of the test ingredient for 24, 48, 72 hours (h), and 6 days (D) were assessed by MTS assay. Dose response curves were obtained using GraphPad. **, $p \leq 0.0001$ (vs. nontreated control). n=5-6 replicates per concentration.

Table 62. Viability (%) of DPCs treated with Guar.

		0%	0.0001%	0.0005%	0.001%	0.005%	0.01%	0.02%	0.04%	0.08%	0.1%	IC50	R^2
24h	mean ± SD	100 ± 4.35	99.1 ± 4.6	94.61 ± 0.73	94.76 ± 4.89	92.21 ± 3.85	92.06 ± 3.65	95.85 ± 3.38	93.96 ± 2.27	87.31 ± 3.52	81.52 ± 5.22	N/A	N/A
	adj. p-value		>0.9999	0.0581	0.0726	0.0009	0.0007	0.3206	0.021	<0.0001	<0.0001		
48h	mean ± SD	100 ± 1.7	95.2 ± 3.68	95.89 ± 3.24	90.7 ± 3.75	91.26 ± 5.36	90.83 ± 3.19	94.12 ± 3.54	90.44 ± 2.17	73.43 ± 3.21	63.83 ± 2.19	N/A	N/A
	adj. p-value		0.1361	0.335	<0.0001	0.0001	<0.0001	0.0272	<0.0001	<0.0001	<0.0001		
72h	mean ± SD	100 ± 3.04	99.74 ± 2.77	95.13 ± 3.16	91.45 ± 2.99	88.09 ± 3.3	90.25 ± 2.88	90.71 ± 5.16	84.38 ± 3.84	53.55 ± 3.12	**39.1 ± 3.75**	0.100	N/A
	adj. p-value		>0.9999	0.1235	0.0002	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
6 D	mean ± SD	100 ± 2.61	106.56 ± 3.24	102.49 ± 5.17	99.29 ± 1.98	96.01 ± 1.22	100.26 ± 1.16	106.67 ± 3.29	100.45 ± 2.75	**49.26 ± 1.74**	**21.24 ± 2.7**	0.078	0.9791
	adj. p-value		0.0087	>0.9999	>0.9999	0.3876	>0.9999	0.0073	>0.9999	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. Highlighted values correspond to cell viability< 50%. N/A, not available.

Table 63. Viability (%) of NHEKs treated with Guar.

		0%	0.0001%	0.0005%	0.001%	0.005%	0.01%	0.02%	0.04%	0.08%	0.1%	IC50	R^2
24h	mean ± SD	100 ± 5.57	89 ± 9.35	82.01 ± 10.47	87.06 ± 10.79	92.42 ± 8.61	99.85 ± 8.67	106.69 ± 5.43	104.76 ± 3.77	109.81 ± 5.45	108.92 ± 8.4	N/A	N/A
	adj. p-value		0.1007	0.0004	0.0265	0.7123	>0.9999	>0.9999	>0.9999	0.2109	0.3524		
48h	mean ± SD	100 ± 4.02	92.08 ± 5.22	92.86 ± 4.52	92.86 ± 4.46	92.47 ± 8.81	89.35 ± 9.53	90.03 ± 6.22	75.86 ± 8.64	70.28 ± 5.02	54.06 ± 6.11	N/A	N/A
	adj. p-value		0.6021	0.8856	0.8856	0.7328	0.1259	0.1921	<0.0001	<0.0001	<0.0001		
72h	mean ± SD	100 ± 7.05	93.41 ± 13.42	89.14 ± 12.54	86.28 ± 9.01	84.45 ± 10.17	74.51 ± 10.03	69.63 ± 12.01	53.22 ± 7.49	**42.67 ± 4.71**	**32.92 ± 5.74**	N/A	N/A
	adj. p-value		>0.9999	0.1108	0.0147	0.0034	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
6 D	mean ± SD	100 ± 6.44	94.68 ± 3.74	96.72 ± 4.68	89.05 ± 5.25	73.32 ± 5.34	52.26 ± 4.06	**36.02 ± 6.65**	**17.01 ± 4.25**	**11.58 ± 3**	**9.61 ± 1.1**	0.011	0.9821
	adj. p-value		>0.9999	>0.9999	0.1045	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. Highlighted values correspond to cell viability< 50%. N/A, not available.

Helianthus Annuus (Sunflower seed) Oil

Sunflower oil is widely used as the base oil in hair care products due to its anti-freezing and odorless properties at ambient temperature. Sunflower seed oil caused overall increases in cell viability in both DPCs and NHEKs, with the maximum increase occurring at 24h **(Fig.42, Tables 64 & 65)**.

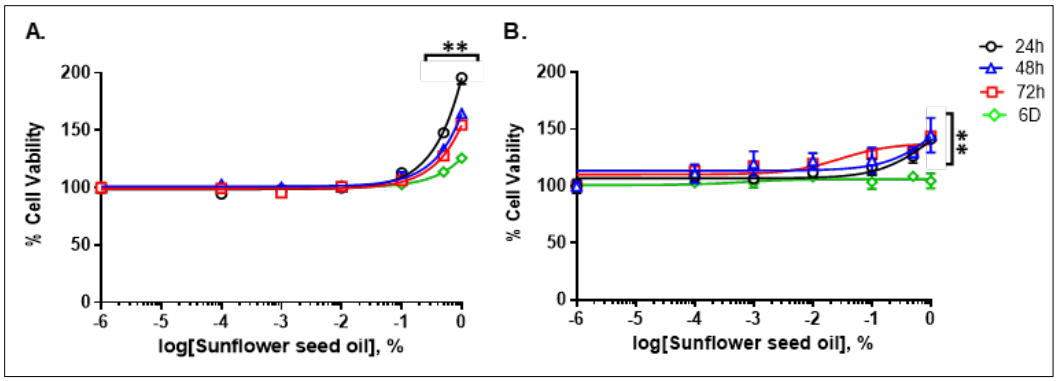


Figure 42. Cytotoxicity assessment of Sunflower seed oil in DPCs (A) and NHEKs (B). Cells cultured in the presence of the test ingredient for 24, 48, 72 hours (h), and 6 days (D) were assessed by MTS assay. Dose response curves were obtained using GraphPad. **, $p \leq 0.0001$ (vs. nontreated control). n=5-6 replicates per concentration.

Table 64. Viability (%) of DPCs treated with Sunflower seed oil

		0%	0.0001%	0.001%	0.01%	0.1%	0.5%	1%	IC50	R^2
24h	**mean ± SD**	100 ± 3.82	94.43 ± 2.79	98.73 ± 3.62	99.72 ± 3.73	113.12 ± 3.7	147.89 ± 3.77	196 ± 5.76	N/A	0.9857
	adj. p-value		0.0263	>0.9999	>0.9999	<0.0001	<0.0001	<0.0001		
48h	**mean ± SD**	100 ± 2.21	103 ± 2.98	100.5 ± 3.11	100.65 ± 1.78	109.19 ± 4.36	133.35 ± 3.03	164.6 ± 3.88	N/A	0.982
	adj. p-value		0.7304	>0.9999	>0.9999	<0.0001	<0.0001	<0.0001		
72h	**mean ± SD**	100 ± 4.47	99.46 ± 2.4	95.45 ± 2.97	100.95 ± 2.59	106.17 ± 2.83	127.73 ± 2.78	154.56 ± 3.74	N/A	0.9735
	adj. p-value		>0.9999	0.1159	>0.9999	0.01	<0.0001	<0.0001		
6 D	**mean ± SD**	100 ± 3.65	100.82 ± 3.13	98.56 ± 2.42	101.94 ± 1.65	102.69 ± 2.51	113.83 ± 2.53	125.74 ± 3.9	N/A	0.9146
	adj. p-value		>0.9999	>0.9999	>0.9999	0.9896	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. N/A, not available.

Table 65. Viability (%) of NHEKs treated with Sunflower seed oil.

		0%	0.0001%	0.001%	0.01%	0.1%	0.5%	1%	IC50	R^2
24h	**mean ± SD**	100 ± 6	108.44 ± 4.62	106.44 ± 3.04	112.15 ± 4.08	116.72 ± 6.87	126.23 ± 5.91	141.49 ± 3.89	N/A	N/A
	adj. p-value		0.4836	>0.9999	0.1092	0.004	<0.0001	<0.0001		
48h	**mean ± SD**	100 ± 4.2	110.89 ± 8.27	119.69 ± 10.81	122.16 ± 6.93	122.63 ± 11.29	129.42 ± 4.81	144.71 ± 15.18	N/A	N/A
	adj. p-value		0.0844	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
72h	**mean ± SD**	100 ± 4.04	113.52 ± 7.23	117.78 ± 8.69	119.71 ± 10.62	128.16 ± 8.74	131.05 ± 10.11	143.85 ± 13.91	N/A	N/A
	adj. p-value		0.0333	0.0018	0.0004	<0.0001	<0.0001	<0.0001		
6 D	**mean ± SD**	100 ± 5.23	103.13 ± 3.74	102.91 ± 4.11	108.2 ± 1.55	103.63 ± 6.05	108.57 ± 3.11	104.57 ± 6.46	N/A	N/A
	adj. p-value		>0.9999	>0.9999	0.3786	>0.9999	0.3125	>0.9999		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. N/A, not available.

Lavandula Angustifolia (Lavender) Oil

Lavender oil has been implicated in promoting hair growth, and despite a lack of clinical evidence, it has been anecdotally used to treat alopecia for more than 100 years. It is used in cosmetic products in amounts ranging from 1 to 5%. In a small preclinical study, topical application of lavender oil (3 - 5%) was implicated in a modest increase in the number of hair follicles in C57BL/6 mice [70]. However, in our study employing DPCs, lavender oil caused substantial dose- and time-dependent decreases in cell viability. At 0.5% lavender oil, the cell viability was reduced to 48.95% at 24h and 17.12% at day 6 **(Fig. 43A, Table 66)**. While NHEKs treated with lavender oil also showed dose- and time-dependent decreases in cell viability, a substantial decrease was apparent only at day 6, at which time the maximum reduction of 70% was observed at concentrations ≥0.5% **(Fig. 43B, Table 67)**.

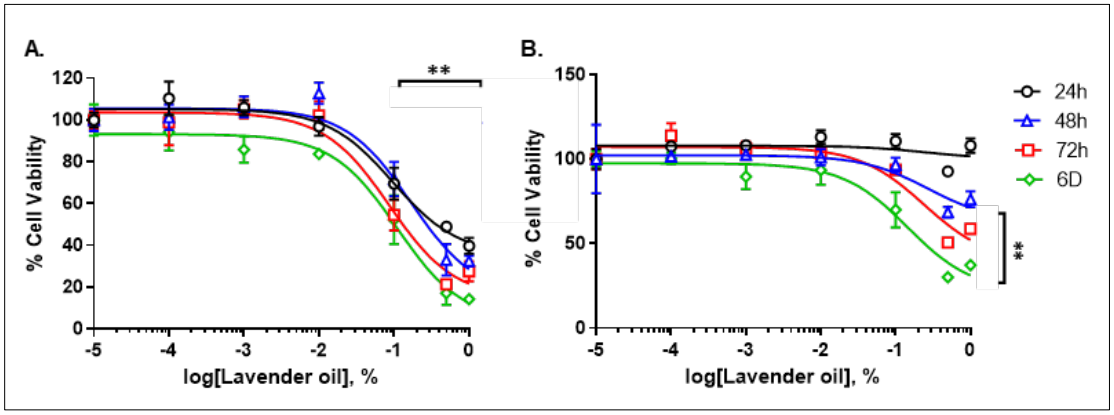


Figure 43. Cytotoxicity assessment of Lavender oil in DPCs (A) and NHEKs (B). Cells cultured in the presence of the test ingredient for 24, 48, 72 hours (h), and 6 days (D) were assessed by MTS assay. Dose response curves were obtained using GraphPad. **, $p \leq 0.0001$ (vs. nontreated control). n=5-6 replicates per concentration.

Table 66. Viability (%) of DPCs treated with Lavender oil.

		0%	0.0001%	0.001%	0.01%	0.1%	0.5%	1%	IC50	R^2
24h	**mean ± SD**	100 ± 3.42	110.33 ± 8.02	105.93 ± 3.62	96.99 ± 4.47	69.51 ± 7.6	**48.95 ± 0.79**	**39.73 ± 3.8**	0.097	0.9576
	adj. p-value		0.0273	0.5992	>0.9999	<0.0001	<0.0001	<0.0001		
48h	**mean ± SD**	100 ± 5.37	101.31 ± 5.99	106.37 ± 5	112.8 ± 5.18	71.72 ± 8.25	**33.06 ± 7.52**	**32.59 ± 2.58**	0.167	0.9407
	adj. p-value		>0.9999	0.551	0.0051	<0.0001	<0.0001	<0.0001		
72h	**mean ± SD**	100 ± 4.46	98.85 ± 10.88	105.16 ± 4.38	102.13 ± 6.8	54.52 ± 7.4	**21.37 ± 2.47**	**27.57 ± 4.68**	0.087	0.9515
	adj. p-value		>0.9999	0.9119	>0.9999	<0.0001	<0.0001	<0.0001		
6 D	**mean ± SD**	100 ± 7.54	94.13 ± 8.74	85.88 ± 6.36	83.74 ± 1.87	52.28 ± 11.78	**17.12 ± 5.59**	**14.22 ± 2.08**	0.109	0.9511
	adj. p-value		0.6188	0.0008	<0.0001	<0.0001	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. Highlighted values correspond to cell viability< 50%. N/A, not available.

