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Cosmetogenomics: A New Approach to Understanding Skin Physiology

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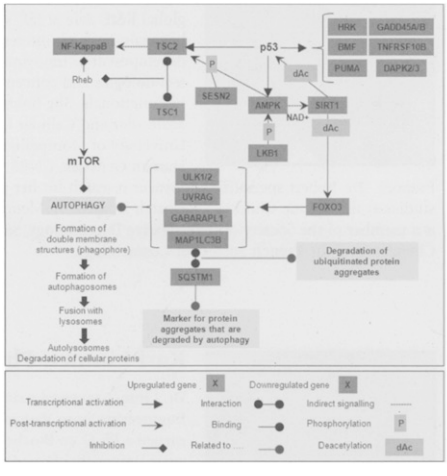
INTRODUCTION: Over the course of the past five years, cosmetogenomics became a recognized field in cosmetic research. More and more products reach the marketplace by claiming activities found to be elicited at the level of gene expression. However, one has to be cautious since gene expression profiling generates a tremendous amount of data and thus, the observation that a gene is modulated is not by itself sufficient for understanding. Indeed, genes function in an orchestrated manner and encoded proteins may participate in different pathways, depending on the types of cells and/or the inducers that initiate their regulation. Gene regulation is complex and integrates positive and negative feedback loops that may cloud and overshadow the global impact of a given compound.

Here, we will address these issues through two examples based on gene expression profiles performed on human pangenomic DNA microarrays. Modulated genes were submitted to PredictSearch™ software, a data mining tool dedicated to identify functional networks.

A. Functional networks involved in the regulation of autophagy: a new concept in cosmetic research

A series of quinolines, including chloroquine and quinine, were identified as potent pigmentation inhibitors (1) by interfering with lysosomal functions and disrupting the intracellular trafficking of key factors in melanogenesis. Moreover, chloroquine, used in the treatment of malaria, has been described inhibiting HCV replication and blocking autophagy, a lysosomal-related process involved in cellular proteins and organelles degradation. During aging, the efficiency of autophagic degradation declines and intracellular waste products accumulate suggesting a relationship between aging and age-related degenerative diseases and autophagocytosis. In order to gain more insight into the mechanism by which chloroquine inhibits autophagy, RNAs extracted from infected HuAP cells treated with chloroquine or left untreated were processed for microarray hybridizations.



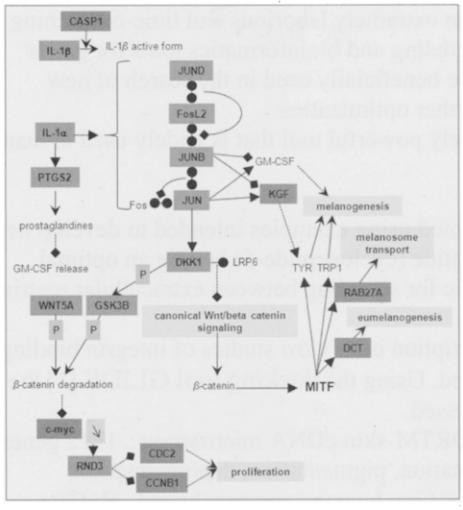
RESULTS: We demonstrate that chloroquine inhibits the transcriptional activation of FoxO3, a transcription factor involved in the expression of key autophagic factors. Activation of FoxO3 is itself regulated by SIRT1, a NAD-dependent deacetylase enzyme, whose activity is positively controlled by AMPK. AMPK is a metabolic fuel gauge that acts to maintain cellular energy stores switching on catabolic pathways that produce ATP, while switching off anabolic pathways that consume ATP. Activation of AMPK depends on its phosphorylation by the serine-threonine kinase LKB1 and inhibits mTOR (target of rapamycin), an inhibitor of autophagy. Interestingly, in addition to the repression observed for most of p53-target genes, chloroquine can reduce the transcription control of p53 on SIRT1, AMPK, LKB1, SESN2, and TSC2 that encode factors known to inhibit mTOR activity.

