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Regulation of epidermal pathways and skin homeostasis through a skin-identical plant-derived vitamin D and cholesterol precursor.

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1. Introduction

7-Dehydrocholestrol (7-DHC), also known as provitamin D3, is largely present in the cell membranes of keratinocytes in the basal and spinous layers of the epidermis. It is a natural precursor in the synthesis of cholesterol and vitamin D3 [1]. 7-DHC is the last precursor which is converted by 7-Dehydrocholesterol reductase to cholesterol. When exposed to UVB, 7-DHC is converted to previtamin D3 which in turn isomerizes to vitamin D3 [2]. Studies on keratinocytes and *in vitro* human skin equivalent models have shown that the conversion of 7-DHC into vitamin D3 occurs at UVB wavelengths of 285 to 315 nm. In addition, the studies showed that the amount of vitamin D3 generated is dependent on concentration of 7-DHC, wavelength and dose of UVB [3], [4]. Multiple skin functions are regulated by vitamin D3 which includes epidermal proliferation and differentiation [5]

However, despite 7-DHC being present in the epidermis and being part of the vitamin D3 pathway, its efficacy in skin is not well characterized. A study with keratinocytes showed that treatment with 7-DHC increases the protein level of heat shock proteins in the cells. This in turn could increase the chance of survival of the cells under harsh conditions [6]. It suggests

that 7-DHC could potentially play a greater role in the efficacy of skin function which needs to be explored.

7-DHC was historically extracted from animal products such as wool, but was now successfully generated from a plant-based process to lead to a skin-identical vegetal sterol. As the source of the 7-DHC could have an impact on its ability to convert and function, characterization of the conversion of vegetal 7-DHC into vitamin D3 and its efficacy in an *in vitro* reconstructed skin model was carried out.

2. Materials and Methods

UV conversion of 7-DHC (activated 7-DHC)

A solution of either 0.1% or 0.01% of 7-DHC in emollient was prepared. The samples were either exposed to a single dose of UVB using a high intensity UV lamp for 90 seconds or a full spectrum solar stimulator with exposure on different days to UV light at 0.2 SED and 0.5 SED respectively. Samples were incubated at 37^{O}C for 24 hours after the last exposure. HPLC analysis was carried out to detect the presence of inactivated and activated 7-DHC in the samples.

Skin Cell Culture

Normal human dermal fibroblast and epidermal keratinocytes isolated from neonatal foreskin were obtained commercially (ThermoFisher). Primary fibroblasts were cultured in CnT-Prime Fibroblast Proliferation Medium (CELLnTEC). Keratinocytes were cultured in EpiLIfe supplemented with EpiLife Defined Growth Supplement (EDGS, ThermoFisher) in Collagen I coated flask. Cells were passaged once reaching 70-90% confluency and maintained at low passage for the production of skin models. All cell culture media were supplemented with 0.1% gentamicin and amphotericin B. Cells were grown at 37^{O}C under 5% CO_2 until desired confluency was achieved.

Skin Model Production

To produce the dermal equivalent human dermal fibroblasts were embedded in a fibrin hydrogel. The hydrogel gel was then submerged for 7 days with CnT-Prime Fibroblast Proliferation Medium (CELLnTEC) supplemented with getamicin, ampothericin B, ascorbic

acid, aprotinin. Media was replaced every second day. Once the hydrogel was ready, human keratinocytes were seeded on the surface of the dermal compartment. From this step, the skin model was cultured in CnT Prime Airlift Media (CELLnTEC) supplemented with getamicin, ampothericin B, ascorbic acid, aprotinin. From day 11 onwards, the skin models were put in culture at the air-liquid interface to ensure keratinocyte differentiation and epidermis formation. Media was changed every other day. A stratified and differentiated epidermis was obtained on day 21 of culture and models were collected.

Treatment of Skin Model

For treatment done under physiological conditions, on day 21, 0.1% or 0.01% (diluted in emollient) of inactivated or activated 7-DHC was topically applied on the skin model. For treatment done under UV B stress, skin models were exposed to UVB 50mJ/cm^2 followed by topical application of 0.1% or 0.01% (diluted in emollient) of inactivated or activated 7-DHC. Treatment at physiological conditions and UV-B stress was carried out every 24 hours for a total of 72 hours. After 72 hours, tissue and supernatant was harvested for downstream processing.