Table 67. Viability (%) of NHEKs treated with Lavender oil.

		0%	0.0001%	0.001%	0.01%	0.1%	0.5%	1%	IC50	R^2
24h	**mean ± SD**	100 ± 5.96	107.46 ± 2.16	108.05 ± 2.32	112.88 ± 4.08	110.52 ± 4.35	92.75 ± 2.92	107.93 ± 4.29	N/A	N/A
	adj. p-value		0.2577	0.175	0.0034	0.0276	0.2947	0.1894		
48h	**mean ± SD**	100 ± 7.09	101.58 ± 2.5	102.53 ± 2.24	101.03 ± 4.81	96.6 ± 4.17	68.57 ± 3.15	76.17 ± 4.82	N/A	N/A
	adj. p-value		>0.9999	>0.9999	>0.9999	>0.9999	<0.0001	<0.0001		
72h	**mean ± SD**	100 ± 6.11	113.86 ± 7.32	106.16 ± 4.09	103.01 ± 3.31	93.51 ± 3	50.6 ± 1.63	58.63 ± 2.74	N/A	N/A
	adj. p-value		0.0007	0.4754	>0.9999	0.3864	<0.0001	<0.0001		
6 D	**mean ± SD**	100 ± 4.52	100.3 ± 3.25	89.61 ± 7.55	93.45 ± 8.7	69.96 ± 10.37	**30.08 ± 1.5**	**37.22 ± 2.06**	0.150	0.9234
	adj. p-value		>0.9999	0.0202	0.373	<0.0001	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. Highlighted values correspond to cell viability< 50%. N/A, not available.

Methylchloroisothiazolinone (5-Chloro-2-methyl-4-isothiazolin-3-one, MCI)
Methylisothiazolinone (MI)

MCI and MI are isothiazolinone derivatives that are commonly used as preservatives in skin Table 67. Viability (%) of NHEKs treated with Lavender oil. and hair care products such as shampoo, conditioner, soap, and body lotion. In addition to their potent biocidal activity, they are characterized as contact allergens. Since an epidemic of allergic contact dermatitis to the combination product (a 3:1 mixture of

MCI/MI, marketed as Kathon® CG) [71], the use of MCI/MI has been limited to 7.5 ppm in the US. However, because MI is assumed to be less allergenic than MCI, MI as a stand-alone preservative is permitted at higher concentrations (up to 100 ppm, 0.01%) [72]. In our study, the cytotoxicity of MCI and MI was assessed separately with concentrations ranging from 0.00001% to 0.1%. MCI was extremely toxic to DPCs, killing >90% of the cells in 24h at ≥ 0.00005% **(Fig. 44A, Table 68)**. MI was also toxic; however, the average IC50 of MCI was 10- to15-fold lower than that of MI (1.8E-05 vs. 2.3E-04%) in DPCs and longer exposure was required for MI to achieve a reduction of cell viability >90% **(Fig. 45A,Table 70)**. In contrast to the acute cytotoxicity observed in DPCs, >60% of NHEKs remained viable at 24h when exposed to >0.005% of MCI **(Fig. 44B,Table 69)** and MI caused no substantial reduction in NHEK viability within 48h **(Fig. 45B,Table 71)**.

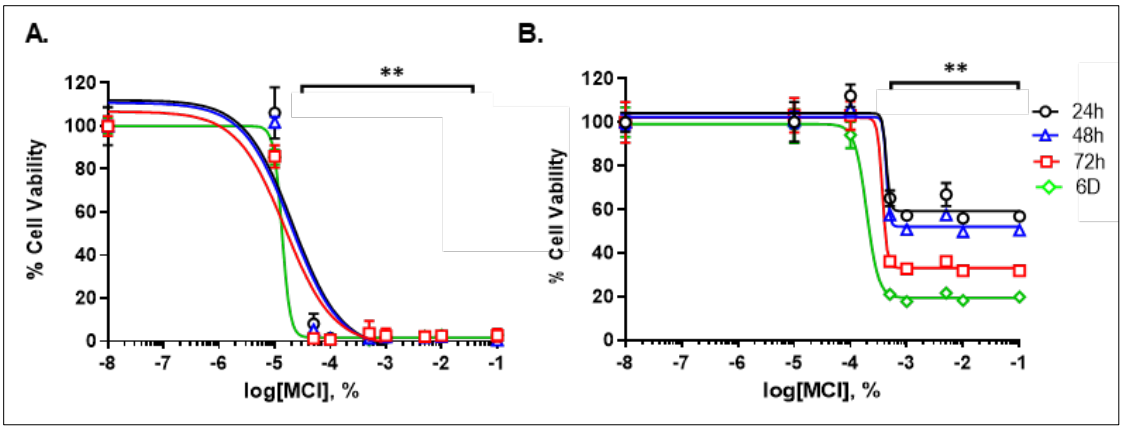


Figure 44. Cytotoxicity assessment of MCI in DPCs (A) and NHEKs (B). Cells cultured in the presence of the test ingredient for 24, 48, 72 hours (h), and 6 days (D) were assessed by MTS assay. Dose response curves were obtained using GraphPad. **, *p ≤ 0.0001* (vs. nontreated control). n=5-6 replicates per concentration.

Table 68. Viability (%) of DPCs treated with MCI.

		0%	0.00001%	0.00005%	0.0001%	0.0005%	0.001%	0.005%	0.01%	0.1%	IC50	R^2
24h	mean ± SD	100 ± 8.83	106.19 ± 11.92	8.19 ± 4.62	1.57 ± 0.89	1.2 ± 1.01	1.69 ± 0.54	2.12 ± 1.04	2.12 ± 0.96	0 ± 0.91	2.13E-05	0.9858
	adj. p-value		0.0138	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
48h	mean ± SD	100 ± 2.57	101.74 ± 2.55	5.38 ± 2.14	1.39 ± 0.49	1.34 ± 0.45	1.94 ± 0.7	1.89 ± 1.05	2.09 ± 0.98	0.5 ± 0.84	1.98E-05	0.9986
	adj. p-value		>0.9999	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
72h	mean ± SD	100 ± 4.63	85.86 ± 5.24	1.21 ± 1.97	0.76 ± 1.35	3.69 ± 5.78	2.55 ± 3.32	2.1 ± 1.01	2.6 ± 1.05	2.8 ± 2.92	1.54E-05	0.9925
	adj. p-value		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
6 D	mean ± SD	100 ± 3.71	85.58 ± 3.51	1.77 ± 1.66	0 ± 0.35	0.51 ± 0.34	1.58 ± 0.59	2.37 ± 0.68	2.81 ± 0.75	2.96 ± 0.87	1.55E-05	0.9974
	adj. p-value		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. Highlighted values correspond to cell viability< 50%. N/A, not available.

Table 69. Viability (%) of NHEKs treated with MCI.

		0%	0.00001%	0.0001%	0.0005%	0.001%	0.005%	0.01%	0.1%	IC50	R^2
4h	mean ± SD	100 ± 4.49	100.16 ± 9.15	112.19 ± 5.27	65.2 ± 3.67	57.26 ± 1.25	66.8 ± 5.3	55.97 ± 1.31	56.86 ± 1.79	0.0003	N/A
	adj. p-value		>0.9999	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
48h	mean ± SD	100 ± 3.66	101.1 ± 3.8	106.03 ± 2.12	57.48 ± 1.35	50.82 ± 1.41	57.4 ± 1.17	49.73 ± 1.64	50.43 ± 1.25	0.00026	N/A
	adj. p-value		>0.9999	0.1027	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
72h	mean ± SD	100 ± 9.33	103.27 ± 7.92	103.04 ± 6.51	36.18 ± 1.81	32.75 ± 0.93	36.14 ± 2.12	31.89 ± 1.04	31.89 ± 1.34	0.00022	N/A
	adj. p-value		>0.9999	>0.9999	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
6 D	mean ± SD	100 ± 6.85	98.27 ± 7.81	94.13 ± 5.99	21.09 ± 1.01	17.79 ± 0.3	21.65 ± 1.37	18.22 ± 0.89	19.9 ± 0.82	0.00019	0.9883
	adj. p-value		>0.9999	0.1227	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. Highlighted values correspond to cell viability< 50%. N/A, not available.

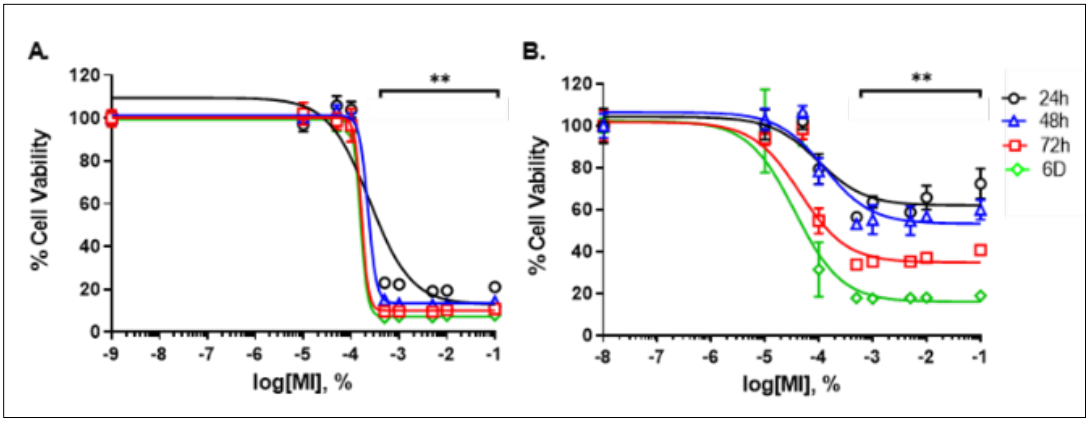


Figure 45. Cytotoxicity assessment of MI in DPCs (A) and NHEKs (B). Cells cultured in the presence of the test ingredient for 24, 48, 72 hours (h), and 6 days (D) were assessed by MTS assay. Dose response curves were obtained using GraphPad. **, $p \leq 0.0001$ (vs. nontreated control). n=5-6 replicates per concentration.

Table 70. Viability (%) of DPCs treated with MI.

		0%	0.00001%	0.00005%	0.0001%	0.0005%	0.001%	0.005%	0.01%	0.1%	IC50	R^2
24h	mean ± SD	100 ± 2.61	97.03 ± 3.44	105.81 ± 4.52	103.93 ± 3.69	23.07 ± 1.42	22.34 ± 0.96	19.13 ± 1.03	19.5 ± 1	21.19 ± 0.94	0.0003	0.993
	adj. p-value		0.5058	0.0026	0.1126	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
48h	mean ± SD	100 ± 2.55	100.43 ± 3.4	103.26 ± 2.95	99.57 ± 2.72	15.15 ± 0.29	13.5 ± 0.88	12.85 ± 0.86	13.19 ± 0.4	14.37 ± 1.04	0.0002	0.9977
	adj. p-value		>0.9999	0.3326	>0.9999	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
72h	mean ± SD	100 ± 3.88	102.06 ± 5.17	98.61 ± 3.98	96.69 ± 7.62	9.76 ± 0.66	9.72 ± 0.87	9.58 ± 1.12	10.43 ± 1.29	10.9 ± 1.73	0.0002	0.9939
	adj. p-value		>0.9999	>0.9999	0.3087	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
6 D	mean ± SD	100 ± 3.35	100.21 ± 3.8	98.11 ± 4.24	93.61 ± 1.48	6.76 ± 0.49	7.22 ± 0.76	7.08 ± 1.73	7.72 ± 1.63	7.8 ± 1.13	0.0002	0.9974
	adj. p-value		>0.9999	>0.9999	0.0007	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. Highlighted values correspond to cell viability< 50%. N/A, not available.

Table 71. Viability (%) of NHEKs treated with MI.