Thus, our findings suggest that chloroquine blocks, as one of the most upstream target, p53, considered as the "guardian of the cellular genome". It is possible that the inhibitory effect of chloroquine on p53-target genes might result at least in part, in the cytoplasmic sequestration of p53 since p53 needs to be translocated into the nucleus to elicit its transcriptional activity. Although the best-studied functions of p53 relate to its control of cell-cycle arrest and cell death, increasing evidence suggests that this protein represents a central node in stress- and nutritional-response networks. While p53 activation was found to induce autophagy, it has been shown that basal levels of p53 inhibit autophagy (2). Stimulation of autophagy by p53 occurs when cells are subjected to oncogenic activation or genotoxic stress and p53 is activated. In contrast, p53 loss induces autophagy in the absence of stress signals, suggesting that basal levels of p53 activity (rather than activated p53) inhibit autophagy. Constitutive autophagic activity decreases with age in living cells, thus increasing the accumulation of toxic materials and inefficient cellular components that will affect lifespan. However, it is thus far unknown whether there is indeed a cause-effect relationship between the increase of autophagy and healthy aging in humans. Such a causal relationship would revolutionize the entire field of aging research.

B. Discovering pathways that inhibit melanogenesis

Melanogenesis is a complex process, with at least 125 distinct genes involved in its regulation either directly or indirectly (3) in which the tyrosinase gene family plays a pivotal role. It consists of TRP-1 (tyrosinase-related protein 1), DCT/TRP-2 (tyrosinase-related protein 2) and TYR (tyrosinase), a bifunctional enzyme that modulates melanin production. The three types of melanins produced depend on the function of melanogenic enzymes. The overall regulation of melanogenesis does not concern only melanocytes but also is influenced by factors secreted by neighbouring keratinocytes and fibroblasts.

In the following example, gene expression profiles were performed using RNAs extracted from pigmented epidermal equivalents either left untreated or treated with a compound selected for its activities on keratinocyte differentiation. The rational of the experience was to investigate whether a global impact of these activities in pigmented epidermal equivalents can be deduced.



RESULTS: We find that the expression of IL-1α and IL-1-β encoding genes are transiently but strongly up-regulated upon treatment. As it can be expected, some of IL-1-transcriptional targets such as PTGS2, JUN, JUNB, JUND and FosL2 are in turn induced. Furthermore, the use of PredictSearch software leads to determine functional correlations between several modulated genes and terms and/or concepts related to melanogenesis. Investigating these relationships, we can integrate key elements engaged in a functional network leading to modulate melanogenesis. Indeed, several reports indicate that prostaglandins produced by PTGS2 suppress the secretion of GM-CSF (granulocyte-macrophage colony-stimulating factor), a cytokine that stimulates stem cells to produce granulocytes and monocytes. In ultraviolet induced epidermal pigmentation, the paracrine cytokine linkage between keratinocytes and melanocytes within the epidermis is documented to play an essential role in accentuating the proliferation and melanogenesis of melanocytes. As KGF (keratinocyte growth factor), GM-CSF is known to stimulate melanogenesis (4). Furthermore, JUN and JUNB antagonistically control cytokine-regulated mesenchymal-epidermal interaction in skin and have opposite effect on the expression of GM-CSF and KGF. Accordingly to our results, it can be hypothesized that the compound inhibits GM-CSF secretion via the production of prostaglandins by PTGS2 and stimulates a negative feedback loop in which JUNB, interacting with JUN, blocks the ability of JUN to induce GM-CSF and KGF expression. JUN and JUNB form AP1 complexes, that consist either in homodimers of Jun family proteins or in heterodimers of Jun/Fos family proteins. Three Jun proteins (Jun, JunB, JunD) and at least four Fos proteins (Fos, FosB, Fra-1, FosL2) are found in AP1 complexes. Therefore the regulation of AP1 target genes might depend on some specific sites within their promoters as well as on the components forming the complexes. Supporting an inhibitory activity of the compound on melanogenesis, induction of DKK1 is observed. This Jun- regulated gene encodes the dickkopf1 protein that represses the activity of the transcriptional factor MITF (microphthalmia-associated transcription factor) via the inhibition of the Wnt/β-catenin signalling pathway. The repression of MITF is also supported by the decreased expression of DCT and RAB27A that are regulated by MITF. As TRP1, DCT acts in the last step of eumelanogenesis and RAB27A encodes a small GTPase protein involved in melanosomes transport. Interestingly, over-expression of Wnt5a (wingles-type MMTV integration site family, member 5A) as phosphorylation of GSK3B (glycogen synthase kinase 3β) lead to β-catenin degradation and abrogate MITF activity. These events are associated with a reduction of c-myc inducing in turn RND3 that inhibits melanocyte proliferation via the inhibition of CDC2 and CCNB1 expression.

