Investigative report


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Effects of Hydroxydecine®
(10-hydroxy-2-decenoic acid) on skin barrier structure and function in vitro and clinical efficacy in the treatment of UV-induced xerosis

10-Hydroxy-2-decenoic acid, a natural fatty acid only found in royal jelly, may be of value in correcting skin barrier dysfunction. We evaluated the activity of Hydroxydecine®, its synthetic counterpart, in vitro on the regulation of epidermal differentiation markers, ex vivo on the inflammatory response and restoration of skin barrier function, and in vivo on UV-induced xerosis in healthy human volunteers. In cultured normal human keratinocytes, Hydroxydecine®-induced involucrin, transglutaminase-1 and filaggrin protein production. In topically Hydroxydecine®-treated skin equivalents, immunohistochemical analysis revealed an increase in involucrin, transglutaminase-1 and filaggrin staining. In a model of thymic stromal lymphopoietin (TSLP)-induced inflamed epidermis, a Hydroxydecine®-containing emulsion inhibited TSLP release. In a model of inflammation and barrier impairment involving human skin explants maintained alive, Hydroxydecine® balm restored stratum corneum cohesion and significantly increased filagrin expression, as shown by immunohistochemistry. It also decreased pro-inflammatory cytokine secretion (IL-4, IL-5 and IL-13). In healthy volunteers with UV-induced xerosis, the hydration index increased by +28.8% (p<0.01) and +60.4% (p<0.001) after 7 and 21 days of treatment with Hydroxydecine® cream, respectively. Hydroxydecine® thus proved its efficacy in activating keratinocyte differentiation processes in vitro, restoring skin barrier function and reducing inflammation ex vivo, and hydrating dry skin in vivo.

Key words: 10-hydroxy-2-decenoic acid, skin barrier dysfunction

The skin barrier function is exercised mainly by the stratum corneum (SC, also referred to as the cornified layer), the outermost layer of the skin, which is formed and continuously renewed by the terminal differentiation of keratinocytes into corneocytes [1]. Among the various structural proteins and enzymes involved in this process, involucrin, transglutaminase-1 (TG1) and filaggrin play a significant role and constitute biological markers of the different stages of keratinocyte differentiation and SC formation [2, 3]. In certain dermatoses, including genetic ichthyosis and atopic dermatitis (AD), or in acquired skin disorders such as sebile, winter- or UV-induced xerosis, barrier function is often impaired because of an alteration in SC physiology. In AD, for instance, genetic defects and modifications in gene and protein expression of SC compounds are thought to be responsible for skin barrier impairment, which is characterised by increased water loss [4] and is associated with the development of an inflammatory immune response in the skin [5-8]. Scratching and barrier defects facilitate penetration of allergens and irritants, which in turn reinforce the production of pro-inflammatory cytokines and chemokines. In addition, thymic stromal lymphopoietin (TSLP) has been shown to promote the T helper type 2 (Th2) cell response associated with the pathogenesis of many inflammatory diseases, including AD [9]. In particular, mutations of the filaggrin gene have been shown to significantly predispose individuals to early-onset, severe and persistent AD [10]. Furthermore, involucrin expression is down-regulated by IL-4 and IL-13 TH2 cytokines, which are overexpressed in the skin of atopic patients [2]. In addition, components of intercellular junctions, such as claudins, are critical to the maintenance of tissue integrity and water retention [11]. Recently, it has been shown that an impairment of tight junctions contributes not only to barrier alteration but also to immune dysregulation in AD subjects [12].

10-Hydroxy-2-decenoic acid (10HDA) is a natural fatty acid found only in royal jelly [13]. As the main lipid in the diet of larvae, it plays an important role in the immature stages of the growth and development of honeybees by slowing larval growth [14]. In view of its antiproliferative and differentiation properties in vitro, this compound could be of value in the treatment of skin barrier dysfunction.

barrier function. To this end we assessed the effect of Hydroxydecine® in vitro on the regulation of SC differentiation markers and ex vivo on the regulation of cell cohesion in the SC, using a model of damaged and inflamed skin explants maintained alive. We then evaluated its activity on the inflammatory response induced in this model. More specifically, we also used an inflamed epidermal model with enhanced TSLP release to address the modulatory activity of a Hydroxydecine® formulation on TSLP induction. Finally we assessed the ability of a finished product containing Hydroxydecine® (Ictyane HD® cream, Ducray) to improve skin hydration in vivo in a model of UV-induced xerosis in the skin of healthy human volunteers.