		0%	0.00001%	0.00005%	0.0001%	0.0005%	0.001%	0.005%	0.01%	0.1%	IC50	R^2
24h	mean ± SD	100 ± 8.23	100.41 ± 7.24	102.13 ± 3.82	79.36 ± 7.25	56.43 ± 1.88	63.55 ± 2.72	58.72 ± 1.45	65.77 ± 5.67	72.4 ± 7.21	N/A	N/A
	adj. p-value		>0.9999	>0.9999	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
48h	mean ± SD	100 ± 5.71	102.96 ± 5.09	106.54 ± 2.9	78.29 ± 6.31	53.13 ± 1	55.2 ± 6.92	54.78 ± 6.98	56.71 ± 1.58	59.94 ± 4.68	N/A	N/A
	adj. p-value		>0.9999	0.4593	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
72h	mean ± SD	100 ± 7.22	93.89 ± 3.17	98.31 ± 4.79	54.66 ± 6.03	33.7 ± 0.38	35.28 ± 0.79	35.23 ± 1.07	36.97 ± 1.38	40.65 ± 0.93	4.4E-05	0.9616
	adj. p-value		0.6054	>0.9999	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
6 D	mean ± SD	100 ± 3.33	97.43 ± 19.86	98.05 ± 7.66	31.47 ± 12.99	17.89 ± 2.48	17.55 ± 0.44	17.8 ± 1.35	18.08 ± 0.64	18.97 ± 1.3	3.6E-05	0.9833
	adj. p-value		>0.9999	>0.9999	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. Highlighted values correspond to cell viability< 50%. N/A, not available.

Pisum Sativum (Pea) Peptide

Rich in amino acids, especially lysine, pea peptide appears to have film-forming properties, thereby functioning as a moisturizer in skin and hair care products. It is also considered to reduce oxidative damage and promote scalp and hair follicle health. The typical use level is 1 – 5%. A significant reduction in cell viability was observed in DPCs treated with 2% Pea extract. Concentrations ≥ 2.5% was toxic, killing all cells in 24 hours **(Fig. 46A, Table 72)**. The cytotoxicity profiles between time points were almost identical, suggesting that the effects of Pea extract are predominantly dose-dependent. NHEKs were less sensitive to pea peptide, causing only modest decreases in cell viability. At 24h, >60% of NHEKs remained viable when exposed to ≥2.5% of pea peptide **(Fig. 46B, Table 73)**, while these doses were toxic to DPCs, killing >97% of DPCs.

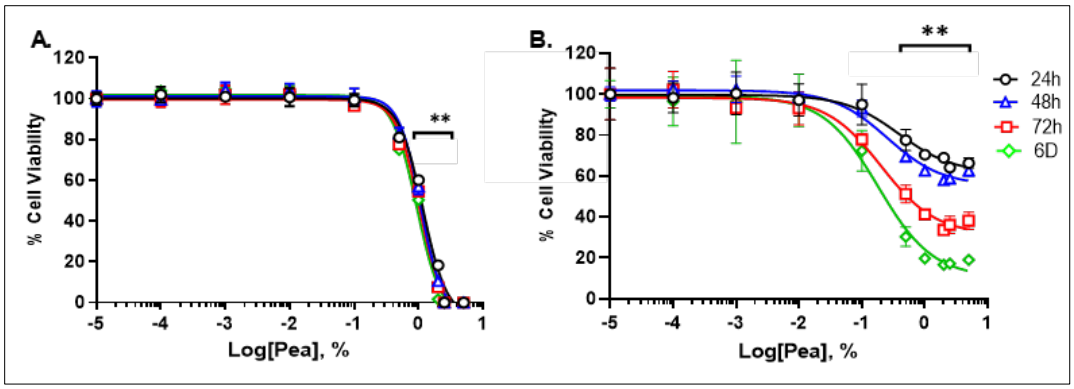


Figure 46. Cytotoxicity assessment of Pea extract in DPCs (A) and NHEKs (B). Cells cultured in the presence of the test ingredient for 24, 48, 72 hours (h), and 6 days (D) were assessed by MTS assay. Dose response curves were obtained using GraphPad. **, *p ≤ 0.0001* (vs. nontreated control). n=5-6 replicates per concentration.

Table 72. Viability (%) of DPCs treated with Pea extract.

		0%	0.0001%	0.001%	0.01%	0.1%	0.5%	1%	2%	2.5%	5%	IC50	R^2
24h	**mean ± SD**	100 ± 2.92	102.1 ± 3.89	101.07 ± 2.06	100.72 ± 4.48	99.33 ± 2.99	81.06 ± 2.46	60.12 ± 1.72	**18.35 ± 0.6**	**0 ± 0.37**	**0 ± 0.36**	1.162	0.9885
	adj. p-value		>0.9999	>0.9999	>0.9999	>0.9999	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
48h	**mean ± SD**	100 ± 3.81	99.54 ± 1.34	104.11 ± 3.88	103.6 ± 3.77	101.06 ± 4.03	82.95 ± 2.84	56.81 ± 3.73	**10.52 ± 0.68**	**0 ± 0.42**	**0 ± 1.59**	1.072	0.9906
	adj. p-value		>0.9999	0.0477	0.129	>0.9999	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
72h	**mean ± SD**	100 ± 3.25	99.31 ± 3.4	101.92 ± 4.52	102.37 ± 2.99	96.51 ± 1.77	77.7 ± 2.11	54.58 ± 2.34	**7.54 ± 1.38**	**0 ± 0.21**	**0 ± 0.27**	1.033	0.9888
	adj. p-value		>0.9999	>0.9999	0.9459	0.1598	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
6 D	**mean ± SD**	100 ± 3.02	103.86 ± 1.89	104.62 ± 2.39	103.29 ± 3.12	98.82 ± 2.32	74.92 ± 1.92	50.37 ± 1	**1.67 ± 0.42**	**0 ± 0.11**	**0.07 ± 0.2**	0.934	0.9883
	adj. p-value		0.0796	0.0161	0.2252	>0.9999	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. Highlighted values correspond to cell viability< 50%.

Table 73. Viability (%) of NHEKs treated with Pea extract.

		0%	0.0001%	0.001%	0.01%	0.1%	0.5%	1%	2%	2.5%	5%	IC50	R^2
24h	**mean ± SD**	100 ± 12.69	98.23 ± 7.3	100.35 ± 10.36	97.17 ± 7.94	94.93 ± 9.89	77.59 ± 5.1	70.4 ± 2.44	68.99 ± 1.66	64.15 ± 1.53	66.39 ± 2.55	N/A	N/A
	adj. p-value		>0.9999	>0.9999	>0.9999	>0.9999	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
48h	**mean ± SD**	100 ± 3.41	102.6 ± 3.83	102.39 ± 6.57	98.37 ± 2.55	93.06 ± 4.07	69.41 ± 1.14	62.69 ± 1.28	58.03 ± 1.9	58.79 ± 2.39	62.69 ± 2.72	N/A	0.9562
	adj. p-value		>0.9999	>0.9999	>0.9999	0.7503	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
72h	**mean ± SD**	100 ± 12.61	102.21 ± 8.92	93.21 ± 3.08	93.07 ± 8.07	77.86 ± 2.55	51.21 ± 4.35	**41.29 ± 1.81**	**33.64 ± 1.62**	**36.14 ± 4.24**	**38.14 ± 4.2**	0.203	0.9496
	adj. p-value		>0.9999	0.8143	0.7555	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
6 D	**mean ± SD**	100 ± 6.75	96.44 ± 11.93	96.29 ± 20.36	96.98 ± 12.84	72.3 ± 9.97	**30.33 ± 4.77**	**19.73 ± 0.82**	**16.63 ± 0.46**	**17.21 ± 0.17**	**19.03 ± 0.84**	0.181	0.9406
	adj. p-value		>0.9999	>0.9999	>0.9999	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were

generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. Highlighted values correspond to cell viability< 50%. N/A, not available.

Polysorbate 60

Polysorbate 60 is obtained by esterification of sorbitol with one or three molecules of a fatty acid including stearic, lauric, oleic, and palmitic acid. It is a nonionic, multi-purpose emulsifying agent and also functions as a thickener. The typical use level is 1–10% in cosmetic products; however, testing concentrations >2.5% was not feasible due to insolubility. Although decreases in cell viability were observed at concentrations >0.5%, its effect was not apparent at later time points (72h and 6D) **(Fig. 47A, Table 74)**. In contrast, polysorbate 60 at concentrations >0.01% was cytotoxic in NHEKs and reduced cell viability to 22.85% at day 6 **(Fig. 47B, Table 75)**.

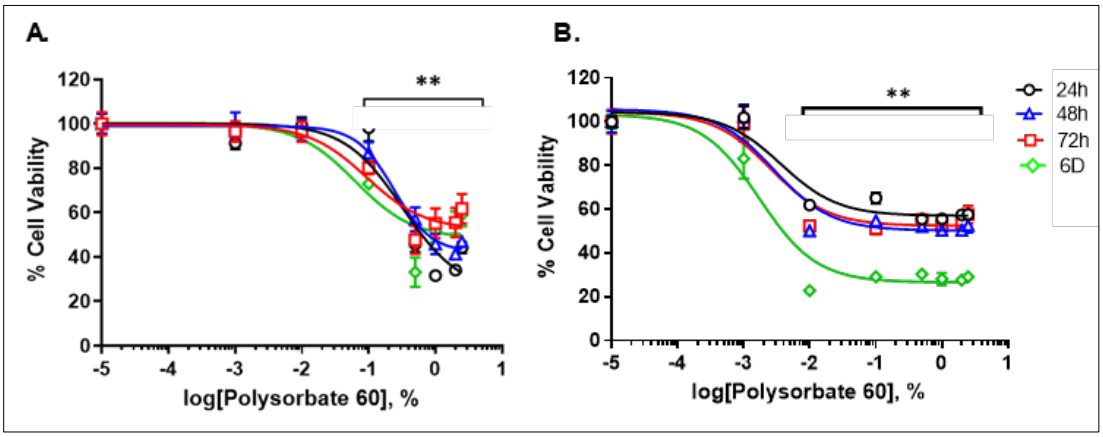


Figure 47. Cytotoxicity assessment of Polysorbate 60 in DPCs (A) and NHEKs (B). Cells cultured in the presence of the test ingredient for 24, 48, 72 hours (h), and 6 days (D) were assessed by MTS assay. Dose response curves were obtained using GraphPad. **, $p \leq 0.0001$ (vs. nontreated control). n=5-6 replicates per concentration.

Table 74. Viability (%) of DPCs treated with Polysorbate 60.

		0%	0.001%	0.01%	0.1%	0.5%	1%	2%	2.5%	IC50	R^2
24h	mean ± SD	100 ± 4.5	91.36 ± 2.85	99.13 ± 3.87	98.26 ± 6.05	**45.84 ± 3.54**	**31.65 ± 2.12**	**34.18 ± 1.75**	**44.25 ± 2.54**	0.322	N/A
	adj. p-value		0.0102	>0.9999	>0.9999	<0.0001	<0.0001	<0.0001	<0.0001		
48h	mean ± SD	100 ± 4.48	99.33 ± 5.77	97.03 ± 4.71	86.9 ± 4.84	56.93 ± 5.38	**45.83 ± 4.63**	**41.35 ± 2.36**	**46.93 ± 1.53**	0.276	0.9682
	adj. p-value		>0.9999	>0.9999	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
72h	mean ± SD	100 ± 5.28	96.6 ± 4.67	96.44 ± 4.66	80.18 ± 2.94	**47.67 ± 6.29**	55.13 ± 6.73	55.5 ± 6.61	61.77 ± 6.62	0.093	N/A
	adj. p-value		>0.9999	>0.9999	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
6 D	mean ± SD	100 ± 4.36	94.81 ± 3.25	98.28 ± 3.67	72.91 ± 2.02	**33.14 ± 6.57**	51.65 ± 4.87	55.4 ± 5.14	58.99 ± 5.12	0.063	N/A
	adj. p-value		0.3755	>0.9999	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. Highlighted values correspond to cell viability< 50%. N/A, not available.

Table 75. Viability (%) of NHEKs treated with Polysorbate 60.

		0%	0.001%	0.01%	0.1%	0.5%	1%	2%	2.5%	IC50	R^2
24h	mean ± SD	100 ± 2.64	102 ± 5.25	61.94 ± 1.45	65.12 ± 2.5	55.59 ± 1.69	55.68 ± 1.63	57.4 ± 1.57	57.67 ± 1.93	0.004	0.9166
	adj. p-value		>0.9999	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
48h	mean ± SD	100 ± 4.92	102.4 ± 5.37	50.19 ± 0.74	54.77 ± 1.17	52.07 ± 1.02	50.34 ± 2.29	50.41 ± 1.83	52.67 ± 3.37	N/A	N/A
	adj. p-value		>0.9999	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
72h	mean ± SD	100 ± 5.58	98.92 ± 8.02	52.41 ± 1.8	51.13 ± 2.69	55.38 ± 2.28	52.26 ± 2.56	53.59 ± 2.97	57.59 ± 4.01	N/A	N/A

		0%	0.001%	0.01%	0.1%	0.5%	1%	2%	2.5%	IC50	R^2
	adj. p-value		>0.9999	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
6 D	**mean ± SD**	100 ± 3.26	83.14 ± 9.17	**22.85 ± 1.19**	**29.17 ± 1.85**	**30.38 ± 1.5**	**28.17 ± 2.69**	**27.6 ± 1.84**	**29.17 ± 2.13**	0.002	0.9341
	adj. p-value		<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. Highlighted values correspond to cell viability< 50%. N/A, not available.