Conclusion: These two examples illustrate the efficiency of transcriptomic analysis, when associated with suitable computational tools, in deciphering the gene expression data to identify functional networks. However, keeping in mind that gene expression reflects only a snapshot, the "velocity of changes" on a wide time window has to be considered to fully understand the global and physiological impact of a given compound.

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The Application of Proteomics and Genomics in Cosmetic Actives Development

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INTRODUCTION

The development of a new product for cosmetics is an extremely laborious and time-consuming process. Computer techniques, such as molecular modeling and bioinformatics tools for genes and protein analyses, can facilitate this process and be beneficially used in the search of new templates and frameworks as lead compounds for further optimization.

Gene expression microarray technology is an extremely powerful tool that is widely used in many areas of pharmaceutical research.

METHODOLOGY

Computational modeling approaches will be presented using examples intended to develop new ingredients, specific to the design of a new cyclic peptide (cyclopeptide-5) having an optimal conformation for binding to integrin receptors specific for signaling between extracellular matrix (ECM) and cells of skin.

Besides the molecular modeling studies and the description of *in vitro* studies of integrin binding activities, cDNA microarray analysis will be presented. Using the docking tool GLIDE [1] the integrin and cyclopeptide-5 interactions will be discussed.

Gene expression analysis was carried out using PIQORTM-skin cDNA microarrays. 1312 genes involved in target pathways related to stress, inflammation, pigmentation, depigmentation, moisturization, anti-aging and hair follicle development ion humans were evaluated. Buffer-treated skin equivalents served as controls.

The cDNA microarray data was analysed using MetaCore™ [2]. MetaCore™ is a set of software for functional analyses of experimental data such as microarray gene expression, metabolomics, proteomics, and pathway analysis (Figure 2).

Treatment was carried out with two concentrations and a control. The selected genes were based on statistical methods ($p < 0.05$, at least 1.5-fold de-regulation).

RESULTS

Computational modeling was used to design a new peptide that would have an optimal conformation for binding with an integrin receptor-selective ligand specific for signaling in the extracelular matrix (ECM) of skin (Figure 1).

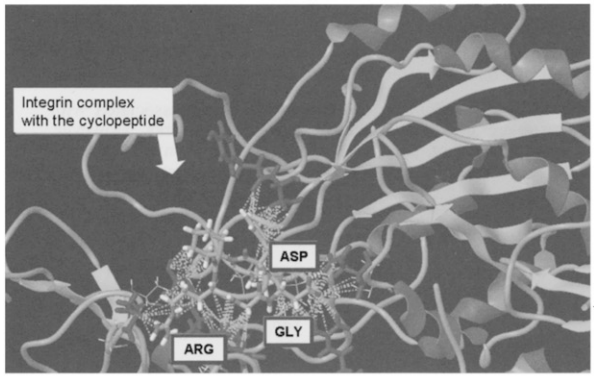

Figure 1: Docking of extracellular segment of integrin avβ3 in complex with cyclopeptide.

Several hundreds of de-regulated genes have been recognized and analyzed using MetaCore software (Figure 2). Treatment of human full thickness skin models show promising results regarding the de-regulation of genes with positive signaling in epidermis development (collagen, elastin and fibronectin enhancement, etc), and hair re-growth.