Histology and Epidermis Thickness Quantification

Samples were fixed in 4% Paraformaldehyde (PFA) for 24 hours, embedded in paraffin and processed for hematoxylin and eosin (H&E) staining. Images were taken using a Zeiss microscope. Multiple images of the sections of the different tissues were captured. Epidermis thickness was quantified from images analysed with Image J software.

ELISA

Supernatant was harvested after 72 hours of treatment of inactivated or activated 7-DHC. ELISA analysis was carried out to determine the levels of Matrix Metalloproteinase 1 (MMP-1) (Abcam) and Superoxide Dismutase 1 (SOD1) (Abcam) present in the supernatant.

Proteomics Analysis

Proteins were extracted from the skin model. BCA assay was carried out for protein quantification. Tryptic digestion was carried out, peptides were separated using nano-exchange liquid chromatography and injected on a mass spectrometer. Bio-informatic analysis

was carried out to identify and compare the proteins and pathways modulated between inactive 7-DHC and active 7-DHC treatment under physiological and stress conditions.

3. Results

A key characteristic of 7-DHC present in the skin is its ability to convert to UV-activated 7-DHC when exposed to UV from the sun. Plant-derived 7-DHC is also able to be converted into the UV-active 7-DHC when exposed to UV by a high intensity UV lamp as seen in figure 1A or a full spectrum solar stimulator as seen in figure 1B. The data from the full spectrum solar stimulator also shows that the conversion is dependent on the concentration of 7-DHC and intensity of UV light.

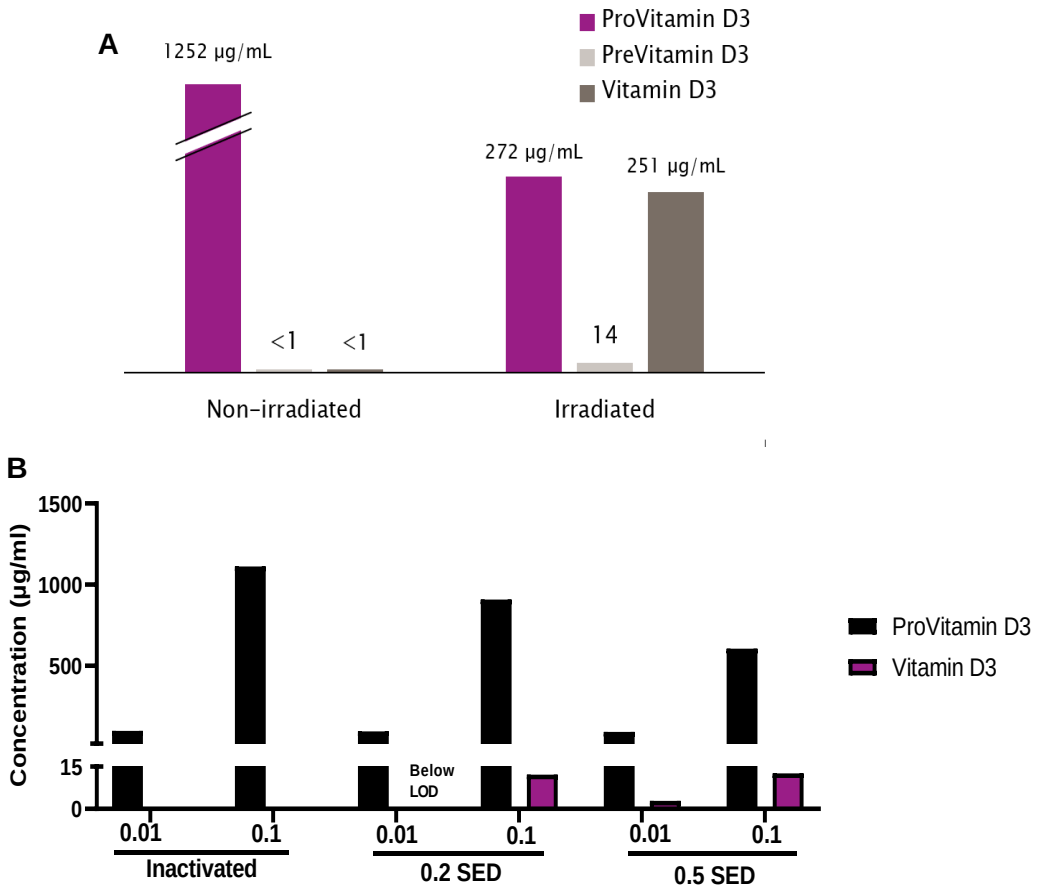

Figure 1: (A) HPLC analysis and quantification of UV conversion of 7-DHC from provitamin D3 to previtamin D3 and vitamin D3 using a high intensity UV lamp. **(B)** HPLC analysis and quantification of UV conversion of 7-DHC from provitamin D3 to previtamin D3 and vitamin D3 using a full spectrum solar stimulator at different dosage. LOD=limit of detection.