### Material, patients and methods

**Effect of Hydroxydecine® on differentiation markers in vitro**

**Keratinocyte differentiation**

Normal human keratinocytes (NHK) were prepared from human breast skin fragments obtained during plastic surgery. NHKs were cultured in low-calcium (0.01 mM), keratinocyte serum-free medium (KSF, Invitrogen, Cergy Pontoise, France), supplemented with 25 μg/mL bovine pituitary extract (BPE, Invitrogen) and 1.5 ng/mL epidermal growth factor (EGF, Invitrogen) at 37°C in 5% CO₂.

To study keratinocyte differentiation, NHKs were grown in KSFM without BPE and EGF and were treated by the addition of 1.2 mM calcium (positive control) or 3 concentrations of Hydroxydecine® (17, 50, and 100 μM) in the medium containing 0.01% DMSO final concentration for 72 h. Non-treated controls were grown in medium with DMSO only.

**mRNA expression by Quantigene technology**

The QuantiGene Plex 2.0 system (Ozyme, Saint Quentin en Yvelines, France) was used to quantify mRNA levels of filaggrin directly from culture cell lysates. The assay was performed according to the manufacturer’s instructions. Cells were lysed with lysis mixture. Cell lysate (80 μl) was added to a 96-well hybridisation plate containing magnetic capture beads and QG_2.0 probe sets. The plate was incubated for ∼20 h at 55°C and agitated at 600 rpm to maintain suspension. Beads were sequentially hybridised (1 h, 50°C) with Pre-Amplifier, Amplifier and label probes and SAPE, with washes in between followed by a final 30-min incubation at 37°C. Bead discrimination and signal detection were performed on a Luminex instrument (Bio-Rad). Data were exported to Excel, where background subtraction and normalisation to two housekeepers (hypoxanthine phosphoribosyl transferase - HPRT - and polymerase (RNA) II polypeptide A - POL2RA -) were performed.

**Total protein extraction and analysis of involucrin, transglutaminase 1 and filaggrin synthesis by Western Blot**

Normal human keratinocyte intracellular proteins were extracted from 2 × 10⁶ cells by supplementing lysis buffer (50 mM Tris-HCl pH 8, 150 mM NaCl, 1% Triton X100, 1% Na deoxycholate, 0.1% SDS, 5 mM EDTA and protease inhibitor cocktail P8340 (Sigma, Saint-Quentin Fallavier, France) with 1 M DTT. The cell suspension was then incubated for 30 min in ice and centrifuged. Total proteins in the supernatant were assayed by the RCDC protein assay (Biorad kit). Twenty-five μg of the total protein content was then separated in 10% SDS-polyacrylamide gel (Promega). After migration, the proteins were transferred to a PVDF membrane and then probed with primary mouse monoclonal antibodies: anti-involucrin antibody (1:1000 dilution; Novo Castra, Rungis, France), anti-TG1 antibody (1:100 dilution; Biochemical Technologies Incorporation (BTI), Stoughten, MA, USA), anti-filaggrin antibody (1:100 dilution; BTI) or anti-TBP (TATA-binding protein) antibody (1:1,000 dilution; Abcam, Paris, France). Blots were washed and incubated with Precision Protein StrepTactin-HRP conjugate (1:10,000 dilution; Bio-Rad) and with goat antimouse IgG secondary antibody (1:20,000 dilution; Bio-Rad). Detection was performed using the Immuno-Star™ HRP Western blotting detection system (RPN2132, BioRad). The expression of involucrin, TG-1 and filaggrin was semiquantified with QuantityOne (Bio-Rad) from a scan analysis of the Western blot bands compared with the untreated control, taking TBP as internal reference.

**Immunohistochemical analysis of involucrin, transglutaminase 1 and filaggrin expression in skin equivalents**

The effect of Hydroxydecine® on filaggrin and involucrin synthesis was assessed using Mimeskin®, a three-dimensional model of skin equivalents developed and prepared by Coletica (Lyon) [18]. The skin equivalents were cultured in a keratinocyte medium containing DMEM, 10% foetal calf serum (FCS), 10 ng/mL EGF, 0.12 IU/mL insulin, 0.4 μg/mL hydrocortisone and antibiotics. The medium was changed and supplemented daily with 50 μg/mL L-ascorbic acid (Sigma). After 8 days of culture, mature skin equivalents were topically treated 4 times for 10 days, either with 40 μL/cm² of Hydroxydecine® diluted to 0.01% in caprylic/capric triglyceride (Myritol® 318, Cognis) as vehicle, or with Myrtil® alone (negative control), or with 10 μmol vitamin D3 (positive control). Four skin equivalent samples per treatment were then harvested for immunohistochemical studies.