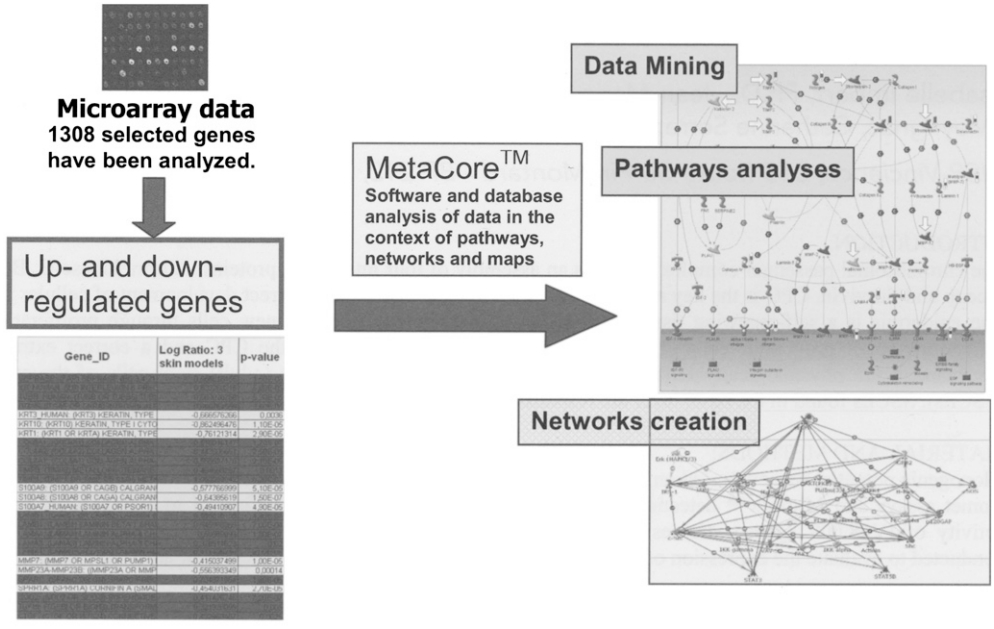

Figure 2: A scheme of data analysis using MetaCore: from genes to the possible biological processes (path-ways and maps).

CONCLUSION

The mentioned modeling and data analyses will be presented in details. Bioinformatic analyses provide insights into the pathways of the given example cyclopeptide-5 that are involved in effects for cosmetic applications such as anti-ageing and hair growth. These methodologies can be used to select appropriate ingredients for cosmetics applications.

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The Epidermal Basal Cellular Environment and the Proteins of the Chromosomal Passenger Complex are Together Involved in the Homeostasis and Protection of the Somatic Stem Cells of the Epidermis

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INTRODUCTION:

The chromosomal passenger complex (CPC) is an assembly of four interacting proteins: Aurora kinase-B, Borealin, Incenp and Survivin. CPC is the key regulatory complex responsible for the correct development of cellular mitosis. The epidermis is a self-renewing tissue that needs to continuously generate new cells through proliferation and differentiation of progenitor cells [1, 2]. Both the mitosis supervision by the CPC and a correct extracellular environment are physiologically required for the homeostasis of the somatic (adult) stem cells of the epidermis (SSCEs). SSCEs found in the basal layer are responsible for the replenishment and maintenance of the epidermis.

MATERIAL AND METHODS:

Clonogenic potential was evaluated on HaCaT and RA keratinocytes (Rapidly Adherent on collagen IV) [3].
Comet assays and Mitosox™ detection of mitochondrial superoxides were performed to evaluate the protective activity of IV08.009 against stresses (UVB or H_2O_2). Immunoblotting or immunohistological detections were conducted to evaluate the expression of Exportin-1 (or CRM1) and Survivin on foreskin or adult skin biopsies.

RESULTS:

We previously demonstrated the ability of compound IV08.009 to positively modulate Survivin expression in human keratinocytes (+54%). Interestingly, IV08.009 regulated the expression of Survivin without affecting the clonogenic potential of RA cells (SSCEs-enriched keratinocytes) (Figure 1).

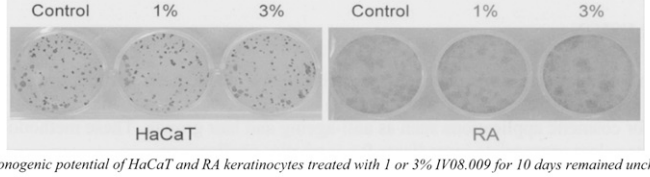

Fig. 1: Clonogenic potential of HaCaT and RA keratinocytes treated with 1 or 3% IV08.009 for 10 days remained unchanged

Comet Assay experiments allowed the efficacy evaluation of IV08.009 in protecting SSCEs from UVB irradiation (60 mJ/cm^2). Results showed a 44.3% reduction of UVB-induced DNA damage in presence of IV08.009 (Figure 2).

