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Use of Reconstructed Skin Specimens to Analyze *Stratum corneum* Remodeling and Epidermal Modifications

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1. Introduction

The epidermis is the epithelial tissue lining the dermis. It is constituted of differentiating keratinocytes distributed in successive layers according to their degree of differentiation. It contains cycling cells with neurological, immune and sensorial functions, such as Langerhans cells, melanocytes, Swan cells, Merkel cells. The upper layers of the epidermis are constituted by fully differentiated corneocytes which constitute the *stratum corneum* or horny layer.

The *stratum corneum* constitutes the interface between the body and the outside world. It modulates the exchanges of oxygen and water vapor with the environment. It harbors enzymes which contribute to its healthy maintenance. Some enzymes help the steady state renewal of the surface by cutting the inter-corneocyte bonds and allowing outer-layer corneocytes to be shed. Others catalyze the synthesis of factors of moisturization, such as urea. Other enzymes such as RNase fight viral infections while the colonization of the skin by film forming microorganisms is averted by proteases like trypsin and chymotrypsin. Defensins, specific peptides in the *stratum corneum* originating from sebaceous gland, exert an efficient anti-bacterial action.

The *stratum corneum* reflects and refracts visible light and is thus partially responsible for the image of the individual in the eyes of the observer. When the *stratum corneum* is not in its optimal state, it is felt as conferring dryness, roughness, lack of flexibility, or general discomfort to the skin. Learning about the structure of the *stratum corneum*, and about ways to modify it to achieve a smooth, supple, flexible, extensible, translucent, shiny surface is one of the objects of surface biochemistry and of cosmetically-oriented scientific research.

Reconstructed skin specimens can be prepared by mixing fibroblasts in a collagen suspension, allowing it to gellify and seeding keratinocytes on the top of it. Air exposed keratinocytes do differentiate and the cells can be kept alive for several days by feeding growth medium underneath the specimen (Prunieras *et al*, 1983).

It is possible to explore the structure of the *stratum corneum* of reconstructed skin and to investigate the chemical or physical-chemical parameters which contribute to its structure. The absence of sebaceous glands, sweat glands, blood vessels or nervous endings makes it

simpler to study the effect of particular chemicals on the structure of the horny layer because of the absence of components difficult to control such as sebum lipids or sweat salts. Reconstructed skin specimens can also be used to assess the synthesis of molecules relevant to the structure and to the physiology of keratinocytes in the latest stages of differentiation. For instance, filaggrin (**fil**ament **aggr**egating prote**in**) is derived from the maturation of Pro-filaggrin which is a major component of keratohyalin granules (Sandiland *et al*, 2009, Chen *et al*, 2008). During differentiation, Pro-filaggrin is dephosphorylated and cleaved by serine proteases to form monomeric filaggrin which then binds to and condenses keratin filaments to trigger squame biogenesis. Within the squames, filaggrin is citrullinated by peptidyl-arginine de-aminases. This promotes its unfolding (from keratin) and further degradation by Caspase 14 to generate hygroscopic amino acids in the biogenesis of the so called Natural Moisturizing Factors, to which urea is eventually added, as the final catabolite of arginine *via* the action of arginase. Reconstructed skin specimens can be treated with biochemical moieties to explore their effect on filaggrin maturation and degradation.

Reconstructed skin specimens can also be prepared to contain melanocytes mixed with keratinocytes (Klausner *et al*, 1995). These specimens (MelanoDerm) allow one to explore the effect of specific treatments on the synthesis, the turnover and the overall accumulation of melanin. Melanocytes in the basal layer spontaneously produce melanin, allowing the specimens to progressively darken.

2. Materials and methods

2.1 Analysis of the stratum corneum

Specimens of reconstructed skin were purchased from MatTek Corporation (Ashland, MA) and were as described (Hayden *et al*, 2003). Upon reception, the specimens were dipped in growth medium EFT-400-ASY as provided by MatTek Corp, and incubated overnight at 37° C in a CO_2 oven. After incubation, the growth medium was removed and the test material, dissolved in water or ethanol, was added (40 µl per specimen) and allowed four hours at room temperature. After this incubation, as much as possible of the 40 µl were removed with a micro-pipette and the specimen fixed with 2.5% glutaraldehyde in water, for 44 hours, then stored in 70% ethanol and processed for histology analysis as described (Sheehan & Hrapchak, 1987, or in the Manual of Histologic Staining Methods of the Armed Forces Institute of Pathology -Third Edition). Upon punching a "biopsy" in the specimen of treated or untreated reconstructed skin, fixed with 2.5% glutaraldehyde, MatTek rafts were removed from ring and placed in a tissue cassette. Samples were processed through Ethanol, xylene, and paraffin for 11 hours on a TissueTek VIP tissue processor. Samples were bisected and embedded in Blue Ribbon Embedding Media (cat# 3801360) from Surgipath (Richmond, IL). Sections were cut at 5 microns on a RM2135 microtome from Leica Microsystems (Bannockburn, IL) and placed on Surgipath Apex glass slides (cat# 3800082). Slides were heated overnight at 70 degrees C. Slides were stained with Hematoxylin and Eosin-Phloxine (cat #s S2697 & S176) from Poly-Scientific (Bay Shore, NY) and coverslipped with resinous mounting media. Microscopy analysis allows one to assess the percent of "damaged" surface which is visible in one microscope field. Figure 1 displays typical histology images. In our experimental conditions (magnification x10), every section was comprised in four fields and eight sections per biopsy were examined. The percent damage in every field (determined as described in the legend of figure 1) were added to yield a number, the parameter of damage or d-value, which is equal to or larger than zero and smaller than or equal to 3,200 ($0 \leq d \leq 3{,}200$).