**Immunofluorescence analysis**

Skin samples were embedded in OCT Tissue-Tek® (Miles, USA). Six-μm frozen sections were obtained with a Frigocut 2800 cryotome (Reichert-Jung), air dried and blocked in phosphate buffered saline containing 1% (w/v) bovine serum albumin. Involucrin, TG1 and filaggrin were detected with mouse monoclonal antibodies directed against human involucrin (1:50 dilution, Nova Castra), human TG1 (1:50 dilution, Harbor Bio-Products, Norwood, MA) and human filaggrin (1:100 dilution, BTI). Goat anti-mouse IgG secondary antibodies (1:50 dilution, Cedarlane, Canada), labelled with fluorescein in isothiocyanate (FITC), were mixed with 0.1% Evans blue to reduce non-specific staining of the sponge network [19]. To control for non-specific labelling, the primary antibody was omitted. Multiple
sections of each specimen (at least 10) were processed to ensure representative samples. Observations were performed double blind by two different persons on at least 10 areas of the section.

**Effect of Hydroxydecine® on inflammatory response and skin barrier function restoration in tissue models**

**Preparation of experimental TSLP-producing and inflamed reconstructed epidermis**

Fully differentiated reconstructed epidermis was maintained in air-liquid interface culture for 7 days, as described by Poumay et al. (2004) [20]. A simple emulsion containing 0.01% Hydroxydecine®, placebo without Hydroxydecine® or the commercially available Ictyane HD® balm was then applied to the reconstructed epidermis (3 mg/cm²). The emulsion contained caprylic/capric triglyceride (Myritol® 318, Cognis, France), Pemulen TR2 (Gattefosse, France) and triethanolamine to adjust the pH to 6.6 (UNIVAR, France). After a 6-hr incubation, a specific inflammatory response was induced by adding double-stranded poly (I:C) RNA (5 μg/mL) and IL-1α (10 ng/mL) to the culture medium. The supernatants were collected 24 hr later and TSLP and IL-8 were assayed by ELISA (TSLP, R&D Systems DY1398; IL-8, PeproTech 900-K18). Triplicate assays were performed using supernatants from three reconstructed epidermis models (n=9 measurements). For each cytokine assay, results were expressed as mean SD. Statistical analysis was done using Student’s t test with an α-risk of 5%.

**Preparation and treatment of experimental inflamed and damaged skin explants**

Normal human skin explants were obtained from plastic surgery (8 donors) and were kept alive according to methods described previously [21, 22]. Briefly, skin explants were cut into 1 cm² fragments and rinsed in phosphate buffer pH 7.4 with added antibiotics. Each fragment was placed with the epidermis uppermost at the air/liquid interface on culture plates. Explants were kept under normal live skin conditions (normal control, untreated and treated inflamed and damaged skin), a mean score for each donor skin explant was obtained from the scores of 10 to 15 selected fields. A global mean value was then calculated from the mean scores of the 8 skin explants.

**Immunohistochemical analysis of filaggrin and claudin-1 expression**

The skin fragments were fixed in formal and embedded in paraffin, then stained with either anti-filaggrin antibodies (1:50 dilution IgG1, 15C10 clone, AbCys) or anti-claudin-1 antibodies (1:100 dilution, rabbit polyclonal, PO4679 clone, AbCys). Immunodetection of filaggrin and claudin-1 was performed using the indirect immunoperoxidase technique on 2 and 3 layers, respectively (ABC peroxidase kit, Vector Laboratories), and detected with 3-amin-9-ethyl carbazole. Filaggrin and claudin-1 expression was quantified by evaluating the staining intensity using a 5-point scale (from 0: negative staining to 4: intense and very significant staining).

**Assay of pro-inflammatory cytokines and nerve growth factor (NGF)**

Cytokines IL-4, IL-5, and IL-13 were assayed in culture supernatants using the ELISA technique (immunoassay kit, USCNLIFE) with spectrophotometric reading of the concentration at 450 nm. NGF assay was performed by the same technique, but skin explants were homogenised in Tris buffer (TrisHCl 100 mM, NaCl 100 mM, 0.1% Triton x100).

**Statistical analysis**

For each parameter results were expressed as mean±SD. Statistical analysis was done using Student’s t test for matched pairs with an α-risk of 5%.

**In-vivo study of the effect of Hydroxydecine® on UV-induced xerosis**

**Patient selection and study design**

This single-centre, non-interventional study evaluated the moisturising effect of Hydroxydecine® in vivo in a model of UV-induced xerosis. In this study, Hydroxydecine® was evaluated as the main active substance in a finished product, a Hydroxydecine® cream (Ictyane® HD intensive cream, Ducray) containing 0.01% Hydroxydecine® in a vaseline/glycerine complex. The study was performed in the Pierre Fabre Skin Research Centre (licensed by the health authorities), Toulouse, France. Written, informed consent was obtained in accordance with the guidelines of the Declaration of Helsinki from the 10 volunteers included (7 women, 3 men), aged 32.9±5.5 years (range 25-42 years), with healthy skin of
phototypes II and IIIa, not using any topical skin treatments and not having sunbathed (natural or artificial sunlight) in the month prior to the study. Pregnant and breast-feeding women were excluded.