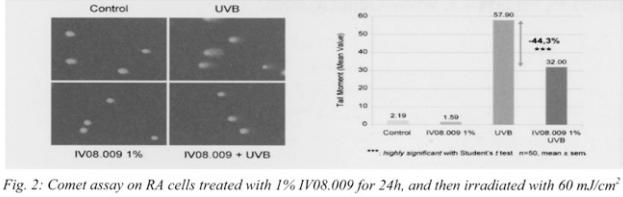

Fig. 2: Comet assay on RA cells treated with 1% IV08.009 for 24h, and then irradiated with 60 mJ/cm^2 UVB

We next investigated the protection conferred by IV08.009 (48h, 1%) against mitochondrial superoxide production induced by UVB (100 mJ/cm^2) or H_2O_2 stress (30 minutes, 5 mM H_2O_2) in RA cells. Following both stresses, a lower level of induced mitochondrial superoxide ions was observed in treated cells, pointing out the role of Survivin in protecting the cells (Figure 3).

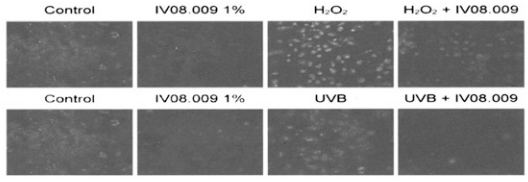

Fig. 3: Mitochondrial Superoxide ions detection with Mitosox™ reagent, in RA cells pre-treated with IV08.009 and then exposed to H2O2 or UVB stress

In parallel, the role of CRM1 was investigated. CRM1 is a key player in the regulation of Survivin localization, and is expressed in all supra-basal layers of the epidermis. IV08.009 was able to reverse the effect of leptomycin B, a compound known to inhibit CRM1-Survivin interactions. IV08.009 restored CRM1 expression profile in skin (Figure 4).

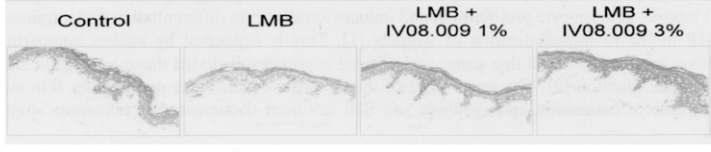

Fig. 4: Immuno-labelling of CRM1 in skin biopsies treated with IV08.009 for 48h, then stressed with 1µM of Leptomycin B

Finally, we studied the protection conferred by IV08.009, against UVB irradiation. Adult human skin or foreskin biopsies were treated or not with 1 or 3 % IV08.009 for 24 hours, then stressed with UVB (Figures 5 and 6).

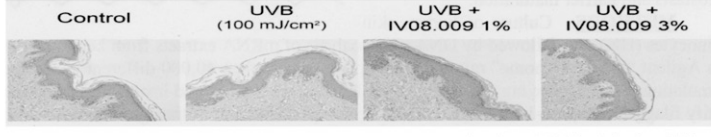

Fig. 5: Immuno-histological detection of Survivin in foreskin biopsies treated with IV08.009 for 24h, then UVB-irradiated

Survivin expression was detected by immunohistological staining, DAB revelation and H&E counterstain.
In both foreskin and adult skin, IV08.009 offered a significant protection of the epidermis from UVB-induced basal damages. As the SSCEs essentially reside in this basal layer, these results strongly suggest the high protective effect of IV08.009 on SSCEs.

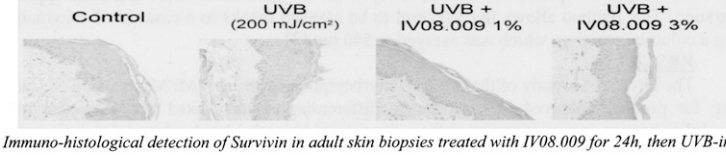

Fig. 6: Immuno-histological detection of Survivin in adult skin biopsies treated with IV08.009 for 24h, then UVB-irradiated

CONCLUSION:
These studies demonstrate the interest of modulating Survivin expression in order to help protect SSCEs-containing epidermal basal layer from UVB stress and suggest the ability of protecting SSCEs essential renewing potential.
All together, these new data should be of particular interest to investigate future development of biofunctionals targeting adult epidermal stem cells protection.