As there is no study on the effect of plant derived 7-DHC applied to skin, efficacy characterization of this compound was carried out. Its effect was tested under physiology and UV-B-stressconditions in reconstructed skin models. In the UV-B stress models, markers MMP-1 (matrix metalloproteinase 1) and SOD1 (superoxide dismutase 1) which are known to be upregulated by UV irradiation were analyzed by ELISA. Figure 2 shows that the inactivated version of 7-DHC reduced UVB-induced MMP-1 and SOD-1 levels compared to the vehicle control.

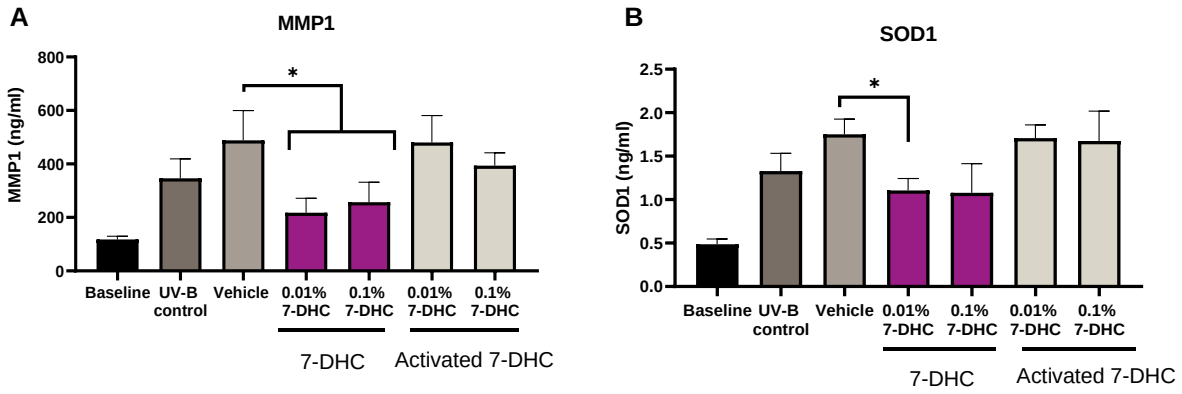

Figure 2: (A) Quantification of MMP-1 from the supernatant of the inactivated and UV-activated 7-DHC treated skin model. **(B)** Quantification of SOD-1 from the supernatant of the inactivated and UV-activated 7-DHC treated skin model. MMP-1 or SOD-1 ELISA performed on supernatant harvested from skin model at the end of treatment in baseline (no UV-B stress) and UV-B stress conditions with 0.01% or 0.1% 7-DHC inactivated or activated. Data analyzed using one-way ANOVA. *p<0.05 vs vehicle control.

Additionally. histological assessment using H&E staining was carried out on the tissue treated with 0.01% inactivated and UV-activated 7-DHC in physiological conditions. As seen in figure 3 a thicker epidermis can be observed in skin tissues treated with activated 7-DHC when compared to the vehicle control (figure 3A). The quantification based on image analysis shows a significant increase in epidermal thickness in tissue treated with activated 7-DHC compared to vehicle control as well as the inactivated version of 7-DHC (figure 3B).