The study took place over 5 weeks, including one 12-day period of xerosis induction and one 3-week period of treatment with or without Hydroxydecine® cream. On the day of inclusion (D1) a skin area delineated on the subjects’ back was exposed to UV treatment to determine the minimal erythema dose (MED) the next day (D2). One MED for a patient with a fairly sensitive skin is equivalent to 20 mJ/cm² of effective UV. The UV source used in this study was a xenon arc solar simulator (Multiport 601, 300 Watts, Solar Light Company, Philadelphia, PA, USA) filtered with a dichroic mirror and UV shaping filters (Schott WG320, 1-mm thick short cut-off filter to eliminate the UVC contribution and a 1-mm thick UG11 filter to minimise the visible and IR contributions to the spectral output). The UV source and the complete procedure comply with the protocol described by the COLIPA guideline (COLIPA SPF test method. Ref 94/289. 1994). On D2, two other well-defined skin areas were exposed to a 2-MED dose of UV and a third area was kept as a non-irradiated control. The effect of UV exposure on the onset of xerosis was monitored on D5 and D12 by measuring the moisturising index by corneometry (CM825PC corneometer, Courage and Khazaka).

Treatment was initiated on D12, once xerosis was completely established. Hydroxydecine® cream (2 mg/cm²) was applied topically twice daily, with at least a 6-hour interval between the two applications, for three weeks (from D12 to D30), five days per week, to one of the two irradiated areas in a randomised Latin square design, the other area serving as an untreated irradiated control.

The same experiment was performed to determine the level of natural moisturising factor (NMF). However, as skin from a given area could not be sampled more than once a week for NMF determination, the number of areas had to be tripled to allow sampling at D12, D26 and D33.

Evaluation criteria
The main efficacy criterion was the measurement of the moisturising index of the outer epidermal layers of the skin areas by corneometry, determined on D2, at the beginning of each treatment week, on D12, D19 and D26, and at the end of treatment (D33).

As a secondary criterion the level of NMF in SC was assessed by assaying pyrrolidone carboxylic acid (PCA) in skin surface samples before (D12) and after 2 and 3 weeks of treatment (D26 and D33). At each time point (D12, D26 and D33), a sample was taken from one untreated non-irradiated area, one untreated irradiated area and one treated irradiated area. PCA was assayed by HPLC after derivatisation with para-nitrobenzyl diisopropylurea.

Statistical analysis
For the descriptive analysis of the data, all quantitative variables were expressed as sample size and mean±standard deviation (SD) and qualitative variables as percentage of sample size and frequency. Within-group comparisons were performed by a two-factor analysis of variance (ANOVA) and the time effect was analysed by paired Student’s t or Wilcoxon’s tests. Between-group comparisons were performed by a two-factor ANOVA and the treatment effect was analysed by Bonferroni’s test. For all the analyses a 5% level of statistical significance was used.

Results

Effect of Hydroxydecine® on keratinocyte differentiation markers in vitro

Effect of Hydroxydecine® on the modulation of filaggrin mRNA and protein, and TG1 and involucrin protein expression in a model of keratinocyte differentiation

In our keratinocyte differentiation model, increasing concentrations of Hydroxydecine® of 17 to 100 μM induced filaggrin mRNA expression dose-dependently (figure 1A). Under our conditions, 100 μM Hydroxydecine® increased filaggrin mRNA expression as much as the positive control (1.2 mM calcium).

![Figure 1. Hydroxydecine®-induced filaggrin, involucrin and transglutaminase 1 expression.](image-url)
Western blot analysis showed a dose-dependent increase of filaggrin protein production in NHK treated with 17, 50 and 100 μM Hydroxydecine® compared with untreated cells (1.3-, 2.3- and 3-fold increase, respectively) (figure 1B). Similarly, Hydroxydecine® dose-dependently increased the production of TG1 protein 0.8-, 1.2- and 1.4-fold at 17, 50 and 100 μM, respectively (figure 1B). Calcium at 1.2 mM as a positive control also induced filaggrin and TG1 production 1.3- and 1.9-fold, respectively.

As shown in Figure 1b, the two highest concentrations of Hydroxydecine®, 50 and 100 μM, were capable of markedly increasing involucrin protein expression (1.3- and 1.2-fold increase, respectively), whereas under our experimental conditions calcium-treated cells were not. TATA-binding protein (TBP) expression was used for semi-quantification as a loading control [23].