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Genomic and Proteomic Investigations of a Novel Mechanism to Stimulate Skin Moisture Regulation

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INTRODUCTION:

The view that sphingolipids are essentially passive structural components of cellular membranes, especially in the *stratum corneum* has yielded to the realization that metabolism of sphingomyelin releases various highly active and specific lipid mediators. One such sphingomyelin derivate, sphingosine-1-phosphate (S1P) inhibits keratinocyte proliferation and induces keratinocyte differentiation and migration, suggesting a role for S1P in the re-epithelialization of wounds [1]. This is supported by studies comparing atopic dermatitis patients where it was found that genes with altered expression included those relevant to skin barrier formation and immune function [2]. The importance of intact barrier function for maintaining skin moisture homeostasis and the role of ceramides, sphingolipids and S1P has been documented in numerous studies over the last 20 years.

OBJECTIVE OF THE STUDY:

To study genetic and metabolic reactions of human keratinocytes and 3D skin models to sphingosine-1-phosphate and/or its analog myristylphosphomalate (MPM) and to better understand the complex interactions of enzymes (filaggrinase, caspase-14, transglutaminase...) involved in apoptosis, skin homeostasis and barrier maturation.

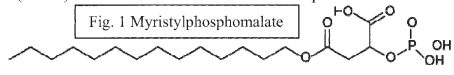

Fig. 1 Myristylphosphomalate

METHODS: Culture of human skin keratinocytes (HK) was followed by DNA chip analysis of mRNA extracts from keratinocytes and 3D epidermis on an Agilent "Whole Genome" microchip comprising more than 40,000 different gene sequences, in response to stimulation; fluorescence tagging and immunolabeling techniques and image analysis allowed us to detect and quantify filaggrin, caspase-14, filaggrinase, loricrine; quantification of transglutaminase was carried out by qRT-PCR and enzymology; ceramides and other skin lipids were assayed by thin layer chromatography.

Clinical confirmatory studies assaying Caspase-14 *in vivo* and Glycerin content in the epidermis of human volunteers were performed, also including TEWL and impedance measurements. We developed an original method for assaying the activity of caspase-14 from strips collected from the forearms of volunteers. These strips (6 DSquame® in total) underwent extraction in a neutral pH buffer. This solution was then placed in contact with a synthetic substrate cleaved by caspase-14; the fluorescence resulting from this process was recorded using a fluorescence reader. In parallel to the caspase-14 assay, we used the same extracts to evaluate the quantity of glycerol present in the upper layers of the epidermis before and after application of the MPM preparation. This method allows free glycerol to be assayed thanks to a cascade of enzymatic reactions, finally giving a coloured reaction, which was assayed at 540 nm [3]

RESULTS:

The DNA array study of the effect of myristylphosphomalate (MPM) revealed the induction of all genes coding for proteins involved in keratinocyte differentiation and linked to the protection of the epidermal permeability barrier, just as is expected from the natural bioactive lipid sphingosine-1-phosphate. Almost all the genes induced in the keratinocyte culture were also induced in the reconstructed 3D epidermis model.

Three distinct networks of interacting and complementary genes appeared from this analysis:

The first network identified by PredicSearch® datamining software is linked to formation of the protein barrier, as the transglutaminase-1 (TGM1) and involucrin (IVL) genes were induced by MPM. Interestingly an increase in the gene expression of the *Grained-like 3* (GRLHL3) protein, the inactivation of which reduces TGM1 production and epidermal barrier protection with a marked increase in insensitive water loss was also observed. The S100P protein gene, which can bind various divalent cations including calcium, essential for transglutaminase-1, and the CASP14 gene, which codes for caspase-14, are also induced in the presence of MPM. This latter enzyme is specific and essential for filaggrin metabolism and the formation of the cornified cell envelope.