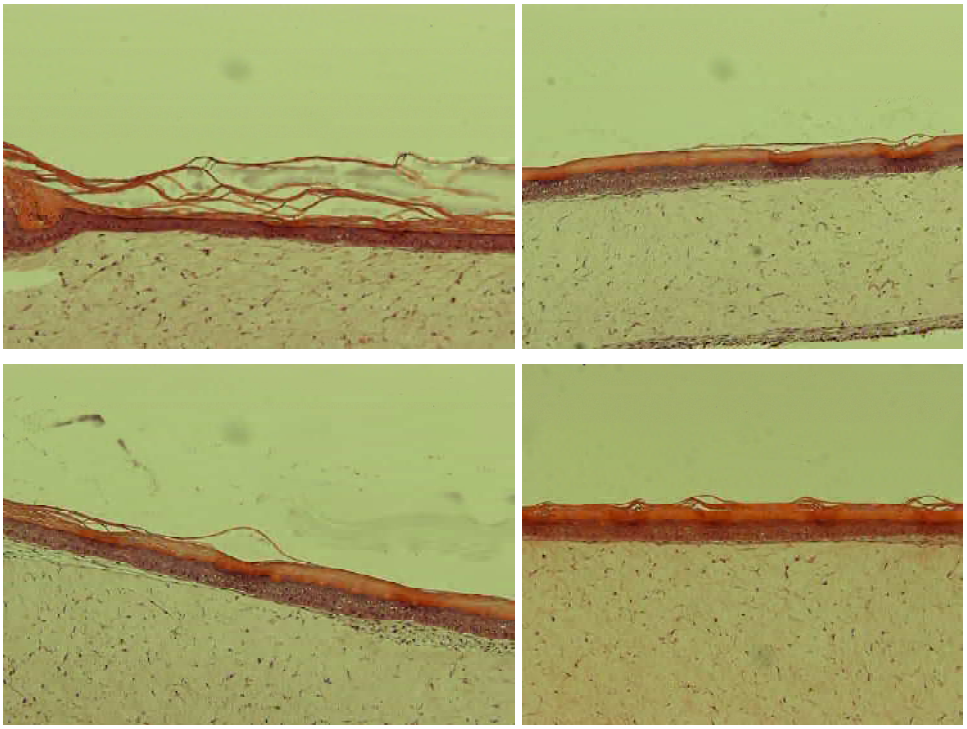


Fig. 1. Calculating d-value from histology images. The percent of surface damage in a field can be determined while observing the sections under the microscope. The top-left image has 100% surface damage, the top-right image has ~57% surface damage, the bottom-left image has ~52% damage, and the bottom-right image has 42% damage. The sum of the percent damage per field, summed over the 32 fields of eight sections, yields the damage parameter or d-value.

2.2 Surface remodeling

2.2.1 Chemicals used for surface remodeling experiments

Acrylates: Daitosol 5000 AD, Daitosol 5000 SJ, Daitosol 4000 SJT and Daitosol 5000 STY (Acrylates/Ethylhexyl Acrylate copolymers and Acrylates co-polymers) were from Kobo (South Plainfield, NJ). Galactorabinan was from Elemente (Jericho, NY). Styleze was from International Specialty Products (Wayne, NJ) and Simulgel was from Seppic (Paris, France).
Alkylated silicones. SF1632 and SF 16 42 were from Kobo (South Plainfield, NJ).
Ceramides. ω-3 ceramides from linen, ω-6 ceramides from wheat, primrose, cotton and safflower, as well as ω-9 ceramides were from Solabia (Pantin, France) and Lipowheat (total ceramides from wheat) were from Hitex-Lavipharm (Pentaparc, France).
Polysaccharides and muchopolysaccharides. Hyaluronic acid was from CPN (Dolni Dobrouc, Czech Republic). Unitamuron (poly-saccharide), and Unichondrin (mucho-poly-saccharide) were from Induchem (Volketswil, Switzerland). Fucogel, Glycofilm, Glycolift, Fucocert were from Solabia (Pantin, France), Fucoidan YSK was from Yaizu Suisankagaku Industries (Shizuoka, Japan) Crystalhyal, Stimulhyal and Soligel were from Soliance

(Allendale, NJ), Clearogel (scleroglucan) was from MMP (Plainfield, New Jersey). Maltrin (maltodextrin) was from GPC (Muscatine, Iowa).

Hydrolyzed proteins. Sericin, Silk Peptides, Silk Powder and Silk Amino Acids were from Sandream Enterprises (Chatam, NJ).

Proteins and Protein/polysaccharide mixtures Uniprosyn (oat proteins) was from Induchem (Volketswil, Switzerland). Reductine and Tensine were from Silab (Brive, France). Argatensyl was from Cognis (Pulnoy, France). Vegetensor was from Alban Muller (Vincennes, France). Functional keratin was from Keratech (Christchurch, NZ).

Hydrogenated poly-isobutenes. Perleam and Perleam 4 were from Rossow USA (Highland, NJ)

Lecithins and Hydrogenated Lecithins. Cerasome and Lipoid SLM were from Lipoid GmbH (Ludwigshafen, Germany). Liposomes with Green Tea, White Tea, Aloe Vera, Guarana, White Hibiscus were from Cosmetochem (Steinhausen, Switzerland), Merospheres were from AGI-dermatics (Freeport, NY). LipidureA, Lipidure B, Lipidure PMP,Lipidure NR and Lipidure NA were from Rossow USA (Highlands, NJ). Edemin was from Res Pharma (Trezzo d' Adda, Italy).

Exfoliators. Salicylic acid encapsulated in liposomes under the trade names Catezomes (~20% salicylic acid) or Salisomes (~10% Salicylic acid) was from BASF (Florham Park, NJ)

Lipids and Lipido-peptides Pellicer was fromAsahi Kasei Corp (Tokyo, Japan). N-Acetyl-L-Hydroxyproline was from Kyowa Hakko Bio Co.-Presperse (Somerset, NJ). Vitaskin E was from Solabia (Pantin, France). Buckwheat (capric/caprylic acid triglyceride) was from Barnet (Englewood Cliffs, NJ), Linoleamide was from Solabia (Pantin, France). Skinmimics was from Evonik (Essen, Germany). Lutein was from Kemin, Zenigloss was from Ultra (Red bank, NJ). Phytantriol (tetra-methyl-hexadecane) was from DSM (Kaiseraugst, Switzerland). Koboguard 5400 SQ (hydrogenated polycyclopentadiene) was from Kobo (South Plainfield, NJ).