Rosmarinus Officinalis Leaf Extract (Rosemary Oleoresin, ROE)

Rosemary is a woody perennial that is grown widely around the world. Its fresh leaves and flowering buds contain rosmarinic acid, caffeic acid, chlorogenic acid, carnosic acid, rosmanol, carnosol, diterpenes as well as many other natural antioxidants [73]. Similar to minoxidil, rosemary has been shown to improve blood circulation and vascularity [73], and in C57BL/6 mice, the topical administration of rosemary leaf extract (2 mg/day/mouse) has been shown to improve hair regrowth after testosterone-induced interruption of hair growth [74]. According to CIR report, it is considered safe at concentrations ≤0.2% in cosmetics [75]. In DPCs, rosemary extract resulted in decreases in cell viability; however, this decrease was apparent only in cells treated with the highest concentration (0.01%) for > 48h, at which the maximum reduction of 85.94% was observed at day 6 **(Fig. 48A, Table 76)**. On the other hand, rosemary extract increased NHEK viability at early time points. The cell viability was maximal at 24h and then diminished gradually to 34.89% at day 6 in cells treated with the highest concentration (0.01%) **(Fig. 48B, Table 77)**.

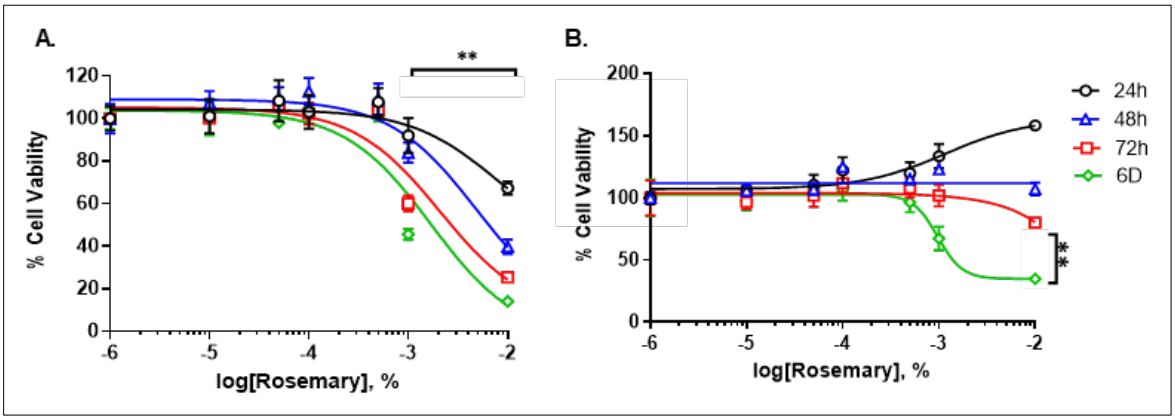


Figure 48. Cytotoxicity assessment of Rosemary leaf extract in DPCs (A) and NHEKs (B). Cells cultured in the presence of the test ingredient for 24, 48, 72 hours (h), and 6 days (D) were assessed by MTS assay. Dose response curves were obtained using GraphPad. **, *p ≤ 0.0001* (vs. nontreated control). n=5-6 replicates per concentration.

Table 76. Viability (%) of DPCs treated with Rosemary extract.

		0%	0.00001%	0.00005%	0.0001%	0.0005%	0.001%	0.005%	IC50	R^2
24h	**mean ± SD**	100 ± 5.79	101.09 ± 8	108.35 ± 9.66	103.01 ± 7.89	107.73 ± 6.53	91.96 ± 8.22	67.26 ± 3.11	N/A	N/A
	adj. p-value		>0.9999	0.0438	>0.9999	0.0768	0.0582	<0.0001		
48h	**mean ± SD**	100 ± 6.92	107.36 ± 5.53	109.37 ± 5.39	112.79 ± 6.3	109.18 ± 7.28	83.97 ± 4.7	**39.77 ± 3.44**	0.001	0.9331
	adj. p-value		0.106	0.0162	0.0003	0.0196	<0.0001	<0.0001		
72h	**mean ± SD**	100 ± 2.12	100.18 ± 1.48	106.13 ± 1.18	102.23 ± 5.16	103.44 ± 2.59	60.05 ± 3.7	**25.35 ± 1.61**	0.001	0.9877
	adj. p-value		>0.9999	0.2851	>0.9999	>0.9999	<0.0001	<0.0001		
6 D	**mean ± SD**	100 ± 4.99	99.96 ± 7.82	97.98 ± 2.25	101.58 ± 3.93	102.09 ± 3.42	**45.59 ± 2.57**	**14.06 ± 0.38**	0.0009	0.9859
	adj. p-value		>0.9999	>0.9999	>0.9999	>0.9999	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. Highlighted values correspond to cell viability< 50%. N/A, not available.

Table 77. Viability (%) of NHEKs treated with Rosemary extract.

		0%	0.00001%	0.00005%	0.0001%	0.0005%	0.001%	0.01%	IC50	R^2
24h	**mean ± SD**	100 ± 4.87	107 ± 4.15	110.77 ± 7.83	122.34 ± 10.59	120.05 ± 8.82	133.78 ± 9.77	158.55 ± 1.94	N/A	N/A
	adj. p-value		0.8553	0.1494	<0.0001	0.0003	<0.0001	<0.0001		
48h	**mean ± SD**	100 ± 2.93	106.08 ± 4.28	107.17 ± 4.47	125 ± 2.02	115.44 ± 2.15	123.11 ± 2.12	107.17 ± 5.23	N/A	N/A
	adj. p-value		>0.9999	0.7982	<0.0001	0.0087	<0.0001	0.7982		
72h	**mean ± SD**	100 ± 14.1	96.79 ± 5.51	101.86 ± 9.02	112.07 ± 7.86	108.14 ± 7.6	102.07 ± 8.73	80.21 ± 2.99	N/A	N/A
	adj. p-value		>0.9999	>0.9999	0.0367	0.3739	>0.9999	<0.0001		
6 D	**mean ± SD**	100 ± 14.72	97.71 ± 7.56	107.95 ± 4.12	106.16 ± 8.3	96.53 ± 8.05	67.34 ± 9.53	**34.89 ± 1.93**	N/A	N/A
	adj. p-value		>0.9999	0.4119	0.9435	>0.9999	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. Highlighted values correspond to cell viability< 50%. N/A, not available.

Solanum Lycopersicum (Tomato) Seed Oil

Tomato seed oil is used as an emollient and carrier in the cosmetic industry. It is implicated in improving scalp and hair health. Treating DPCs with Tomato seed oil led to significant dose-dependent increases in cell viability, with the maximum increase of 70.19% occurring 24h after treatment followed by gradual decreases, returning to the basal level at day 6 **(Fig. 49A, Table 78)**. In NHEKs, a 16.17% increase in cell viability was observed at 48h when treated with 1% Tomato seed oil **(Fig. 49B, Table 79)**.

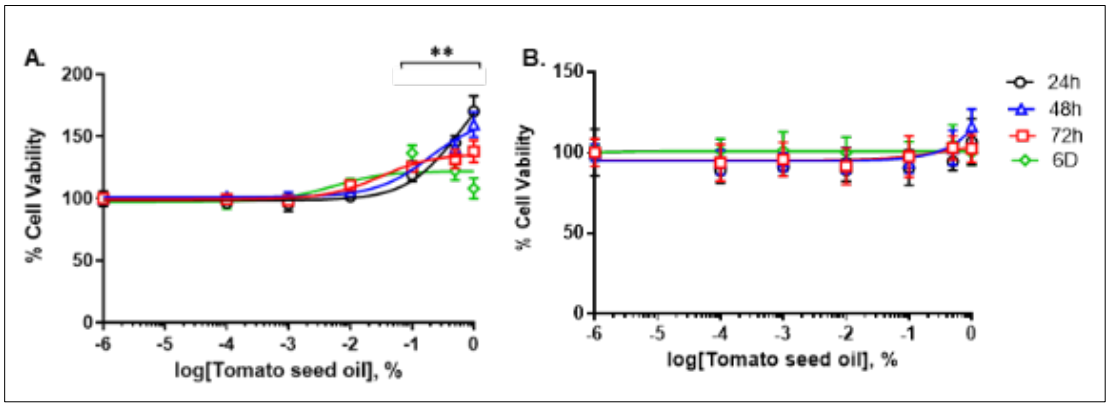


Figure 49. Cytotoxicity assessment of Tomato seed oil in DPCs (A) and NHEKs (B). Cells cultured in the presence of the test ingredient for 24, 48, 72 hours (h), and 6 days (D) were assessed by MTS assay. Dose response curves were obtained using GraphPad. **, $p \leq 0.0001$ (vs. nontreated control). n=5-6 replicates per concentration.

Table 78. Viability (%) of DPCs treated with Tomato seed oil.

		0%	0.0001%	0.001%	0.01%	0.1%	0.5%	1%	IC50	R^2
24h	**mean ± SD**	100 ± 6.16	96.39 ± 3.77	95.65 ± 6.09	101.62 ± 2.66	119.08 ± 5.09	144.86 ± 5.77	170.19 ± 12.38	N/A	0.9393
	adj. p-value		0.7648	0.6053	0.9929	<0.0001	<0.0001	<0.0001		
48h	**mean ± SD**	100 ± 2.41	101.44 ± 4.09	100.94 ± 4.26	105.45 ± 2.19	126.44 ± 2.44	140.41 ± 6.89	159.57 ± 10.21	N/A	0.9274
	adj. p-value		0.9955	0.9996	0.377	<0.0001	<0.0001	<0.0001		

		0%	0.0001%	0.001%	0.01%	0.1%	0.5%	1%	IC50	R^2
72h	**mean ± SD**	100 ± 2.89	99.49 ± 3.45	98.38 ± 2.68	110.55 ± 3.23	125.61 ± 2.98	131.7 ± 7.51	137.99 ± 8.84	N/A	0.9027
	adj. p-value		0.9998	0.9928	0.0097	<0.0001	<0.0001	<0.0001		
6 D	**mean ± SD**	100 ± 1.34	96.51 ± 5.01	100.07 ± 4.68	111.57 ± 4.38	136.64 ± 6.14	122.27 ± 7.57	108.11 ± 8.37	N/A	N/A
	adj. p-value		0.7883	>0.9999	0.0036	<0.0001	<0.0001	0.0738		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. N/A, not available.

Table 79. Viability (%) of NHEKs treated with Tomato seed oil.

		0%	0.0001%	0.001%	0.01%	0.1%	0.5%	1%	IC50	R^2
24h	**mean ± SD**	100 ± 14.56	88.83 ± 7.62	90.86 ± 2.31	88.96 ± 6.96	90.61 ± 10.89	94.54 ± 5.65	106.73 ± 14.25	N/A	N/A
	adj. p-value		0.2476	0.565	0.2616	0.5127	>0.9999	>0.9999		
48h	**mean ± SD**	100 ± 5.33	93.95 ± 7.69	94.22 ± 5.68	94.94 ± 8.23	93.58 ± 6.32	102.89 ± 10.8	116.17 ± 10.86	N/A	N/A
	adj. p-value		>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	0.0202		
72h	**mean ± SD**	100 ± 8.49	93.64 ± 11.44	95.82 ± 10.56	91.55 ± 11.39	97.59 ± 12.73	102.9 ± 7.24	102.41 ± 8.52	N/A	N/A
	adj. p-value		>0.9999	>0.9999	0.7282	>0.9999	>0.9999	>0.9999		
6 D	**mean ± SD**	100 ± 4.78	101.12 ± 7.49	101.4 ± 11.4	99.86 ± 9.82	97.44 ± 9.53	105.13 ± 12.03	100 ± 7.59	N/A	N/A
	adj. p-value		>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. N/A, not available.