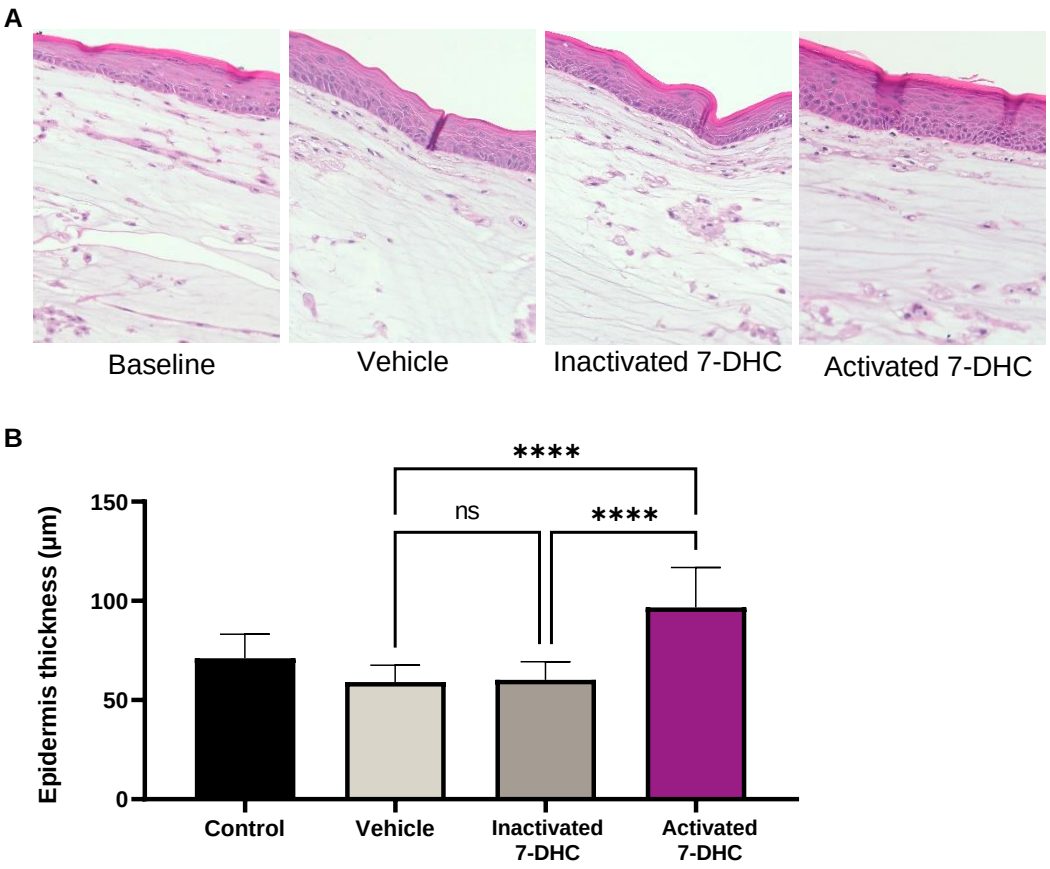

Figure 3: (A) Tissue morphology of skin model treated with inactivated and activated 7-DHC under physiological conditions. Representative images of H&E staining of the tissues for each condition**. (B)** Quantification of epidermal thickness of skin model treated with inactivated and activated 7-DHC. Data analyzed using one-way ANOVA. p values ****<0.0001.

The experiments conducted in reconstructed skin models provided some insights into the effects of inactivated and activated 7-DHC on skin. In addition, a proteomics analysis was carried out to further elucidate and characterize the overall effects of inactivated and activated 7-DHC in skin models. The results revealed a broad array of proteins and pathways beneficial to skin function, that were significantly modulated by 7-DHC. Approximately 1000 differentially expressed proteins were identified in treated conditions in comparison to the control. Table 1 summarizes pre-selected proteins of high interest identified in the proteomics analysis.

Protein Name	Percent Change (%)	
	7-DHC	UV-Activated 7-DHC
Collagen alpha-1 (III),	+46	ns
Collagen alpha -1 (XV)	+45	+35
Collagen alpha-1 (XIV)	+45	ns
Collagen alpha-1 (XII)	ns	+39
Metalloproteinase Inhibitor 1	+49	ns
Vimentin	+31	ns
MMP-9	-36	-73
Collagen alpha-1 (IV),	-29	ns
Collagen alpha-1 (VIII)	-31	ns
Kallikrein-6	+34	+121
Kallikrein-7	ns	+53
Kallikrein-14	-33	-64
Filaggrin	-83	-132
Filaggrin-2	-84	-117
SOD3	+75	+114
Glutathione Peroxidase 1	+31	ns
Thioredoxin	+55	ns
Thioredoxin Reductase	+129	ns
Glutathione Transferase	+30	+431
Glutathione Peroxidase 3	ns	+36
Carbonyl Reductase 1	ns	+48

Table 1: Summary of protein of interest from proteomics study that differentially expressed in comparison to control. ns=no significant change.