**Figure 2.** Immunohistochemical analysis of involucrin, TG1 and filaggrin expression in skin equivalents. Immunofluorescence staining of fixed frozen sections with anti-human involucrin monoclonal antibodies (1:50 dilution), anti-TG1 (1:50 dilution) and filaggrin monoclonal antibodies (1:100 dilution) shows an increase in involucrin, TG1 and filaggrin staining in mature skin equivalents topically treated 4 times within 10 days with 10 μmol vitamin D3 (vit D3) or 0.01% HD in Myritol® (HD), compared with skin equivalents treated with 40 μL/cm² Myritol® alone (vehicle) or untreated (control) (scale bar: 100 μm).
Figure 3. Hydroxydecine® alters TSLP release during IL-1α/Poly (I:C)-driven inflammatory response in human reconstructed epidermis.

Human reconstructed epidermis was stimulated with poly (I:C) RNA (5 μg/mL) and IL-1α (10 ng/mL) for 24h. Increased levels of secreted thymic stromal lymphopoietin (TSLP) were detected from stimulated control reconstructed epidermis. TSLP levels were undetectable from unstimulated epidermis. The levels of released TSLP were significantly decreased when reconstructed epidermis was treated with either 0.01% Hydroxydecine® or Ictyane HD® balm containing 0.01% Hydroxydecine® compared with the stimulated control. Shown are triplicate assays performed using supernatants from three epidermal models. Bar: SEM.

IL-1α/Poly (I:C)-stimulated reconstructed epidermis released increased levels of TSLP up to 150±22 pg/mL, whereas TSLP was undetectable in unstimulated epidermis. Pretreatment of the reconstructed epidermis with 0.01% Hydroxydecine® emulsion or the commercially available Ictyane HD® balm containing 0.01% Hydroxydecine® significantly decreased TSLP concentration to 79±9 pg/mL and 83±9 pg/mL, respectively, compared with the stimulated control (p<0.05). By contrast, placebo emulsion induced a slight, non-significant decrease to 99±17 pg/mL. On the other hand, both 0.01% Hydroxydecine® and placebo emulsions significantly decreased IL-8 release induced by IL-1α/Poly (I:C) (data not shown).

Effect of Hydroxydecine® balm on modulation of stratum corneum cohesion

The results of the histological analysis of SC cohesion in normal and in inflamed and damaged skin explants, untreated and treated with Hydroxydecine® balm, are presented in figure 4. Semiquantitative evaluation showed that SC cohesion was significantly decreased in inflamed and damaged as opposed to normal skin explants, with a mean cohesion score of 2.23 ± 0.4 versus 1.26 ± 0.5 (p=0.0003). After treatment of inflamed and damaged skin explants with Hydroxydecine® balm, SC cohesion was restored (1.47±0.5 vs 2.23±0.4; p= 0.0001).

Figure 4. Histological analysis of SC cohesion in normal and inflamed and damaged skin explants.
A) Normal skin explant.
B) Inflamed and damaged skin explant: normal skin explant treated with VIP and SLS.
C) Inflamed and damaged skin explants treated with Hydroxydecine® balm. 5 μm sections were cut, deparaffinised and stained with haemalum-eosin. SC cohesion was evaluated by means of semiquantitative scores on a 6-point scale (0: no decrease in stratum corneum cohesion; 1: slight, 2: moderate, 3: important, 4: severe decrease; and 5: exfoliation of the stratum corneum with separation of the stratum corneum from the granular layer) (scale bar: 20 μm).

Effect of Hydroxydecine® on claudin-1 and filaggrin expression in situ

The results of the immunohistochemical analysis of claudin-1 and filaggrin expression are presented in figure 5. In inflamed and damaged vs normal skin explants, claudin-1 expression was slightly decreased in the granular layer and upper epithelium, and treatment of inflamed and damaged explants with Hydroxydecine® balm tended to restore normal claudin-1 expression levels (figure 5A, B, C). However, this tendency was not statistically significant on
Figure 5. Histological analysis of claudin-1 and filaggrin expression in normal and in inflamed and damaged skin explants, treated or untreated with Hydroxydecine® balm. Immunoperoxidase staining of the fixed skin explant sections with anti-claudin-1 (left-hand column) or anti-filaggrin antibodies (right-hand column).

A) Normal skin explant.
B) Inflamed and damaged skin explant: normal skin explant treated with VIP and SLS. c) Inflamed and damaged skin explants treated with Hydroxydecine® balm for 4 days. (scale bar: 20 µm).