Trifolium Pratense (Red Clover) Blossom Extract

Despite lacking sufficient scientific evidence regarding its efficacy, red clover extract is used for many health conditions including high cholesterol, osteoporosis, menopause syndromes, hair loss, and cancer. It is possibly safe for most people when used in medicinal amounts. However, it can cause skin rash, muscle pain, and headaches. Reproductive failure has also been noted in animals, possibly due to isoflavones present in the extract. The recommended use level of red clover extract is 5–10% in skincare products. In DPCs, dose- and time-dependent reduction in cell viability was apparent at concentrations ≥8%**.** At 8%, red clover extract decreased the viability of DPCs to 71% and 33.82% at 24h and 72h, respectively **(Fig. 50A, Table 80)**, while increasing the viability of NHEKs by 40.87% at 24h **(Fig. 50B, Table 81).**

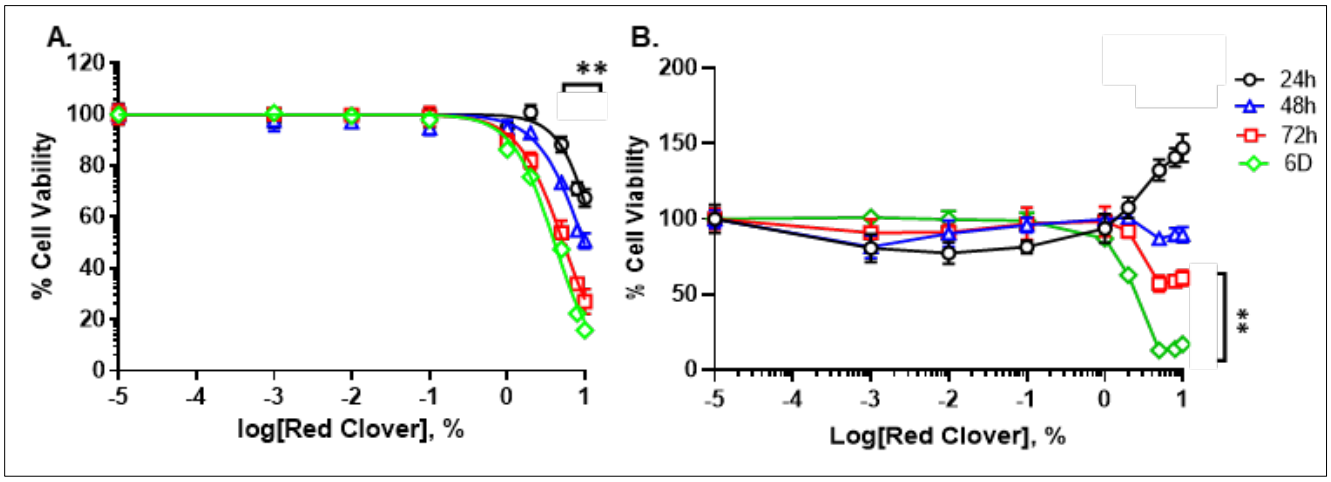


Figure 50. Cytotoxicity assessment of Red clover extract in DPCs (A) and NHEKs (B). Cells cultured in the presence of the test ingredient for 24, 48, 72 hours (h), and 6 days (D) were assessed

by MTS assay. Dose response curves were obtained using GraphPad. **, $p \leq 0.0001$ (vs. nontreated control). n=5-6 replicates per concentration.

Table 80. Viability (%) of DPCs treated with Red clover flower extract.

		0%	0.001%	0.01%	0.1%	1%	2%	5%	8%	10%	IC50	R^2
24h	**mean ± SD**	100 ± 4.09	97.52 ± 2.74	99.02 ± 2.13	98.63 ± 3.86	95.05 ± 2.03	100.85 ± 2.85	88.27 ± 3.04	71 ± 2.54	67.48 ± 3.46	14.090	0.9114
	adj. p-value		>0.9999	>0.9999	>0.9999	0.0292	>0.9999	<0.0001	<0.0001	<0.0001		
48h	**mean ± SD**	100 ± 3.73	97.67 ± 4.28	97.06 ± 2.29	94.66 ± 2.67	94.54 ± 3.13	92.78 ± 2.45	73.35 ± 2.32	54.8 ± 1.96	50.64 ± 2.97	9.751	0.96
	adj. p-value		>0.9999	0.6566	0.0139	0.0111	0.0002	<0.0001	<0.0001	<0.0001		
72h	**mean ± SD**	100 ± 3.86	100.1 ± 2.01	99.77 ± 2.08	99.11 ± 3.87	89.03 ± 3.13	81.85 ± 3.21	53.78 ± 4.83	**33.82 ± 1.68**	**26.96 ± 4.88**	5.259	0.9852
	adj. p-value		>0.9999	>0.9999	>0.9999	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		
6 D	**mean ± SD**	100 ± 2.66	100.58 ± 3.02	99.42 ± 0.9	97.84 ± 2.44	86.29 ± 1.48	75.51 ± 1.82	**47.28 ± 2.2**	**22.22 ± 0.87**	**15.79 ± 0.96**	4.041	0.9894
	adj. p-value		>0.9999	>0.9999	>0.9999	<0.0001	<0.0001	<0.0001	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. Highlighted values correspond to cell viability< 50%.

Table 81. Viability (%) of NHEKs treated with Red clover flower extract.

		0%	0.001%	0.01%	0.1%	1%	2%	5%	8%	10%	IC50	R^2
24h	**mean ± SD**	100 ± 9.22	80.8 ± 9.36	77.46 ± 6.96	81.55 ± 4.24	93.81 ± 9.51	107.55 ± 6.83	132.45 ± 6.98	140.87 ± 5.98	147.06 ± 8.97	N/A	N/A
	adj. p-value		<0.0001	<0.0001	<0.0001	0.6674	0.2804	<0.0001	<0.0001	<0.0001		
48h	**mean ± SD**	100 ± 5.87	81.62 ± 7.47	90.39 ± 8.71	96 ± 5.03	99.91 ± 3.52	101.11 ± 3.04	87.07 ± 3.99	89.79 ± 4.3	89.53 ± 5.1	N/A	N/A
	adj. p-value		<0.0001	0.0602	>0.9999	>0.9999	>0.9999	0.0029	0.0367	0.0295		
72h	**mean ± SD**	100 ± 7.05	90.89 ± 8.82	91.25 ± 4.82	96.86 ± 10.72	98.4 ± 9.86	92.06 ± 4.51	57.26 ± 5.39	58.74 ± 4.08	60.89 ± 5.06	N/A	N/A
	adj. p-value		0.0897	0.1191	>0.9999	>0.9999	0.214	<0.0001	<0.0001	<0.0001		
6 D	**mean ± SD**	100 ± 2.72	101.14 ± 3.66	99.74 ± 5.45	98.93 ± 5.1	87.17 ± 3.69	62.9 ± 2.17	**13.04 ± 0.26**	**13.89 ± 0.37**	**17.06 ± 1.43**	2.141	0.9895
	adj. p-value		>0.9999	>0.9999	>0.9999	0.0032	<0.0001	<0.0001	<0.0001	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. $R^2 > 0.9$ is indicated. Highlighted values correspond to cell viability< 50%. N/A, not available.

Vegetable Oil (Olus oil)

Olus oil has similar properties as petrolatum and is typically used up to 10% in formulating creams and lotions. Due to the insolubility of Olus oil, we were not able to test concentrations higher than 0.5%. At 0.5%, we observed a slight decrease in cell viability in DPCs **(Fig. 51A, Table 82)**. In NHEKs, although not substantial, slight increases in viability were observed when treated at concentrations >0.1% **(Fig. 51B, Table 83)**.

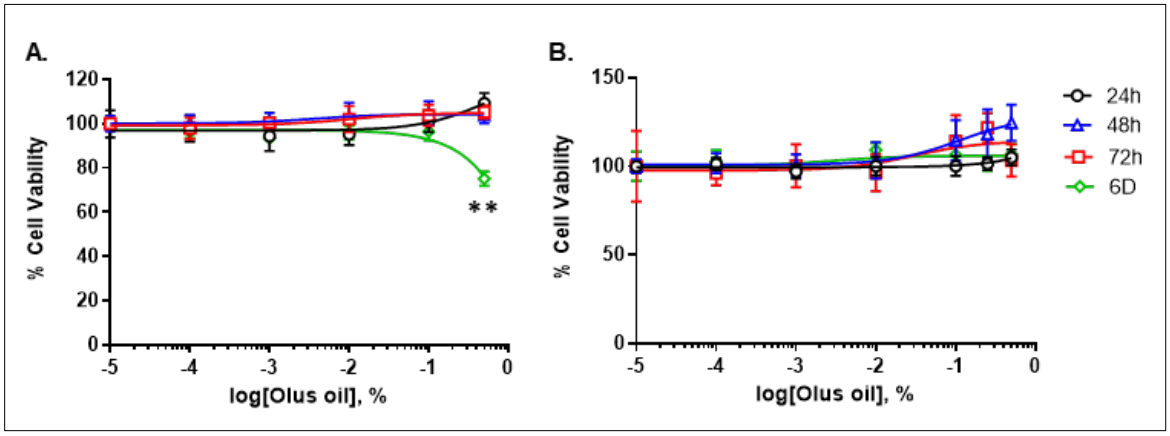


Figure 51. Cytotoxicity assessment of Olus oil in DPCs (A) and NHEKs (B). Cells cultured in the presence of the test ingredient for 24, 48, 72 hours (h), and 6 days (D) were assessed by MTS assay. Dose response curves were obtained using GraphPad. **, $p \leq 0.0001$ (vs. nontreated control). n=5-6 replicates per concentration.

Table 82. Viability (%) of DPCs treated with Olus oil.

		0%	0.0001%	0.001%	0.01%	0.1%	0.5%	IC50	R^2
24h	**mean ± SD**	100 ± 6.16	97.87 ± 5.87	94.47 ± 6.85	95.26 ± 4.93	101.03 ± 4.8	109.25 ± 4.59	N/A	N/A
	adj. p-value		>0.9999	0.161	0.3285	>0.9999	0.0021		
48h	**mean ± SD**	100 ± 3.6	100.13 ± 3.72	101.22 ± 3.62	102.96 ± 6.54	104.63 ± 5.42	103.99 ± 3.88	N/A	N/A
	adj. p-value		>0.9999	>0.9999	>0.9999	0.3614	0.6052		
72h	**mean ± SD**	100 ± 2.27	97.96 ± 4.86	100.45 ± 1.77	102.04 ± 5.92	103.75 ± 4.78	105.28 ± 2.94	N/A	N/A
	adj. p-value		>0.9999	>0.9999	>0.9999	0.7203	0.2036		
6 D	**mean ± SD**	100 ± 2.35	96.14 ± 2.75	93.42 ± 2.35	95.8 ± 3.12	96.41 ± 4.17	75.16 ± 3.19	N/A	N/A
	adj. p-value		0.6678	0.0559	0.5143	0.8104	<0.0001		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. N/A, not available.

Table 83. Viability (%) of NHEKs treated with Olus oil.

		0%	0.0001%	0.001%	0.01%	0.1%	0.25%	0.5%	IC50	R^2
24h	**mean ± SD**	100 ± 2.49	101.3 ± 3.57	97.06 ± 4.03	100.09 ± 5.22	100.09 ± 5.57	101.38 ± 3.69	104.93 ± 4.48	N/A	N/A
	adj. p-value		>0.9999	>0.9999	>0.9999	>0.9999	>0.9999	>0.9999		
48h	**mean ± SD**	100 ± 3.97	101.69 ± 5.58	100.44 ± 6.2	103.32 ± 10.26	114.74 ± 11.45	118.13 ± 14.01	124.61 ± 10.15	N/A	N/A
	adj. p-value		>0.9999	>0.9999	>0.9999	0.0202	0.0021	<0.0001		
72h	**mean ± SD**	100 ± 10.53	96.01 ± 6.99	100.17 ± 12.12	96.36 ± 10.47	114.06 ± 14.94	121.69 ± 8.22	103.34 ± 9.1	N/A	N/A
	adj. p-value		>0.9999	>0.9999	>0.9999	0.0305	0.0001	>0.9999		
6 D	**mean ± SD**	100 ± 8.33	103 ± 6.25	97.31 ± 3.04	109.18 ± 3.93	106.61 ± 5.62	101.53 ± 4.23	106.76 ± 5.96	N/A	N/A
	adj. p-value		>0.9999	>0.9999	0.3918	>0.9999	>0.9999	>0.9999		

Cell viability (% relative to nontreated control), IC50, and R^2 were obtained using GraphPad. Data are represented as the mean ± SD; n=5-6 replicates per concentration. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparison test. N/A, not available.

5.3.5 Summary (Table 84)

- All test products demonstrated significant cytotoxicity within 24h in DPCs; WEN product showed cytotoxicity only at the highest concentration (0.1%) at 24 h.
- In NHEKs, the cytotoxic effects of Monat, Aquaphor, and DevaCurl were apparent from 48h.
- Compared to the other test products, Monat was the most cytotoxic in both DPCs and NHEKs. WEN was the least cytotoxic in DPCs and non-cytotoxic in NHEKs.
- Of the 20 test ingredients, 11 test ingredients demonstrated varying degrees of cytotoxicity in DPCs within 72h. Of these, seven induced acute cytotoxicity in DPCs, decreasing cell viability by more than 50% within 24h. These included MCI, MI, CAPB, Lavender oil, Polysorbate 60, CATC, and Pea extract.
- Of all ingredients tested, MCI and MI were the most cytotoxic, with IC50 values of 2.13E-05% and 0.0003%, respectively, at 24h, followed by Rosemary extract, which had an IC50 value of 0.001% at 48h.
- Coconut oil, Dextrans 40 and 70, Lemon peel oil, Pequi oil, Sunflower seed oil, Tomato seed oil, and Vegetable oil were noncytotoxic and produced overall increases in cell viability in both DPCs and NHEKs.