Saccharides. Pulpactyl, Cohesium and Recoverine were from Silab (Brive, France)

Surfactants and Emulsifiers. Arlacel 165 (glyceryl stearate) was from Uniqema (New Castle, De). Olivoil glutamate was from Kalichem (Botticino Sera, Italy). Net-Won (tetra-alkyl-ammonium hectoride, polyglyceryl isostearate, polyglyceryl-6-polyricinoleate) was from Barnet (Edgewood Cliffs, NJ). Neogloss (isodecyl neopentanoate) and Crystalcast (ß-sitosterol, cetyl alcohol, stearyl alcohol, sucrose stearate/distearate) were from MMP (Plainfield, NJ). Dermofat (octadecanoic acid) was from Alzo International (Sayreville, NJ).

Gums. Easyliance (Acacia Senegal Gum and Hydrolyzed Rhizobian Gum) was from Soliance (Allendale, NJ)

Succinamides. Chitosan succinamide was from MMP (South Plainfield, NJ)

Poly-imides. Aquaflex was from International Specialty Products (Wayne, NJ)

Silanol-polysaccharides and PEG-silicones. Zenester was from Ultra (Red Bank, NJ). Epidermosil was from Exsymol (Montecarlo, Monaco).

Polyurethanes. Baycusan 1000, Baycusan 1003 and Baycusan 1004 were from Bayer (Leverkusen, Germany).

Plant aqueous extracts Dansonyl, Dulcemin and Vegeseryl were from Cognis (Pulnoy, France), Caviar Lime, Pepperberry, Tasmanian pepper, Riberry, Kakadu, Illiwara and Bush Plum were from Southern Cross Botanical (Knockrow, NSW, Australia). Dragon was from IBR (Rehovot, Israel).Hydra Kanzu and Ejitsu Rose were from Barnet (Englewood Cliffs, NJ)

2.2.2 Procedure to test

The test materials were applied on the top of MatTek specimens at concentrations ranging between 1 and 3 % in water (w/v). In some instances concentrations were kept lower (0.1%) to avoid excessive viscosity or gels. In some instances (e.g. with poly-acrylates) dose-response curves were generated, with concentrations ranging from 3% to 15%. Plant-derived ceramides were not soluble in water or in ethanol, so the tested material was the supernatant of a dispersion of those materials at 1% in ethanol.

2.3 Analysis of filaggrin catabolism and metabolism

Altermonas macleodi exopolysacharrides were from Unipex Innovations (Quebec, Canada) and Aquaflex (water extract of *Citrullus vulgaris*) was from Barnet (Englewood, NJ). They were dissolved at 1% or 3% in growth medium and were applied on the top of the MatTek specimens in a 100 µl volume

Upon incubation at 37 C for 2-24 hours, the specimens were fixed with 10% Formalin for 24 hours at room temperature and sections were prepared for immunohistochemistry analysis. Sections were treated with citrate buffer and heated to achieve antigen retrieval and incubated with commercially available anti-filaggrin antibody from Abcam (Cambridge, UK) followed by incubation with the secondary antibody, Vulcan Red alkphos chromagen and finally counterstained with Hematoxylin/Eosine.

2.4 Analysis of melanocyte-containing reconstructed skin

2.4.1 Tissue culture

Melanocyte-containing epidermis on dermis equivalent MelanoDerm) was as described (Klausner *et al*, 1995, Klausner, 1997). MelanoDerm B inserts (Lot #10790, Kit E & Lot #11625, Kit B) from MatTek (Ashland, MA) were stored at 4°C upon receipt. The inserts were incubated in 1 mL of maintenance media (Cat#EPI-100-NMM-113, Lot# 012309TTF & Lot# 040909TTE, MatTek) for 1 h at 37°C in a 5% CO_2 humidified incubator. Then the inserts were placed on top of culture stands (Cat# MEL-STND, MatTek) in 5 mL maintenance media in 6 well plates. The inserts were kept in the incubator when not receiving treatments or being photographed. Maintenance media were changed every other day.

2.4.2 Chemicals and treatments

Cyclohexadecanol was purchased from Barnet (Englewood, NJ). Kojic acid was from Sigma (Saint Louis, MO). The inserts were treated with non-ionic, oil in water emulsions containing 1% Cyclohexadecanol or 0.5% Cyclohexadecanol or 2% Kojic Acid daily (except for weekends) for 21 days. 2.3 µl of each emulsion were pipetted so that the amount of lotion left on the insert was 2 µl after application with the tip of a sterile glass rod. Prior to application, the inserts were washed by vigorously pipetting Phosphate Buffered Saline (PBS) (Cat#DPBS-100, Lot# 043007tvka, MatTek) on the surface, with two changes of PBS.

2.4.3 Microscopy

Each day the inserts were observed under 300x magnification with a Nikon Diaphot (Ontario, NY) microscope. Pictures were taken daily after washing and prior to treatment. Each insert was placed in a sterile 24-well plate, a small amount of PBS was pipetted below

the insert, and then the plate was placed on to the microscope stage. The intensity of the melanin in the melanocytes was analyzed with the D.E. Light program. Macroscopic photographs were also taken with an Olympus SZH10 stereo microscope at 0.7X magnification.

2.4.5 Histology

After 14 days the inserts were rinsed in PBS and then fixed by incubating overnight in 10% formalin (Cat# HT501128) from Sigma (Saint Louis, MO) at 4°C. Then the inserts were placed in small containers filled with PBS and sent to Paragon Bioservices Inc (Baltimore, MD. for sectioning and staining with the Fontana-Masson stain. Pictures were taken at 400X with an Olympus BX60 microscope. The area of the section occupied by melanin was determined with the D.E. Light program.