The expression of filaggrin in SC of inflamed and damaged explants also tended to decrease compared with normal skin explants (figure 5A, B), without significant difference between the two conditions (table 1). By contrast, after application of Hydroxydecine® balm to inflamed and damaged explants, a statistically significant increase of filaggrin expression was achieved with a mean intensity score of 3.45±0.5 vs 2.05±0.86 for the untreated inflamed and damaged explants (p<0.01) (figure 5C, table 1).

Table 1. Semiquantitative immunohistochemical analysis of claudin-1 and filaggrin expression in normal and in inflamed and damaged skin explants, treated or untreated with Hydroxydecine® balm.

<table>
<thead>
<tr>
<th></th>
<th>Claudin-1</th>
<th>Filaggrin</th>
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<tbody>
<tr>
<td>Normal skin explants</td>
<td>2.54 ± 1.0</td>
<td>2.26 ± 1.0</td>
</tr>
<tr>
<td>Inflamed and damaged skin explants</td>
<td>1.40 ± 1.2</td>
<td>2.05 ± 0.86</td>
</tr>
<tr>
<td>Inflamed and damaged skin explants + HD balm</td>
<td>1.86 ± 0.78</td>
<td>3.45 ± 0.50**</td>
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</table>

** p < 0.01 Student’s t test comparison with untreated atopic skin explants.

Inflamed and damaged skin explants were obtained by treating normal skin explants with VIP and SLS. Hydroxydecine® (HD) balm was applied for 4 days to the surface of inflamed and damaged skin explants. Fixed skin explant sections were stained by immunofluorescence with anti-claudin-1 (1:100) and anti-filaggrin (1:50) antibodies. Results are expressed as mean scores ± SD of staining intensity calculated on a 5-point scale (0: no staining to 4: very intense staining). Semiquantification results from the skin explants of 8 different donors.

Effect of Hydroxydecine® on secretion of NGF and pro-inflammatory cytokines IL-4, IL-5 and IL-13

A significant increase in IL-4 and IL-5 secretion was observed in inflamed and damaged skin explants compared with normal skin (p=0.016 and p=0.02, respectively), whereas IL-13 secretion did not vary (table 2). After applying Hydroxydecine® balm, a statistically significant decrease of the 3 cytokines was achieved relative to untreated inflamed and damaged skin (p<0.05 in all cases). NGF secretion was also significantly increased in inflamed and damaged versus normal skin explants (p<0.01) and application of Hydroxydecine® balm to inflamed and damaged skin explants decreased this secretion slightly but non-significantly (table 2).

In vivo study of the hydrating effect of Hydroxydecine® cream in a model of UV-induced xerosis

Effect of Hydroxydecine® cream on skin hydration

No significant difference in skin hydration was observed between the different measurement areas before UV induction of xerosis (D2). After xerosis induction, a significant time effect was observed for the hydration index at D5 and D12 in the irradiated areas, but not in the untreated control area (figure 6). The hydration index of irradiated areas decreased from D2 until D12, corresponding to the onset of UV-induced xerosis.

From the beginning of the treatment period with Hydroxydecine® cream (D12), the hydration index of the treated skin significantly improved with a mean increase of +28.8% on D19 (p<0.01) and continued to increase over time, reaching +60.4% on D33, the end of the study (p<0.001) (figure 6). Compared with the untreated irradiated control, hydration of the treated skin from D12 was 9.1% higher after one week of treatment (p<0.05) and remained significantly higher at D26 (+12.2%) and D33 (+10.1%) (p<0.05 for both).

Assay of PCA concentration

After UV irradiation, the PCA concentration in the superficial layers of stratum corneum increased over time. At D12, compared with the untreated non-irradiated area, the PCA concentration in the untreated irradiated skin was 16.6% higher (0.70±0.22 vs 0.60±0.23 µg/µg total...
Table 2. Assessment of secretion of NGF and pro-inflammatory cytokines IL4, IL5 and IL13 by normal and by inflamed and damaged skin explants, treated or untreated with Hydroxydecine® balm.

<table>
<thead>
<tr>
<th></th>
<th>IL-4 (pg/mL)</th>
<th>IL-5 (pg/mL)</th>
<th>IL-13 (pg/mL)</th>
<th>NG-F (pg/mL)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Normal skin explants</td>
<td>14.2±9.0</td>
<td>0.25±0.29</td>
<td>2.00±0.25</td>
<td>38.1±7.6</td>
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<tr>
<td>Inflamed and damaged skin explants</td>
<td>22.2±12.9*</td>
<td>1.35±0.92#</td>
<td>1.97±0.28</td>
<td>56.1±12.3#</td>
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<tr>
<td>Inflamed and damaged skin explants+HD balm</td>
<td>18.8±10.8#</td>
<td>0.21±0.18*</td>
<td>1.66±0.29</td>
<td>43.9±20.1</td>
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# p<0.05, paired Student’s t test comparison with normal skin explants.
* p<0.05, paired Student’s t test comparison with untreated atopic skin explants.