Table 84. Summary of the cell viability assessment.

1. Test Products

Cytotoxicity Status*	DPCs: Test Product	DPCs: IC50 (%)	NEHKs: Test Product	NEHKs: IC50 (%)
Cytotoxic at 24h	Monat Aquaphor DevaCurl WEN	0.035 0.067 0.079 0.408		
Cytotoxic at 48h			Monat Aquaphor DevaCurl	0.001 0.003 0.005
Non-cytotoxic at 48h			WEN	N/A

2. Test Ingredients

Cytotoxicity Status*	DPCs: Test Ingredient	DPCs: IC50 (%)	NEHKs: Test Ingredient	NEHKs: IC50 (%)
Cytotoxic at 24h	MCI MI CAPB Lavender oil Polysorbate 60 CATC Pea extract	2.13E-05 0.0003 0.067 0.097 0.322 0.573 1.162		
Cytotoxic at 48h	Rosemary extract	0.001	MCI CATC	2.60E-04 0.134
Cytotoxic at 72h	Guar Calendula extract Red clover extract	0.1 0.19 5.26	MI CAPB Pea extract	4.4E-05 9.00E-04 0.203
Cytotoxic at Day 6	Acetyl tetrapeptide-3	0.864	Polysorbate 60 Guar Acetyl tetrapeptide-3 Lavender oil Calendula extract Red clover extract Rosemary extract	0.002 0.01 0.086 0.15 0.237 2.141 N/A
Non-cytotoxic	Coconut oil Dextran 40 Dextran 70 Lemon peel oil Pequi oil Sunflower seed oil Tomato seed oil Vegetable (Olus) oil		Coconut oil Dextran 40 Dextran 70 Lemon peel oil Pequi oil Sunflower seed oil Tomato seed oil Vegetable (Olus) oil	

*, Cytotoxicity was defined as < 50% cell viability at a given experimental condition.
N/A, Not available.

5.4 Apoptosis Assessment

Apoptosis (programmed/active cell death) and necrosis (passive cell death) constitute the major types of cell demise, and the identification of the type of demise can provide valuable information about the cell types and stimuli studied, as well as the dose- and time-dependency of the cytotoxic action [7]. In the present study, in vitro apoptosis/necrosis assays were conducted to identify the potential mechanisms of cytotoxicity.

5.4.1 Selection of Assay Platform

Apoptosis is characterized by defined morphological changes (e.g., cell shrinkage, chromatin condensation, plasma membrane blebbing, apoptotic body formation), and eventual phagocytosis of the dying cells by neighboring cells, while the organelle integrity is maintained within an intact plasma membrane. It is an energy-dependent process that requires active metabolism and involves a complex cascade of biochemical events including the activation of a group of caspases (cysteine proteases), cytochrome c release from mitochondria, externalization of phosphatidylserine (PS) on the cell membrane, poly (ADPribose) polymerase (PARP) cleavage, and internucleosomal DNA fragmentation. In contrast to apoptosis, cell death by necrosis is an energy-independent process that involves cytoplasmic swelling, disruption of the plasma membrane, and the subsequent release of intracellular content into the extracellular environment, triggering inflammatory responses in the surrounding tissue. Both cell death mechanisms can occur simultaneously in different subpopulations of cells and – in most cases – are dependent on a wide variety of factors (e.g., drug concentration, exposure time, cell type, etc.).

We evaluated three in vitro assay platforms in DPCs: 1) the Click-iT TUNEL assay (Thermo Fisher), a modified TUNEL assay that incorporates an alkyne-modified dUTP at the 3'-OH ends of fragmented DNA and enables microscope imaging of apoptotic cells; 2) the Caspase-Glo 3/7 assay system (Promega) which detects Caspase 3/7 activity using a proluminescent DEVD-aminoluciferin substrate; and 3) the RealTime-Glo Annexin V Apoptosis and Necrosis Assay (Promega) which measures the real-time PS exposure on the outer leaflets of cell membranes during the apoptotic process.

The TUNEL assay involves imaging analysis; hence, the quantitation of apoptotic cells was cumbersome and not robust. Both the Caspase 3/7 and Annexin V Apoptosis and Necrosis assays detect luminescence-based apoptosis; however, the caspase 3/7 assay was less sensitive than the annexin V and necrosis assay, and further optimization was needed. For these reasons, we chose the annexin V apoptosis and necrosis assay, which enables continuous monitoring of apoptosis and fluorescence-based detection of necrosis that depends on the loss of cell membrane integrity.

5.4.2 Assay Principle

The assay used in this study allows the measurements of luminescent signals generated by apoptotic cells and fluorescent signals generated by necrotic cells.

Phospholipids of the cell membrane are asymmetrically distributed on the inner and outer leaflets of the lipid bilayers. PS is located on the inner leaflets, and other phospholipids (e.g., phosphatidylcholine, sphingomyelin) are located on the external leaflets. During apoptosis, PS externalizes to the outside surfaces of cell membranes. The cell membrane is still intact during this process. Because PS has a high affinity for the anticoagulant protein annexin V, the binding of PS to various annexin V conjugates is commonly used to detect apoptosis.

The luminescence-based apoptosis assay utilized two annexin fusion V proteins containing complementary subunits of luciferase. In cells undergoing apoptosis, annexin V–luciferase conjugates bind to the externalized PS, producing luminescent signals **(Table 85)**.

The fluorescence-based necrosis assay measured exposed DNA that occurs because of loss of cell membrane integrity and subsequent cell lysis. The assay system uses a cell-impermeable, profluorescent DNA dye that is excluded from viable cells but preferentially stains the dead cells' DNA. When the dye binds to DNA, the dye's fluorescent properties are substantially enhanced. Therefore, the fluorescent signal produced by the dye binding to the dead cells' DNA is proportional to the necrosis.

In vitro, cultured cells that are undergoing apoptosis eventually lose membrane integrity and release their cytoplasmic contents into the culture medium (post-apoptotic necrosis). In post-apoptotic necrosis, luminescence is preceded by temporal increases in fluorescence. The concurrent increases in luminescence and fluorescence indicate necrotic cell death, in which the luminescent signal is generated by the non-specific binding of annexin V to PS present in cell debris. In general, the fluorescence signal indicates necrosis.

Table 85. Apoptosis detection based on PS externalization.

Cell Death Mode	Cell Membrane Integrity & Location of PS	Signal Detected	Outcome	Interpretation
None (viable)	Cell membrane intact PS confined to the inner leaflet	None	Annexin V – negative DNA dye – negative	No cell death
Apoptosis	Cell membrane intact PS translocation to the outer leaflet	Luminescence	Annexin V – positive DNA dye – negative	Ongoing apoptosis
Post-apoptotic Necrosis	Cell membrane disruption PS on the inner and outer leaflets	Luminescence proceeding Fluorescence	Annexin V – positive DNA dye – positive	Increases in DNA dye after Annexin V binding reflect post-apoptotic necrosis
Necrosis	Cell membrane disruption PS on the inner and outer leaflets	Luminescence Fluorescence	Annexin V – positive DNA dye – positive	Concurrent increases in both Annexin V binding and DNA dye reflect necrosis.
Necrosis	Cell membrane disruption PS on the inner and outer leaflets	Fluorescence	Annexin V – negative DNA dye – positive	Necrosis, or the absence of Annexin V binding due to a limited time window for Annexin V binding

5.4.3 Test ingredients

The test ingredients associated with decreases in DPC viability of $\geq$ 50% within 72h were assessed for apoptosis induction in DPCs **(Table 86)**.

Table 86. Experimental design for Annexin V binding assay in DPCs.

1. Test Ingredients

	Test Ingredients*	Vehicle	C1 (%)	C2 (%)	C3 (%)	No. of treatments	Treatment Duration (hours)
1	Calendula Officinalis Extract	Medium	0.1	0.2	0.4	1	48, 72
2	Cinnamidopropyltrimonium Chloride (CRODASORB UV-283), CATC	Medium	0.285	0.57	1.14	1	48, 72
3	Cocamidopropyl Betaine (SurfProTM CAPB)	Medium	0.035	0.07	0.14	1	15, 24, 48, 72
4	Guar Hydroxypropyltrimonium Chloride	Medium	0.04	0.08	0.16	1	48, 72
5	Lavandula Angustifolia (Lavender) Oil	1% Ethanol	0.05	0.1	0.2	1	48, 72
6	Methylchloroisothiazolinone (MCI)	Medium	0.00001	0.00002	0.00004	1	15, 24, 48, 72
7	Methylisothiazolinone (MI)	Medium	0.00015	0.0003	0.0006	1	15, 24, 48, 72
8	Pisum Sativum (Pea) Peptide	Medium	0.58	1.16	2.32	1	48, 72

	Test Ingredients*	Vehicle	C1 (%)	C2 (%)	C3 (%)	No. of treatments	Treatment Duration (hours)
9	Polysorbate 60	20% Ethanol**	0.16	0.32	0.64	1	15, 24, 48, 72
10	Rosmarinus Officinalis Leaf Extract (Rosemary Oleoresin, ROE)	2.5% DMSO	0.0005	0.001	0.002	1	48, 72
11	Trifolium Pratense (Red Clover) Blossom Extract	Medium	2.65	5.3	10.6	1	15, 24, 48, 72

2. Assay Controls

	Assay Controls	Vehicle	Concentration	No. of treatments	Treatment Duration (hours)
12	Minoxidil	100% Ethanol**	3 µm	1	24, 48, 72
13	Cisplatin	DMSO	100 µm	1	24, 48, 72

*, Annexin V binding assay was done only for ingredients shown to inhibit DPC viability within 72h by MTS assay.
**, Refer to Table 4 for the preparation of the stock solutions.

5.4.4 Assay Protocol

Experiments were performed using the annexin V apoptosis and necrosis assay (JA1011, Promega) following the manufacturer's protocol.[2] Each test ingredient was tested at three concentrations in DPCs: one that was two-fold lower than the IC50 (C1); IC50 (C2), determined based on the MTS results; and a concentration that was two-fold higher than the IC50 (C3) **(Table 86)**. The assay included nontreated DPCs (C0) and those treated with cisplatin (100 µm) or minoxidil (3 µm) as controls. The luminescence (resulting from annexin V binding to PS) and fluorescence (resulting from a cell-impermeant DNA dye binding to dead cells' DNA) were measured using a multimode plate reader (Tecan infinite 200Pro). Four replicates (n=4) were tested per concentration per timepoint. The experiments were performed twice.

5.4.5 Statistical Methods & Data Presentation

Statistical analysis was performed using two-way analysis of variance (ANOVA) with Bonferroni multiple comparison test (GraphPad, version 9.4.1.681, GraphPad Software, Inc.). Data are presented as fold changes in luminescence (indicating annexin V binding/apoptosis) or in fluorescence (indicating DNA staining/necrosis) relative to the nontreated control (mean ± SD; n=4 replicates per concentration per timepoint). Adjusted *p* values generated from Bonferroni tests are included in the tables. $p \leq 0.05$ was considered statistically significant. The luminescence and fluorescence signal intensities that are lower than those of the nontreated controls (C0) or that are detectable only at the background level suggest no activity at a given concentration and time point. Such values are indicated as "ND" (not detectable) in the tables.

5.4.6 Assay Validation

DPCs treated with cisplatin showed time-dependent increases in annexin V binding, while the fluorescence resulting from the DNA staining of necrotic cells remained at a level comparable to that of the nontreated control. Consistent with the MTS results, cisplatin-induced apoptosis was detectable in cells treated for ≥ 48h and showed 2- and 9-fold increases (vs. the nontreated control) at 48h and 72h, respectively **(Table 87)**. In line with the growth-promoting effects of minoxidil in DPCs, minoxidil-treated DPCs showed neither detectable annexin V binding nor necrosis **(Table 88)**. These data validate the

[2] *RealTime-Glo™ Annexin V Apoptosis and Necrosis Assay,* Technical Manual # TM507, Promega Corporation

utility of this assay in assessing apoptosis in DPCs. No luminescent and fluorescent signals were detectable at 24h in DPCs treated with cisplatin. Therefore, apoptosis and necrosis were assessed at 48h and 72h.

Table 87. Cisplatin

Treatment Duration		Apoptosis (0 μM)	Apoptosis (100 μM)	Necrosis (0 μM)	Necrosis (100 μM)
24h	**mean ± SD**	ND	ND	ND	ND
	adj. p-value				
48h	**mean ± SD**	1 ± 0.03	**2.09 ± 0.19**	1 ± 0.74	ND
	adj. p-value		<0.0001		
72h	**mean ± SD**	1 ± 0.23	**9.12 ± 0.31**	1 ± 0.29	ND
	p-value		<0.0001		

Data are represented as the mean ± SD; n=4. *p* values were generated using Bonferroni multiple comparisons test. Highlighted values correspond to a fold change > 2. ND, not detectable.