2.4.6 Viability

The alamar blue viability test was performed as described (Ahmed *et al*, 1994; Hamid *et al*, 2004). A 10% alamar blue solution was made by combining 12 mL alamar blue (Cat#DAL1100, Lot# 149661SA) from Invitrogen) (Carlsbad, CA) with 108 mL maintenance media. The maintenance media was removed from each well and replaced with 5 mL of 10% alamar blue in each well. The inserts were incubated in 10% alamar blue for 2 h and then removed. The fluorescence of the medium in each well was measured on the Spectra Max Gemini XPS fluorescent plate reader (Molecular Devices, Sunnyvale, CA) at 530 nm excitation and 590 nm emission.
The viability test was done every week.

2.4.7 Melanin quantitative assay

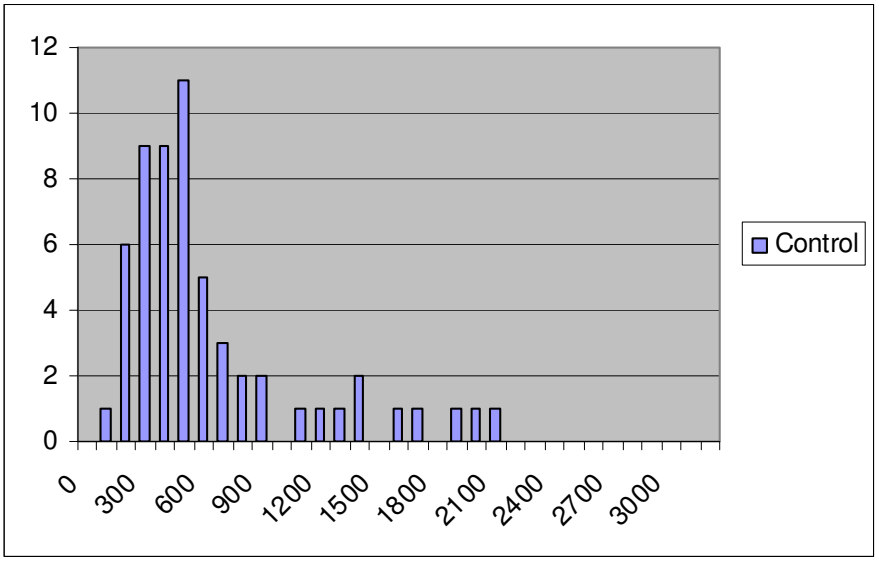
After treatment with test materials and incubation for several days (up to three weeks) the specimens were removed from the plastic insert and placed in 250 µl Solvable (Cat#6NE9100, lot#140-090101) from Perkin Elmer (Waltham, MA) in a 1.7 mL microfuge tube. Tissues were incubated overnight at 60°C. Samples were vortexed and then centrifuged at 13,000 rpm for 5 minutes. 200 µl of the samples were pipetted into a 96-well plate read at 490 nm on the spectrophotometer and measured against melanin standards.
For spectro-photometrical measurements of melanin, tissues were removed from the plastic insert and placed in 250 µl Solvable in a 1.7 mL microfuge tube, incubated overnight at 60°C, vortexed and then centrifuged at 13,000 rpm for 5 minutes. 200 µl of the supernatant of every sample were transferred into the wells of a 96-well plate and read at 490 nm on the spectrophotometer Spectramax 190 (Molecular Devices, Sunnyvale, CA) and measured against melanin standards.

3. Results

3.1 Structure of the *stratum corneum*

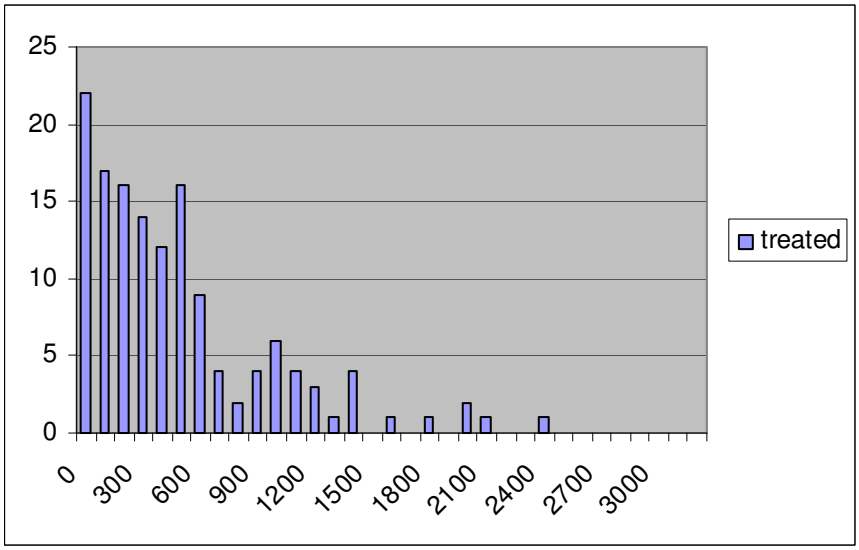
The d-values of the *stratum corneum* of 58 untreated specimens displayed a unimodal distribution centered around a d-value = 500 with a 90 % confidence interval situated between 200 and 1,500 (Figure 2). Treating the specimens with water or ethanol did not result in striking differences as far as the d-values are concerned (data not shown). The distribution histogram for all the treated specimens (n=140) is reported in Figure 3. This

histogram represents a multi-modal distribution centered at 0-100, 500 and 1100. This result seems to indicate that certain treatments might indeed reduce the d-value of the untreated specimens, whereas other treatments might damage the surface or leave it as in the control. In order to point out treatments which efficiently reduce the d-values of the surface, the distribution histograms of the d-values after treatment with substances belonging to the same chemical family, have been plotted (Figures 4 - 8). Treating the specimens with specific chemical families such as some polysaccharides, hydrogenated lecithins, plant-derived ceramides and proteins associated to polysaccharides resulted in a reduction of the d-values to values comprised between 0 and 200, as displayed in Figures 4-7. Two alkylated silicones gave d-values d=0. (data not shown). The addition of salicylic acid-containing liposomes (an exfoliator) also dramatically reduces the d-value (data not shown). On the other hand, acrylates or polyacrylates (Figure 8), hydrolyzed proteins, mono- or oligosaccharides, hydrogenated poly-isobutenes, styrene-acrylate co-polymers (data not shown) do not reduce the d-value, and sometimes they increase it. In some instances, detergents and silicones provide d-values close to zero, but detergents also dramatically reduce the thickness of the *stratum corneum* and the treatment with some silicones seems to provoke pyknotic nuclei and periplasmic edema in a majority of keratinocytes below the horny layers (data not shown).