Inflamed and damaged skin explants were obtained by treating normal skin explants with VIP and SLS. Hydroxydecine® (HD) balm was applied for 4 days to the surface of inflamed and damaged skin explants. Cytokines were assayed in culture supernatants and NGF in a skin explant homogenate by ELISA. Results are the mean of skin explants from 8 different donors.

Discussion

Taken together, our studies demonstrated the effects of Hydroxydecine® on skin barrier restoration and epidermal skin layer hydration. Our results also suggest that it may be of potential value in the treatment of certain dermatoses associated with skin barrier dysfunction and inflammatory dysregulation. In an in vitro model of cellular differentiation of human keratinocytes, Hydroxydecine® was capable of promoting the differentiation of keratinocytes into functional corneocytes. It stimulated involucrin, TG1 and filaggrin synthesis in the same proportion as calcium used as a positive control. These results demonstrate that Hydroxydecine® can induce NHK differentiation dose-dependently in a cellular differentiation model in vitro.

The stimulant effect of Hydroxydecine® on epidermal differentiation was confirmed in another in vitro model of reconstructed skin. Immunohistochemical analysis of SC of reconstructed skin showed that topically applied Hydroxydecine® could promote a similar increase in involucrin, TG1 and filaggrin protein detection compared with the vehicle. These 3 molecules are normally localised in skin layers for their correct functionality in tissue. Filaggrin, involucrin and TG1 are synthesised in the terminal phases of keratinocyte differentiation and play a major role in skin barrier function. They are involved in the formation of the cornified cell envelope (CE), a scaffold-like structure consisting of a layered protein aggregate beneath the corneocyte plasma membrane that forms an effective skin barrier [3, 25]. Filaggrin is responsible for aggregation of keratin intermediate filaments, which causes the cells to collapse and results in the formation of flattened corneocytes [3]. Concurrently, involucrin and other structural proteins (loricrin, plakins, etc.) are synthesised and subsequently cross-linked by the action of transglutaminases to reinforce the CE [26]. TG1 catalyses the formation of intermolecular (γ-glutamyl) lysine bonds and its absence is associated with impaired expression of the different proteins in epidermal differentiation [27, 28]. Involucrin is adjacent to the cell membrane and serves as a substrate for the covalent attachment of ceramides catalysed by TG1 to form the outer surface of the CE [28].

![Figure 6](image-url)  

**Figure 6.** Time course of xerosis in treated and untreated irradiated skin areas in healthy volunteers. Xerosis was induced by UV-treatment on D1 and D2. Treatment was initiated on D12, once xerosis was established, and continued until D33. The hydration index was measured by corneometry at all time points.
Consequently, by stimulating the expression of these 3 proteins, Hydroxydecine® may increase keratinocyte differentiation and CE formation, thereby improving skin barrier function. By activating filaggrin synthesis, it may also maintain a sufficiently hydrating environment. Indeed, filaggrin, which has a 6-hour half-life, is degraded mostly into free amino acids in cornified cells [3]. These hydrophilic amino acids are components of NMF. Their high concentration is therefore essential for the retention of water and contributes to the osmolarity and flexibility of the cornified layer [3].

The value of Ictyane Hydroxydecine® balm in the treatment of barrier dysfunction and water loss associated with dermatoses was then assessed in an experimental model of inflamed and damaged skin, using surviving human skin. The results showed that the balm containing Hydroxydecine® was able to restore SC cohesion, which was significantly decreased in inflamed and damaged surviving skin compared with normal skin. Treatment with SLS and VIP, both conditions which impaired skin barrier and induced inflammation, promoted a decrease of claudin-1, another structural protein involved in tight junctions. This protein contributes to the barrier function of stratified epithelia by regulating the selective permeability of the paracellular pathway [11, 29]. It is expressed in differentiating keratinocytes and accumulates in tight junctions, where it forms one of the major cell adhesion molecules [30]. Furthermore, a recent clinical study suggested that tight junction impairment might be involved in cutaneous barrier and immune dysfunction in AD patients [12]. This phenomenon may be mediated in part by claudin-1, as claudin-1 expression was only reduced in AD patients as opposed to healthy subjects and claudin-1 expression was negatively correlated with Th-2 markers [12].