Table 88. Minoxidil

Treatment Duration		Apoptosis (0 μM)	Apoptosis (3 μM)	Necrosis (0 μM)	Necrosis (3 μM)
24h	**mean ± SD**	ND	ND	ND	ND
	adj. p-value				
48h	**mean ± SD**	1 ± 0.05	ND	1 ± 0.01	1 ± 0.01
	p-value				>0.9999
72h	**mean ± SD**	1 ± 0.04	ND	1 ± 0.02	1 ± 0.01
	adj. p-value				>0.9999

Data are represented as the mean ± SD; n=4. *p* values were generated using Bonferroni multiple comparisons test. ND, not detectable.

5.4.7 Results

Potential apoptosis inducers

Rosmarinus Officinalis (Rosemary) Leaf Extract, Lavandula Angustifolia (Lavender) Oil, and Guar Hydroxypropyltrimonium Chloride (Guar)

Rosemary extract resulted in dose- and time-dependent increases in annexin V-positive/necrosis-negative cells **(Table 89).** Compared to the nontreated cells (C0), an IC50 dose (C2) of rosemary extract caused a 2.16-fold (*p<0.0001*) increase in annexin V binding at 48h and a 4.89-fold (*p<0.0001*) increase at 72h. The highest concentration tested (C3, 0.002%) showed the most apoptotic activity, with a 6.49-fold increase (*p<0.0001*, 72h) **(Table 89)**. The IC50 doses of lavender oil and Guar also induced 2.4-fold (*p<0.0001*) and 3.4-fold (*p<0.0001*) increases in annexin V binding, respectively, at 72h **(Tables 90 & 91)**. The absence of necrosis signals indicated that apoptosis was likely the primary mechanism of cells death induced by these ingredients.

Table 89. Rosmarinus Officinalis Leaf Extract

		Apoptosis	Apoptosis	Apoptosis	Apoptosis	Necrosis	Necrosis	Necrosis	Necrosis
		C0	C1	C2	C3	C0	C1	C2	C3
48h	**mean ± SD**	1 ± 0.03	1.95 ± 0.02	**2.16 ± 0.04**	**2.12 ± 0.02**	1 ± 0.01	ND	ND	ND
	adj. p-value		<0.0001	<0.0001	<0.0001				
72h	**mean ± SD**	1 ± 0.04	**3.66 ± 0.08**	**4.89 ± 0.07**	**6.49 ± 0.13**	1 ± 0.01	ND	ND	ND
	adj. p-value		<0.0001	<0.0001	<0.0001				

Data are represented as the mean ± SD; n=4. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparisons test. Highlighted values correspond to a fold change > 2. ND, not detectable.

Table 90. Lavandula Angustifolia (Lavender) Oil

		Apoptosis	Apoptosis	Apoptosis	Apoptosis	Necrosis	Necrosis	Necrosis	Necrosis
		C0	C1	C2	C3	C0	C1	C2	C3
48h	mean ± SD	1 ± 0.03	ND	ND	ND	1 ± 0.01	ND	ND	ND
	adj. p-value								
72h	mean ± SD	1 ± 0.04	**3.7 ± 0.42**	**2.36 ± 0.16**	1.99 ± 0.3	1 ± 0.01	ND	ND	ND
	adj. p-value		<0.0001	<0.0001	<0.0001				

Data are represented as the mean ± SD; n=4. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparisons test. Highlighted values correspond to a fold change > 2. ND, not detectable.

Table 91. Guar Hydroxypropyltrimonium Chloride

		Apoptosis	Apoptosis	Apoptosis	Apoptosis	Necrosis	Necrosis	Necrosis	Necrosis
		C0	C1	C2	C3	C0	C1	C2	C3
48h	mean ± SD	1 ± 0.04	1.12 ± 0.09	1.25 ± 0.13	1.66 ± 0.04	1 ± 0.02	ND	ND	ND
	adj. p-value		0.2668	0.0025	<0.0001				
72h	mean ± SD	1 ± 0.04	**2.15 ± 0.05**	**3.44 ± 0.17**	**8.48 ± 0.13**	1 ± 0.02	ND	ND	ND
	adj. p-value		<0.0001	<0.0001	<0.0001				

Data are represented as the mean ± SD; n=4. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparisons test. Highlighted values correspond to a fold change > 2. ND, not detectable.

Potential necrosis inducers

Cinnamidopropyltrimonium chloride (CATC), Pea Extract, and Calendula Extract

At the lowest concentration tested (C1, 0.285%), CATC caused 8.5-fold and 61.03-fold increases in annexin V binding at 48h and 72h, respectively **(Table 92)**. However, despite these increases in annexin V binding, concurrent increases in necrosis signals at even the lowest concentration (9.69-fold increase at 48h, *p<0.0001*) suggested that CATC primarily induces necrosis in DPCs. Pea extract caused similar robust increases in necrosis signals. In DPCs treated with the lowest concentration (C1), the necrosis signal was 16.6-fold higher than the annexin V binding at 48h (2.25 annexin V binding vs. 37.43 necrosis), while annexin V-binding was undetectable at the highest concentration (C3) **(Table 93**). This data, coupled with >120-fold increases in necrosis observed at C3, suggested a dose-dependent mechanism of cell death. Consistent with this observation, a variety of stimuli such as heat, radiation, hypoxia, and cytotoxic anticancer drugs have been shown to induce apoptosis at low doses but result in necrosis at higher doses [76]. Compared to CATC and Pea extract, DPCs treated with Calendula extract had barely detectable levels of annexin V binding, with a 3.63-fold at C1 (*p<0.0001*) and 5.04-fold at C2 (*p<0.0001*) increases in necrosis at 48h **(Table 94)**.

Table 92. Cinnamidopropyltrimonium chloride (Crodasorb™ UV-283), CATC

		Apoptosis	Apoptosis	Apoptosis	Apoptosis	Necrosis	Necrosis	Necrosis	Necrosis
		C0	C1	C2	C3	C0	C1	C2	C3
48h	mean ± SD	1 ± 0.03	**8.50 ± 1.1**	**8.18 ± 1.64**	ND	1 ± 0.74	**9.69 ± 0.59**	**25.85 ± 0.83**	**60.54 ± 1.18**
	adj. p-value		<0.0001	<0.0001			<0.0001	<0.0001	<0.0001
72h	mean ± SD	1 ± 0.24	**61.03 ± 0.5**	**43.08 ± 4.44**	15.64 ± 4	1 ± 0.3	**8.70 ± 0.52**	**24.83 ± 0.71**	**57.90 ± 0.19**
	adj. p-value		<0.0001	<0.0001	<0.0001		<0.0001	<0.0001	<0.0001

Data are represented as the mean ± SD; n=4. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparisons test. Highlighted values correspond to a fold change > 2. ND, not detectable.

Table 93. Pisum Sativum (Pea) Extract

		Apoptosis	Apoptosis	Apoptosis	Apoptosis	Necrosis	Necrosis	Necrosis	Necrosis
		C0	C1	C2	C3	C0	C1	C2	C3
48h	mean ± SD	1 ± 0.03	**2.25 ± 0.1**	ND	ND	1 ± 0.74	**37.43 ± 0.98**	**70.37 ± 1.62**	**129.58 ± 3.65**
	adj. p-value		<0.0001				<0.0001	<0.0001	<0.0001
72h	mean ± SD	1 ± 0.23	**7.69 ± 0.52**	**4.98 ± 0.31**	ND	1 ± 0.29	**36.39 ± 0.49**	**69.22 ± 1.55**	**121.77 ± 0.56**
	adj. p-value		<0.0001	<0.0001			<0.0001	<0.0001	<0.0001

Data are represented as the mean ± SD; n=4. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparisons test. Highlighted values correspond to a fold change > 2. ND, not detectable.

Table 94. Calendula Extract

		Apoptosis	Apoptosis	Apoptosis	Apoptosis	Necrosis	Necrosis	Necrosis	Necrosis
		C0	C1	C2	C3	C0	C1	C2	C3
48h	mean ± SD	1 ± 0.04	ND	ND	ND	1 ± 0.02	**3.63 ± 0.02**	**5.04 ± 0.07**	**6.31 ± 0.03**
	adj. p-value						<0.0001	<0.0001	<0.0001
72h	mean ± SD	1 ± 0.04	1.95 ± 0.07	1.57 ± 0.04	ND	1 ± 0.02	**3.44 ± 0.02**	**4.78 ± 0.04**	**6.04 ± 0.05**
	adj. p-value		<0.0001	<0.0001			<0.0001	<0.0001	<0.0001

Data are represented as the mean ± SD; n=4. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparisons test. Highlighted values correspond to a fold change > 2. ND, not detectable.

Potential necrosis or alternative mechanisms

Red clover extract, Polysorbate 60, Methylchloroisothiazolinone (MCI), Methylisothiazolinone (MI) Cocamidopropyl Betaine (CAPB)

Red clover extract caused a modest increase in necrosis signals (~2-fold increase at the highest dose), which occurred in the absence of annexin V binding **(Table 95)**. In contrast, in DPCs treated with Polysorbate 60, the necrosis signal was undetectable, while a 5-fold increase in annexin V binding was observed only at the lowest dose **(Table 96)**. In addition, no significant changes in either annexin V binding or necrosis were observed in DPCs treated with MCI, MI, or CAPB **(Tables 97−99)**. Given the acute cytotoxicity observed at 24h with these ingredients, especially with MCI and MI, a shorter window of time for detection may be needed. Alternatively, continuous real-time monitoring may avoid missing a critical time point, as apoptosis markers such as caspase activity and PS exposure may be present only transiently. However, in subsequent experiments, no substantial annexin V binding and necrosis were detectable as early as 15h **(Tables 100-101)**. While the possibility of alternative mechanisms (e.g., proliferation arrest, autophagy, reduced cell viability due to senescence) cannot be entirely excluded, further investigation using additional markers of cell health (e.g., ATP levels, the release of lactate dehydrogenase [LDH], and caspase activity) is needed to better define the cytotoxic mechanisms of these ingredients.

Table 95. Trifolium Pratense (Clover) Flower Extract

		Apoptosis	Apoptosis	Apoptosis	Apoptosis	Necrosis	Necrosis	Necrosis	Necrosis
		C0	C1	C2	C3	C0	C1	C2	C3
48h	mean ± SD	1 ± 0.04	ND	ND	ND	1 ± 0.02	1.18 ± 0.03	1.61 ± 0.01	**2.2 ± 0.01**
	adj. p-value						<0.0001	<0.0001	<0.0001
72h	mean ± SD	1 ± 0.04	ND	ND	1.43 ± 0.07	1 ± 0.02	1.16 ± 0.03	1.57 ± 0.02	**2.1 ± 0.01**
	adj. p-value				<0.0001		<0.0001	<0.0001	<0.0001

Data are represented as the mean ± SD; n=4. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparisons test. Highlighted values correspond to a fold change > 2. ND, not detectable.

Table 96. Polysorbate 60

		Apoptosis	Apoptosis	Apoptosis	Apoptosis	Necrosis	Necrosis	Necrosis	Necrosis
		C0	C1	C2	C3	C0	C1	C2	C3
48h	mean ± SD	1 ± 0.03	ND	ND	ND	1 ± 0.01	ND	ND	1 ± 0.01
	adj. p-value								>0.9999
72h	mean ± SD	1 ± 0.04	**5.08 ± 0.37**	1.89 ± 0.1	ND	1 ± 0.01	ND	ND	1 ± 0.01
	adj. p-value		<0.0001	<0.0001					0.3169

Data are represented as the mean ± SD; n=4. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparisons test. Highlighted values correspond to a fold change > 2. ND, not detectable.

Table 97. Methylchloroisothiazolinone (MCI)

		Apoptosis	Apoptosis	Apoptosis	Apoptosis	Necrosis	Necrosis	Necrosis	Necrosis
		C0	C1	C2	C3	C0	C1	C2	C3
48h	mean ± SD	1 ± 0.05	1.03 ± 0.03	1.08 ± 0.03	1.09 ± 0.02	1 ± 0.01	1 ± 0.01	1 ± 0.01	1 ± 0.004
	adj. p-value		0.6957	0.0023	0.0011		0.0004	0.326	0.0795
72h	mean ± SD	1 ± 0.04	1.01 ± 0.03	1.09 ± 0.03	1.09 ± 0.03	1 ± 0.02	1 ± 0.003	1 ± 0.01	1 ± 0.01
	adj. p-value		>0.9999	0.0008	0.0014		0.0065	0.5303	>0.9999

Data are represented as the mean ± SD; n=4. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparisons test. ND, not detectable.