The plot represents a uni-modal distribution centered at d=500

Fig. 2. Histogram of distribution of d-values for 58 untreated controls.



The plot represents a multi-modal distribution centered at 0-100, 500 and 1100.

Fig. 3. Histogram of distribution of d-values for 140 differently treated samples.

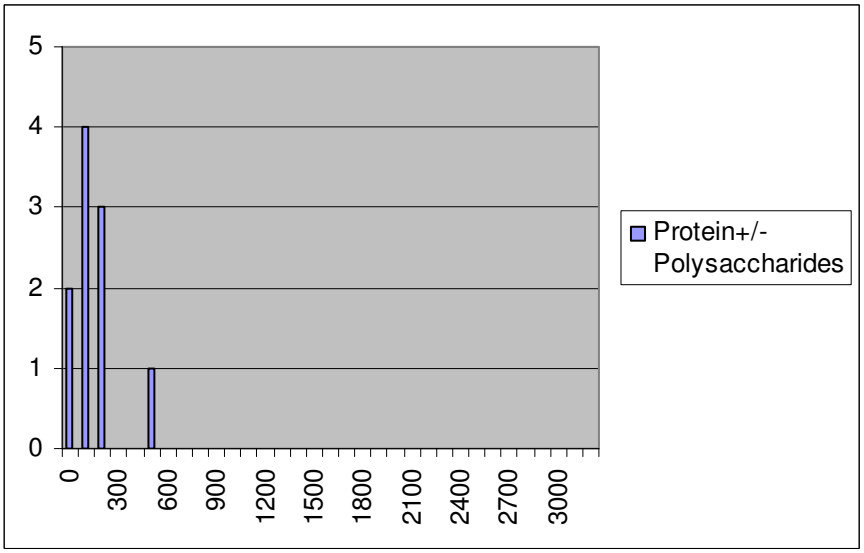


Fig. 4. Histogram of distribution of d-values for specimens treated with ten protein-polysaccharide mixtures.

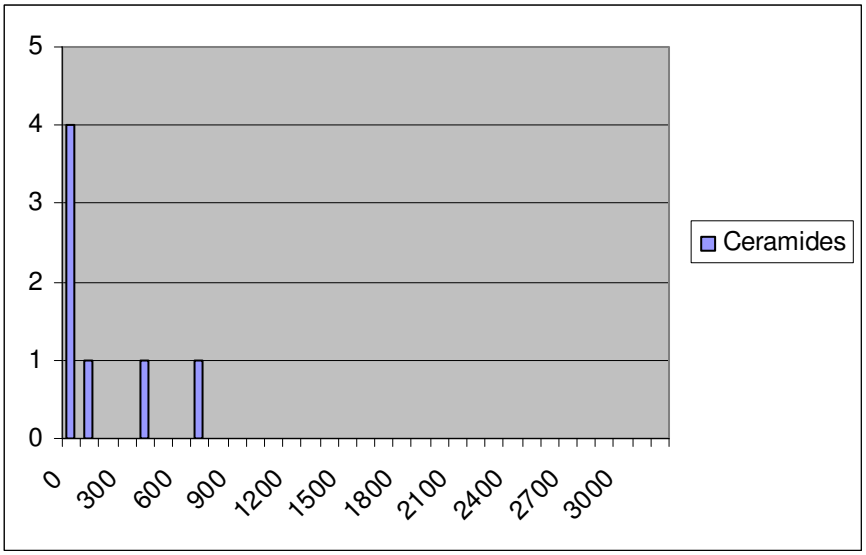


Fig. 5. Histogram of distribution of d-values for specimens treated with seven plant-derived ceramides