In the inflamed and damaged skin explant model, Hydroxydecine® balm tended to restore normal claudin-1 expression. Therefore, Hydroxydecine® balm may be of value in decreasing transepidermal water losses in atopic skin by enhancing claudin expression and reinforcing cellular tight junctions. In this model we also demonstrated the anti-inflammatory effect of Hydroxydecine® balm which significantly decreased the secretion of pro-inflammatory cytokines (IL-4, IL-5 and IL-13) following application of the balm to inflamed and damaged surviving skin. IL-4, IL-5 and IL-13 are overexpressed by Th2 cells that predominate in AD. Th2 cytokines upregulate IgE production and are responsible for the systemic allergic response in atopic patients. Furthermore, recent findings have shown that keratinocytes differentiated in the presence of IL-4 and IL-13 exhibited significantly reduced filaggrin gene expression [31]. This suggested that the atopic immune response could contribute to the defect in filaggrin expression in atopic patients. In our model of inflamed and damaged skin, the increase of Th2 cytokine secretion was correlated with a decrease in filaggrin expression in the SC. Conversely, the decrease of IL-4, IL-13 and IL-5 secretion from inflamed and damaged surviving skin after treatment with Hydroxydecine® balm was associated with an increase in filaggrin and claudin-1 expression in SC. Therefore, as our results suggested that Hydroxydecine® balm could restore normal filaggrin and claudin-1 expression and reduce the production of some pro-inflammatory biomarkers, it may attenuate the amplification cycle in some disorders characterised by epidermal inflammatory xerosis, such as AD.

To further assess the potential value of topically applied Hydroxydecine® in the epidermal responses associated with the pathogenesis of many inflammatory diseases, we used an inflamed epidermal model of TSLP induction. In their review, Ziegler and Artis [32] highlight the pivotal role of this epidermal cytokine in the co-ordination of effector functions of many myeloid lymphoid populations. TSLP can actively drive a Th2 cytokine response, potentially through effects on dendritic cells, granulocytes, natural killer and CD4 + T cells. This TSLP function could make it a likely therapeutic target for the treatment of allergic diseases. In our tissue model, TSLP induction was stimulated by poly (I:C), a TLR3 ligand, and the primary cytokine IL-1α. Indeed, it has been previously demonstrated that a TLR3 ligand associated with different inflammatory cytokines could contribute to skin inflammation, in particular through the increase of TSLP, which plays a critical role in the pathogenesis of AD [24]. Our results showed that when a Hydroxydecine® formulation was topically applied to inflamed epidermis, TSLP release was more extensively hampered than with the vehicle alone. This suggests that topical delivery of Hydroxydecine® to the skin is appropriate for triggering a specific epidermal modulation of TSLP induction. The Ictyane Hydroxydecine® balm used in the ex-vivo model also showed an inhibitory effect on TSLP induction by poly (I:C)-IL-1α.

The results obtained in vivo in a model of UV-induced xerosis developed in healthy skin volunteers corroborated these in-vitro and ex-vivo data by demonstrating the crucial role of filaggrin as a marker of balanced barrier function and the ability of the skin to adapt to pathophysiological or environmental variations. We demonstrated the efficacy of a Hydroxydecine®-based cream in rapidly increasing hydration of UV-treated skin. We also showed that xerosis induced by UV radiation persists for 3 weeks and is associated with an increase in the concentration of PCA, a component of NMF, in the SC. This increase illustrates the normal adaptive response of the skin to xerosis induction in order to restore balanced hydration. Indeed, the skin is a dynamic system and its capacity for response is modulated by environmental factors. To maintain hydration levels, the skin reacts to any desiccating action by increasing NMF production. This phenomenon is observed in aged skin, the main characteristic of which is xerosis [33]. In our study we showed that, by preventing the increase of UV-induced PCA concentration, treatment with a Hydroxydecine®-based cream can promote a better adaptive response of the skin to xerosis induction and incidentally improve cutaneous comfort. Although our in-vivo study does not allow the moisturising effect of Ictyane® HD to be ascribed to Hydroxydecine® with certainty, all in-vitro and ex-vivo data support the hypothesis that this compound may be useful in increasing the adaptive response of the skin to environmental desiccating stress, in particular in AD, ichthyosis vulgaris or acquired xerosis, through improvement of barrier function and epidermal cohesion markers.

Thus, by supporting physiological mechanisms in maintaining a healthy skin barrier and by modulating some
inflammatory components, a Hydroxydecine®-based product may be used for the treatment of xerosis associated with AD, age, and psoriatic conditions. Moreover, as the use of emollient therapy is proposed for skin barrier repair to prevent the onset of cutaneous inflammatory disease [34], Hydroxydecine®-based cream and balm could be appropriate and effective candidates.

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