Table 98. Methylisothiazonlinone (MI)

		Apoptosis	Apoptosis	Apoptosis	Apoptosis	Necrosis	Necrosis	Necrosis	Necrosis
		C0	C1	C2	C3	C0	C1	C2	C3
48h	mean ± SD	1 ± 0.05	1.23 ± 0.02	1.39 ± 0.02	1.48 ± 0.03	1 ± 0.01	1.55 ± 0.02	**2.23 ± 0.03**	1.85 ± 0.02
	adj. p-value		0.6957	0.0023	0.0011		0.0004	0.326	0.0795
72h	mean ± SD	1 ± 0.04	1.72 ± 0.13	1.77 ± 0.09	**2.57 ± 0.21**	1 ± 0.02	1.63 ± 0.04	**2.56 ± 0.03**	1.95 ± 0.03
	adj. p-value		>0.9999	0.0008	0.0014		0.0065	0.5303	>0.9999

Data are represented as the mean ± SD; n=4. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparisons test. Highlighted values correspond to a fold change > 2. ND, not detectable.

Table 99. Cocamidopropyl Betaine (CAPB)

		Apoptosis	Apoptosis	Apoptosis	Apoptosis	Necrosis	Necrosis	Necrosis	Necrosis
		C0	C1	C2	C3	C0	C1	C2	C3
48h	mean ± SD	1 ± 0.05	ND	ND	ND	1 ± 0.01	1.03 ± 0.04	1.08 ± 0.01	1.06 ± 0.001
	adj. p-value						0.2814	0.0005	0.0047
72h	mean ± SD	1 ± 0.04	1.36 ± 0.23	ND	ND	1 ± 0.02	1.06 ± 0.05	1.11 ± 0.01	1.08 ± 0.004
	adj. p-value		<0.0001				0.0061	<0.0001	0.0002

Data are represented as the mean ± SD; n=4. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparisons test. ND, not detectable.

Table 100. Red Clover, Polysorbate 60, MCI, MI, and CAPB at 15h.

		Apoptosis	Apoptosis	Apoptosis	Apoptosis	Necrosis	Necrosis	Necrosis	Necrosis
		C0	C1	C2	C3	C0	C1	C2	C3
Red Clover	mean ± SD	1 ± 0.03	ND	ND	ND	1 ± 0.02	1.34 ± 0.04	1.87 ± 0.03	**2.67 ± 0.02**
	adj. p-value						<0.0001	<0.0001	<0.0001
Polysorbate 60	mean ± SD	1 ± 0.02	ND	ND	ND	1 ± 0.02	ND	ND	1.14 ± 0.01
	adj. p-value								<0.0001
MCI	mean ± SD	1 ± 0.03	ND	ND	ND	1 ± 0.01	ND	ND	ND
	adj. p-value								
MI	mean ± SD	1 ± 0.03	1.02 ± 0.02	1.13 ± 0.02	ND	1 ± 0.01	1.14 ± 0	1.31 ± 0.02	1.25 ± 0.02
	adj. p-value		0.5459	<0.0001			<0.0001	<0.0001	<0.0001
CAPB	mean ± SD	1 ± 0.03	ND	ND	ND	1 ± 0.01	ND	1.02 ± 0.01	1.04 ± 0.01
	adj. p-value							0.4681	0.0181

Data are represented as the mean ± SD; n=4. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparisons test. Highlighted values correspond to a fold change > 2. ND, not detectable.

Table 101. Red Clover, Polysorbate 60, MCI, MI, and CAPB at 24h.

		Apoptosis	Apoptosis	Apoptosis	Apoptosis	Necrosis	Necrosis	Necrosis	Necrosis
		C0	C1	C2	C3	C0	C1	C2	C3
Red Clover	**mean ± SD**	1 ± 0.03	ND	ND	ND	1 ± 0.01	1.26 ± 0.04	1.73 ± 0.02	**2.44 ± 0.01**
	adj. p-value						<0.0001	<0.0001	<0.0001
Polysorbate 60	**mean ± SD**	1 ± 0.02	ND	ND	ND	1 ± 0.02	ND	ND	1.07 ± 0.02
	adj. p-value								<0.0001
MCI	**mean ± SD**	1 ± 0.03	ND	1.05 ± 0.02	1.04 ± 0.01	1 ± 0.01	ND	ND	ND
	adj. p-value			0.0012	0.0174				
MI	**mean ± SD**	1 ± 0.03	1.05 ± 0.01	1.19 ± 0.02	1.15 ± 0.04	1 ± 0.01	1.31 ± 0.01	1.64 ± 0.04	1.48 ± 0.02
	adj. p-value		0.0013	<0.0001	<0.0001		<0.0001	<0.0001	<0.0001
CAPB	**mean ± SD**	1 ± 0.03	ND	ND	ND	1 ± 0.01	ND	1.03 ± 0.01	1.04 ± 0.01
	adj. p-value							0.1598	0.0212

Data are represented as the mean ± SD; n=4. Adjusted (adj.) *p* values were generated using Bonferroni multiple comparisons test. Highlighted values correspond to a fold change > 2. ND, not detectable.

5.4.8 Summary

Table 102 summarizes the results of the apoptosis and necrosis assessment.

Table 102. Fold increases in apoptosis and necrosis at IC50 doses.

Treatment Duration	Test Ingredients	Apoptosis*	Necrosis*	Possible mechanism of cytotoxicity
48h	Rosemary Extract	2.16	ND	Apoptosis
48h	CATC	8.18	25.85	Necrosis (concurrent annexin V binding due to loss of membrane integrity)
48h	Pea Extract	ND	70.37	Necrosis
48h	Calendula Extract	ND	5.04	Necrosis
48h	MI	ND	2.23	Necrosis
72h	Rosemary Extract	4.89	ND	Apoptosis
72h	CATC	43.08	24.83	Necrosis (concurrent annexin V binding due to loss of membrane integrity)
72h	Pea Extract	4.98	69.22	Necrosis (concurrent annexin V binding due to loss of membrane integrity)
72h	Calendula Extract	ND	4.78	Necrosis
72h	MI	ND	2.56	Necrosis
72h	Guar	3.44	ND	Apoptosis
72h	Lavender Oil	2.36	ND	Apoptosis
	CAPB	ND	ND	Need further investigation
	MCI	ND	ND	Need further investigation
	Polysorbate 60	ND	ND	Need further investigation
	Red Clover Extract	ND	ND	Need further investigation

*, ≥ 2-fold increase at IC50 dose, compared to nontreated controls.
ND, not detectable, or <2-fold increase at IC50 dose.
Apoptosis was assessed based on annexin V binding. Necrosis was assessed based on the detection of DNA-binding dye due to loss of membrane integrity.

- Three ingredients (i.e., rosemary extract, guar, lavender oil) induced apoptosis at IC50 doses.
- Rosemary extract showed a time-dependent increase in annexin V binding (2.16-fold at 24h, 4.89-fold at 48h).
- Guar and lavender oil-induced apoptosis was detectable at 48h.

- CATC and pea extract caused robust increases in DNA dye, suggesting that necrosis is a primary mechanism of cell death in DPCs treated with these ingredients.
- Compared to CATC and pea extract, calendula extract, MI, and red clover extract detected a modest increase in necrosis. Furthermore, apoptosis and necrosis were undetectable in DPCs treated with CAPB, MCI, and polysorbate 60. Given the acute cytotoxicity observed with these ingredients, additional tests may be necessary to determine the mode of cytotoxicity.

5.5 Conclusion and Discussion

This study evaluated in vitro cytotoxicity associated with the four test products and 20 test ingredients. Cytotoxicity assessed the test products/ingredients' effects on cell viability in DPCs and NHEKs. Eleven cytotoxic test ingredients were selected and evaluated further in DPCs for apoptosis and necrosis induction. **Table 103** summarizes the results of these experiments.

Table 103. Overall conclusion

Ingredient	AQ	DC	MO	WEN	Cell Viability	Possible mechanism of cytotoxicity*
Guar			✓		decreased	Apoptosis
Lavender Oil					decreased	Apoptosis
Rosemary Extract		✓		✓	decreased	Apoptosis
Calendula Extract		✓		✓	decreased	Necrosis
CATC					decreased	Necrosis
Pea Extract			✓		decreased	Necrosis
Red Clover Extract			✓		decreased	Uncertain
CAPB	✓		✓		decreased	Uncertain
MCI		✓		✓	decreased	Uncertain
MI		✓		✓	decreased	Uncertain
Polysorbate 60		✓		✓	decreased	Uncertain

AQ, Aquaphor Baby Wash & Shampoo; DC, DevaCurl Low-Poo Delight; MO, Monat Renew; WEN, WEN Sweet Almond Mint Cleansing Conditioner
✓, presence of test ingredients in test products.
*, See Table 54.

The MTS assay measures cellular metabolic activity as an indicator of cell viability. However, MTS assessment of cell viability does not directly correlate with cell death; nor does it indicate whether cells are undergoing apoptosis or necrosis. For example, cell growth arrest (e.g., quiescence, senescence) can also result in an overall reduction of cell viability. Therefore, annexin V binding was measured as an indicator of apoptosis, and the binding of DNA dye to dead cells' DNA was measured as a necrosis indicator.

Apoptosis was detected in DPCs treated with guar, lavender oil, or rosemary extract, while calendula extract, CATC, and pea extract primarily induced necrosis.

Concentration-dependent increases in necrosis were detectable in DPCs treated with red clover extract. However, >2-fold increases were detectable only at the highest concentration tested (10%), and necrosis signals remained relatively constant, irrespective of the treatment durations. Given that red clover-induced cytotoxicity in DPCs was apparent when cells were treated >72h at concentrations $\geq$8%, further optimization of the assay condition may be needed.

Despite the acute cytotoxicity observed with CAPB, MCI, MI, and polysorbate 60 within 24h, apoptosis and necrosis were undetectable even as early as 15h.

In addition to apoptosis and necrosis, there exist many forms of cell death modalities, including autophagy, anoikis, necroptosis, and ferroptosis, to mention a few [77]. While the type, intensity, and duration of stimuli determine the mode of cell death, a drug at the same concentration can induce multiple modes of cell death at the same time in different subpopulations of cells. Apoptosis and necrosis can also occur independently, sequentially, and simultaneously [76]. Therefore, additional studies that measure ATP and LDH levels, caspase activities, and/or other methods, such as flow cytometry analysis, are required to discern the mechanisms of cell death induced by these ingredients.

5.6 Potential Limitations and Recommendations

Purity of ingredients
An attempt was made to obtain pure ingredients with as few additives as possible. However, for most test ingredients, the degree of purity was not clearly defined (e.g., lavender oil, red clover extract, pequi oil, sunflower seed oil). In addition, the formulation and composition of an extract might vary greatly depending on the manufacturer. For example, calendula extract used in this study contained preservatives (potassium sorbate, sodium benzoate), rosemary extract contained acetone (<25 ppm) as a solvent, and red clover extract contained glycerin. These variables can considerably interfere with the test results.

Solubility
For ingredients with limited solubility, it was not feasible to test higher concentrations. For example, guar, caused the solution to gel at concentrations higher than 0.1%. Coconut oil, olus oil, pequi oil, and polysorbate 60 needed to be heated to 40°C. Limited solubility may cause ingredients to precipitate or aggregate, leading to an underestimation of their activity.

Apoptosis detection
While the annexin V binding to PS is widely accepted as an indicator of apoptosis, several drawbacks must be considered when interpreting data. PS externalization has been detected under patho/physiological conditions unrelated to apoptosis (e.g., platelet activation), and cell swelling can also occur in response to established apoptotic stimuli [78]. In addition, PS externalization is also known to occur during limited windows of time, which can vary depending on the cell type, concentration, and experimental conditions. Therefore, multiplexing using additional markers of apoptosis, such as caspase activation, or a molecular imaging probe capable of detecting apoptosis during a wider window of time can help confirm the occurrence of apoptosis.

6 Overall Conclusion, Challenges, and Recommendations for Future Studies

The observation of a prolonged duration of the telogen phase in mice treated with DevaCurl or WEN products demonstrates that hair cycle abnormalities could be triggered by repeated applications of hair products. A prolonged telogen may result in a delay in anagen induction and subsequent hair growth. While the majority of ingredients differed between these products, five ingredients that were cytotoxic to DPCs were present in both DevaCurl and WEN (i.e., Rosemary extract, Calendula extract, MCI, MI, and Polysorbate 60). The relevance of these ingredients to alopecia warrants further investigation. Discrepancies were observed between the in vitro and in vivo outcomes of Aquaphor and Monat (i.e., absence of alopecia, despite significant in vitro cytotoxicity). It is important to note that the results generated using in vitro, as well as ex vivo, cultures are not always replicated under in vivo conditions,

as these models lack the complex tissue organization and close circuitry linkage of one cell type with the other. Therefore, while in vitro assessments inform further investigations into the properties of an ingredient, the current study does not provide sufficient information to imply an in vitro–in vivo correlation.

Future in vivo long-term treatment studies to assess for alopecia should include a sufficient number of animals to statistically power the study. In addition, transcriptomic and cytokine profiling of DevaCurl- and WEN-treated skin may help identify molecular signatures and inflammatory responses that contribute to aberrant hair cycling associated with these products.

7 References

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