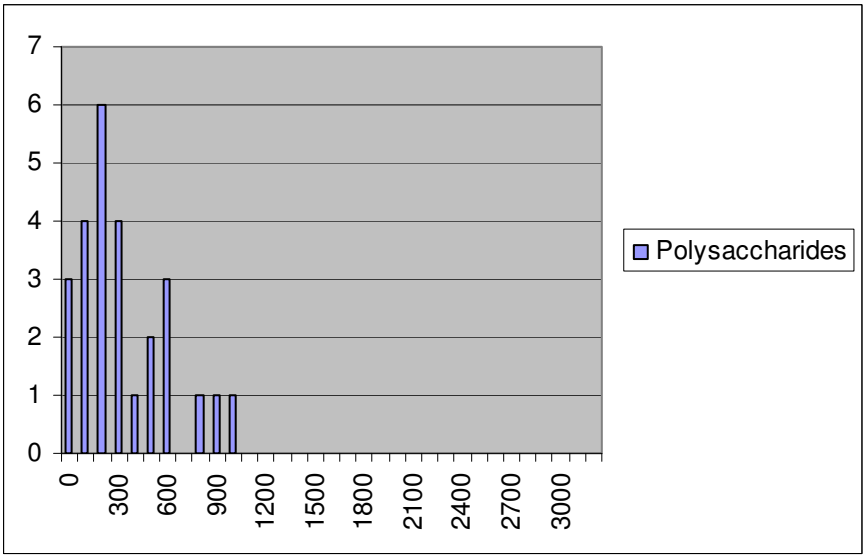
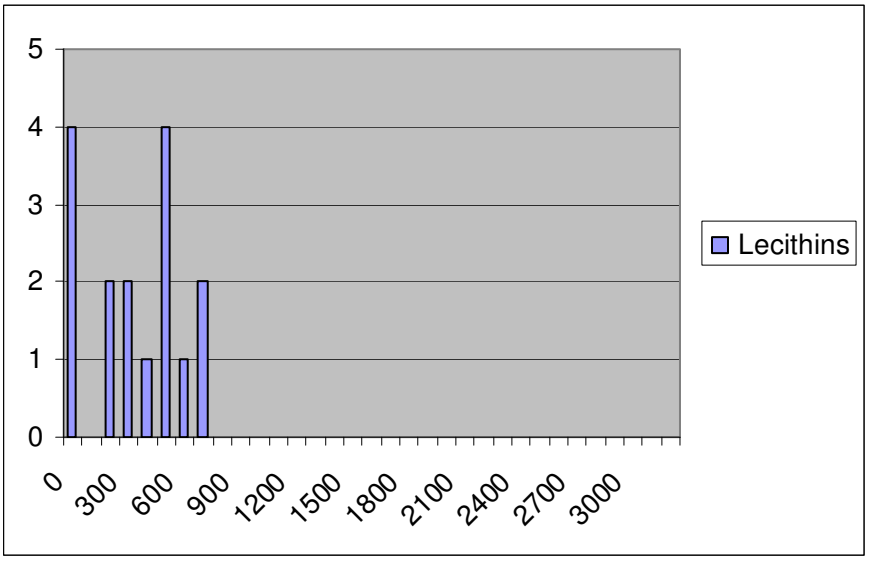


Fig. 6. Histogram of distribution of d-values for specimens treated with 26 polysaccharides



The three hydrogenated lecithins used in this study gave d-values d= 0 or 83 or 252.

Fig. 7. Histogram of distribution of d-values for specimens treated with 16 lecithins.

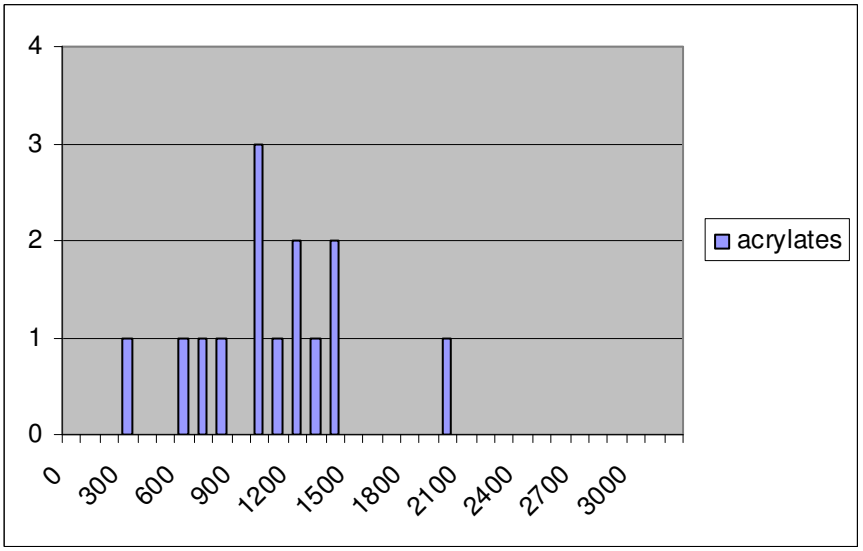


Fig. 8. Histogram of distribution of d-values for 14 specimens treated with acrylates or polyacrylates.

3.2 Analysis of filaggrin

Filaggrin (filament aggregating protein) matures from pro-filaggrin which is a major component of the keratohyalin granule. During the maturation process, pro-filaggrin is dephosphorylated and cleaved by serine proteases to form monomeric filaggrin which binds to and condenses keratin filaments to trigger squame biogenesis (Figure 9a). We have observed that 2 and 24 hours after topical treatment with bacterial exo-polysaccharides, the *stratum corneum* staining associated with filaggrin was dramatically increased (Figure 9b). Similar results were obtained with water extracts from *Citrullus vulgaris* (Figure 9c). These results indicate that the *stratum corneum* can be stained with anti-filaggrin antibodies as early as two hours after treatment, and that this effect lasts for at least twenty four hours. The kinetics aspects of these results are in keeping with the suggestion that bacterial exo-polysaccharide stimulates more the maturation of pro-filaggrin than the induction of the expression of the gene of pro-filaggrin.

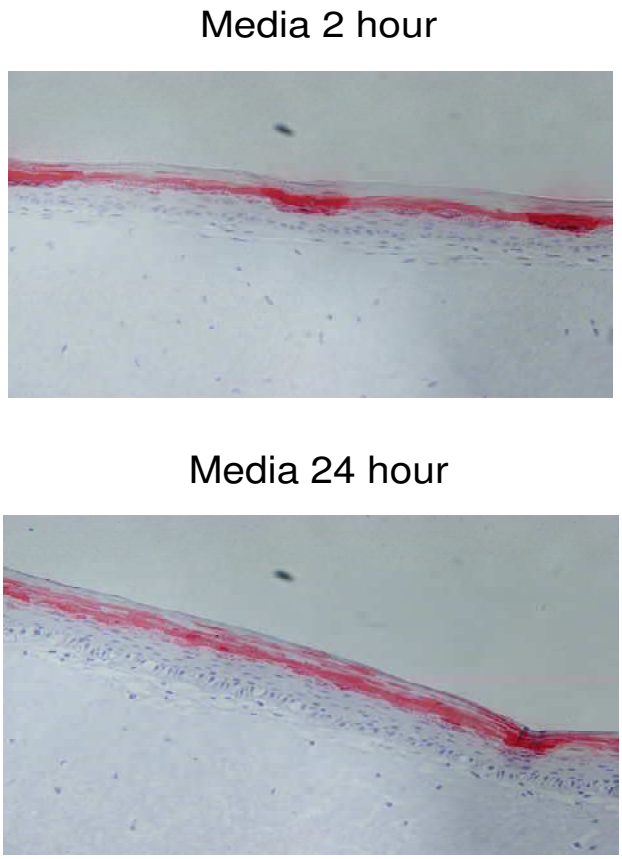


Fig. 9a. Filaggrin in the *stratum corneum* of MatTek specimens after 2 (top panel) or 24 hours (bottom panel). Media-treated control