One of the pathways that is predicted to be activated by 7-DHC and UV-activated 7-DHC is the HIPPO signaling pathway which has been linked to collagen production which potentially alters the characteristics of dermal extracellular matrix (ECM). As shown in Table 1 various proteins that are involved in maitenance of the ECM are modulated by treatment of 7-DHC or UV-activated 7-DHC. Upregulation of collagen alpha-1 (III), collagen alpha -1 (XV) and collagen alpha-1 (XIV) is observed by 7-DHC. The development of ECM in the skin is dependent on organization of collagen fibrils into higher-order structures like fibers. In skin, collagen fibrils are made up of collagen I and III which play important roles in strength and

structure of skin. Collagen XV and XIV are both fibril-associated collagens that have been shown to play a role in collagen fibrillogenesis. Collagen XV has been shown to form a bridge linking collagen fibrils while Collagen XIV has been implicated as a regulator of collagen fibrillogenesis. Additionally, 7- DHC also downregulates collagen alpha-1 (IV), collagen alpha-1 (VIII) which are non-fibrillar collagens. These collagens do not form collagen fibers but play a role in shaping of the ECM. The changes in the expression of the various collagen types, influenced by the treatment with 7-DHC and UV-activated 7-DHC, could potentially result in significant alterations to the structural integrity and functionality of the dermal ECM. By upregulating key fibrillar collagens and downregulating non-fibrillar collagens, these treatments may enhance collagen fibrillogenesis and overall skin resilience.

In additon, Retionoid X receptor (RXR) Beta -β is upregulated by 7-DHC as well as UV-activated 7-DHC. These receptors can form the heterodimers or homodimers with other nuclear receptors such as Liver X Receptor (LXRs) and Vitamin D receptor (VDR). RXR-VDR hetrodimer potentially regulates the expression of genes involved in skin diffrentitation such as kallikerins (KLK) 6 and KLK 7 (Table 1). Kallikriens play an important role in skin desquamation which is the process by which cornified keratinocytes are gradually shed to help maintain epidermal homeostasis and thickness. KLK 6 and KLK 7 have been shown to degrade proteins that form the corneodesmosome which is formed in stratum corneum during the terminal differentiation of keratinocytes. Additionally, KLK7 also plays a role in the profilaggrin processing in the stratum granulosum by activating caspase 14 which is the first step in filaggrin degradation. Furthermore, the proteomics analysis predicted the activation of the Liver X Receptor/Retinoid X Receptor (LXR/RXR pathway) which is known to play a role in lipid metabolism and epidermal differentiation. In summary, this data set demonstrates that there is a link between 7-DHC treatment and epidermal homeostasis in skin not necessarily dependant on the UV-activation of 7-DHC.

Additionally, the analysis identified that especially inactivated 7-DHC upregulates the pathways involved in detoxification of reactive oxygen species (ROS). Both forms activate SOD3

(Table 1) which plays a key role of breaking down ROS species. However, 7-DHC upregulates components of the thioredox in system and glutathione peroxide 1 which play a role in reduction of oxidative stress to prevent cellular damage further demonstrating the potential anti-oxidative properties of this molecule.

4. Discussion

This study showed that the plant derived 7-DHC is able to be converted to provitamin D3 and vitamin D3 at final stage. This conversion was not only seen after high intensity UV lamp exposure but also after full spectrum solar stimulator which shows that conversion can occur under physiological conditions. Additionally, the conversion is shown to be dependent on the concentration of 7-DHC and the intensity of UVB. This suggests that the behavior of plant derived 7-DHC is similar to that of 7-DHC naturally present in the skin. Thus, the ability of 7-DHC to convert to its UV-activated form means that any efficacy on the skin could result from 7-DHC, UV-activated 7-DHC, or both. The presented data showed that the application of either 7-DHC or UV-activated 7-DHC can be beneficial to skin health by modulating tissue remodeling and epidermal biology through for example, collagen and KLK expression. Additonally, it could also play a role in anti-oxidant defense by modulating expression of SOD1 or pathways involved in ROS detoxification. Additionally, the UV-activated 7-DHC not only has its distinct effects on skin, but it is also able to complement the beneficial effects of 7-DHC. The results suggest a dual efficacy from 7-DHC and its UV-activated form on skin health. Further investigation will be needed to explore the mechanism of action of 7-DHC and UV-activated 7-DHC in skin.

5. Conclusion

This study has shown that the plant derived 7-DHC behaves similar to natural present 7-DHC in the epidermis. In addition, the presented data shows that the topical application of 7-DHC to skin would be beneficial to skin function through the modulation of pathways or proteins involved in tissue remodeling and epidermal biology. This is an additional efficacy to their known function in cholesterol synthesis and vitamin D3 production.

6. References

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