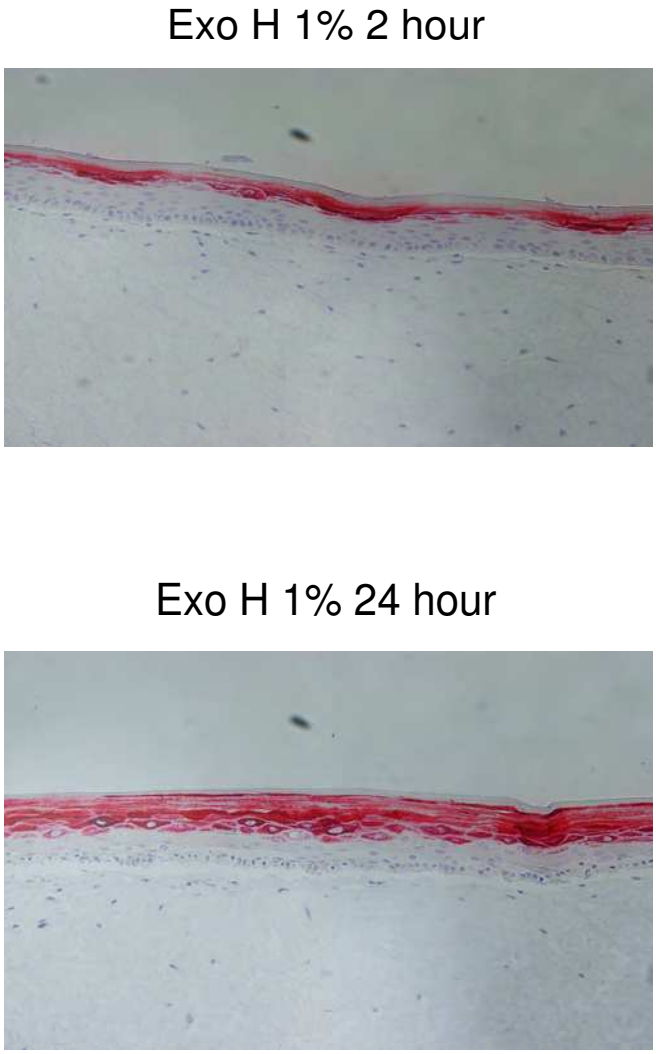


Fig. 9b. Filaggrin in the *stratum corneum* of MatTek specimens after 2 (top panel) or 24 hours (bottom panel). Samples treated with Bacterial Exopolysaccharide H (Exo H). Histology sections from ExoH-treated MatTek specimens, after 2 (top panel) and 24 (bottom panel) hours incubation.

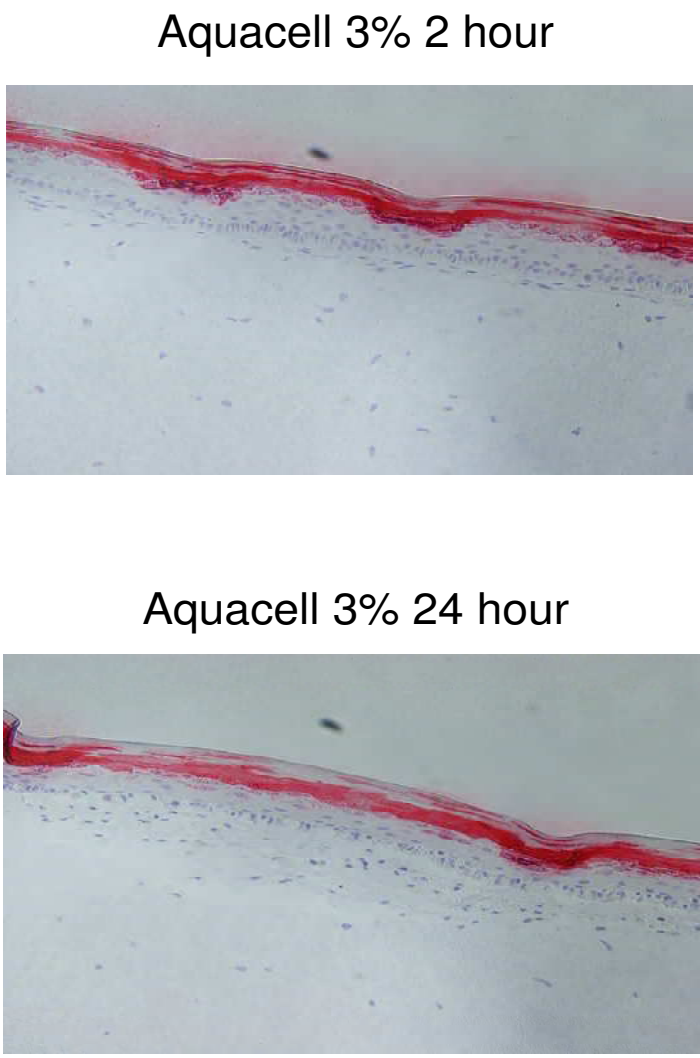


Fig. 9c. Filaggrin in the *stratum corneum* of MatTek specimens after 2 or 24 hours. Histology sections from Aquacell-treated MatTek specimens, after 2 (top panel) and 24 (bottom panel) hours incubation.

3.3 Analysis of pigmentation in melanoderm specimens

3.3.1 Viability

Cyclohexadecanol and kojic acid did not affect the viability at 7, 14, or 21 days (data not shown).

3.3.2 Histology sections

Figure 10 shows histology sections of MelanoDerm B specimens treated with 2% kojic acid or with 0.5% and 1% Cyclohexadecanol

After two weeks of daily treatment with 2% kojic acid or 0.5% or 1% cyclohexadecanol, the surface occupied by melanin in Melanoderm sections stained with Fontana-Masson staining decreased by 20%, 31% and 45% respectively. When melanin is assessed spectrophotometrically, the reduction induced by 0.5% or by 1% cyclohexadecanol is 27% and 29% respectively.

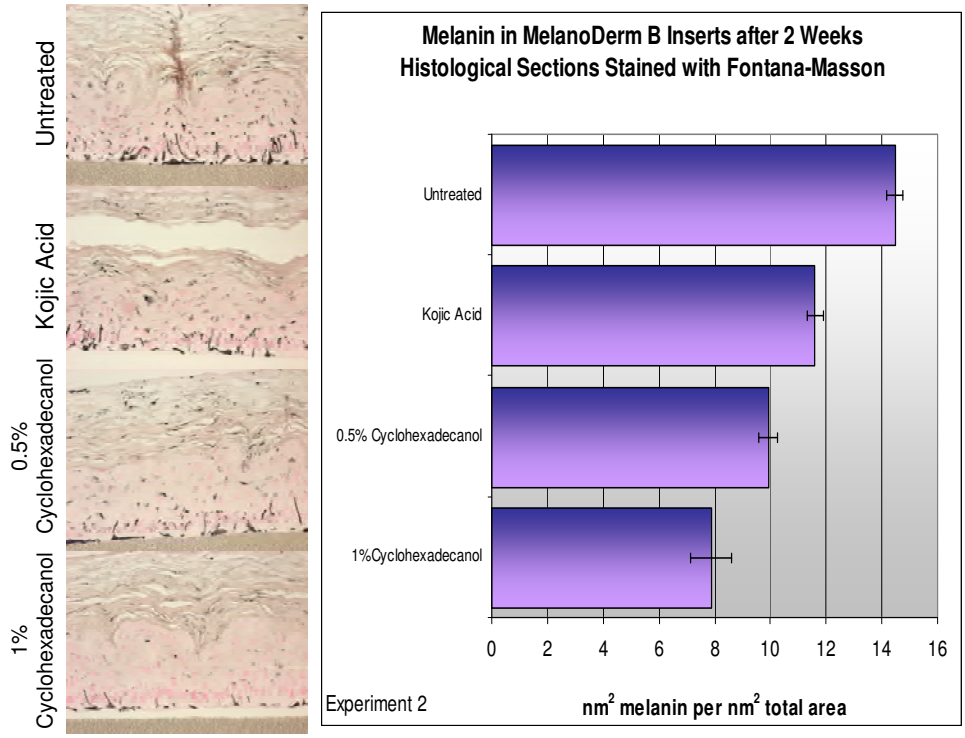


Fig. 10. Determination of melanin in Fontana-Masson stained sections of biopsies from differently treated MelanoDerm specimens

4. Discussion

Models of reconstructed skin have been prepared, which allow one to analyze large number of biopsies without infringing the ethical code which limits the experimentations on human volunteers. Cosmetics address healthy skin and reconstructed skin is suitable for cosmetic studies. The outer layer of the surface of the skin, the *stratum corneum*, plays an important

role in skin smoothness, moisturization and color, it is therefore important to have a reproducible methodology at hand, to study the effects of xenobiotics on its structure and properties. Reconstructed skin is appropriate to undergo biopsy treatment and allows one to screen xenobiotics for their cosmetic properties. The structure of the horny layer is smoother after treatment with substances belonging to defined chemical classes, whereas other chemicals do not reduce the roughness of the surface, and sometimes they increase it. These results are helpful in guiding the formulation chemist in selecting ingredients for cosmetic products aimed at the smoothening of the skin.

Biopsies from specimens treated with bacterial exopolysaccharides or water extracts from *Citrullus vulgaris* increase the *stratum corneum* immuno-staining associated with filaggrin within hours after application, in keeping with an action on the maturation process more than with the induction of gene expression. This method is helpful for the formulation chemist in selecting ingredients for cosmetic products aimed at improving the overall moisturization of the epidermis.

Biopsies punched in melanocytes-containing specimens after two weeks of daily treatment with kojic acid or 0.5% or 1% cyclohexadecanol indicate that the surface occupied by melanin in histology sections stained with Fontana-Masson staining decreased by 20%, 31% and 45% respectively. These results indicate that MelanoDerm is appropriate to experiments aiming at selecting ingredients for topical application to reduce the visibility of surface discoloration

5. References

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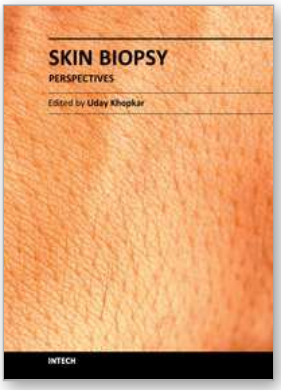

Skin Biopsy - Perspectives

Edited by Dr. Uday Khopkar

ISBN 978-953-307-290-6
Hard cover, 336 pages
Publisher InTech
Published online 02, November, 2011
Published in print edition November, 2011

Skin Biopsy - Perspectives is a comprehensive compilation of articles that relate to the technique and applications of skin biopsy in diagnosing skin diseases. While there have been numerous treatises to date on the interpretation or description of skin biopsy findings in various skin diseases, books dedicated entirely to perfecting the technique of skin biopsy have been few and far between. This book is an attempt to bridge this gap. Though the emphasis of this book is on use of this technique in skin diseases in humans, a few articles on skin biopsy in animals have been included to acquaint the reader to the interrelationship of various scientific disciplines. All aspects of the procedure of skin biopsy have been adequately dealt with so as to improve biopsy outcomes for patients, which is the ultimate goal of this work.

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