In a second network, we find claudine-7 (CLDN7) and claudine-4 (CLDN4) genes - vital proteins for the formation of tight junctions – as well as OCLN and ZO1 genes, which code for occludine and zonula occludens-1, other tight junction components. This conjunction of gene expression highlights the creation of a physical barrier to the passage of water and solutes.

PRSS8, which codes for prostasine and regulates both OCLN and SCNN1A activities (the latter participating in lipid composition maintenance) completes this second network.

In the third network, expression of the FATP4 and FATP6 genes is increased. Both proteins act at the point of entry of long-chain fatty acids in keratinocytes and formation of lamellar bodies. These fatty acids are controlled by two groups of enzymes aiming at extending them in order to render them more insoluble (ELOVL1 and 4-coded elongases both of which are increased), and then to transform them. For this stage, ceramide synthase coded by LASS3 and UGCG together with a FA2H-coded hydrolase, promote the production of hydroxy-ceramides and hydroxy-galactosylceramides. At the same time, it is interesting to emphasise that the LIPE-coding gene for a keratinocyte lipase is induced. This lipase is known to hydrolyse a large range of esters including triacylglycerols, mono- and diglycerides and cholesterol esters, eventually producing glycerol which completes the wetting agents derived from filaggrin and free fatty acids.

The proteomic studies allowed us to confirm these data, as we observe an important stimulation of various elements that play a fundamental role in the synthesis of the NMF: an increase in the synthesis of profilaggrin/filaggrin by +259% ($p<0.01$) is concomitant with an increase in the synthesis of caspase-14 (figure 2: +229%; $p<0.01$), and filaggrinase (+304%; $p<0.01$), enzymes responsible and necessary for metabolism of pro/fillagrin. These results were confirmed by a test on explants of human skin in which an increase in the production of profilaggrin/filaggrin of +33% in the case of normal skin and by +69% in the case of stripped skin, was clearly shown.

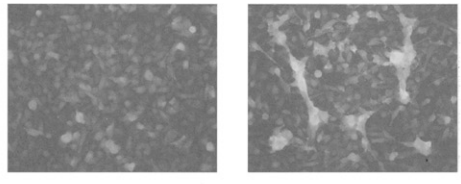

Figure 2: Visualisation of caspase-14: control (left) ; HK incubated with 5ppm MPM (right):

The elements relating to the construction of the cutaneous barrier, pointed out by the genomic approach (DNA array), were also evaluated by proteomic studies. Transglutaminase-1 increased by +101% ($p<0.01$) in quantity and by +124% ($p<0.01$) in activity. Proteins constitutive of the cornified cell envelope, loricrine (+426%; $p<0.01$) and involucrin (DNA-Array), were reinforced. Lastly, the barrier effect was consolidated by stimulation of the synthesis of ceramides (non hydroxylated: +256%, $p<0.01$ and hydroxylated: 7.5 times) and cholesterol (+107%, $p<0.04$) in human keratinocyte culture. Thus the proteomic and downstream analysis perfectly confirms the picture obtained from the genomic studies.

Nevertheless, final proof for the validity of the concept comes from clinical studies with topical application of the material. Whereas Sphingosine-1-phosphate has been the object of a number of *in vivo* studies, although mostly related to various pathologies, the present analog of MPM, so similar in activity *in vitro* to its natural homolog, needed human data for verification.

A clinical study carried out on a mixed panel for 2 months and one week (regression) confirmed the results observed *in vitro*. The method described above made it possible to show, in a non-invasive way on human volunteers, that the increase in caspase-14 and in glycerol content in the superior layers of the skin (+34.8% and +327% compared to placebo, respectively) was perfectly in line with the genomic and proteomic data. This analytical result is then corroborated by the functional data of moisturization (fig. 3) and TEWL (50% reduction after stripping insult), reflecting the improved barrier integrity we expected from the increase in both tight junction proteins, cornified envelope proteins, the NMF components and the increased epidermal lipids (cholesterol, ceramides). It should be remembered that MPM is not a direct moisturizer but stimulates or triggers the synthesis of natural wetting agents and promotes homeostasis of the skin whilst having a positive effect on the barrier.

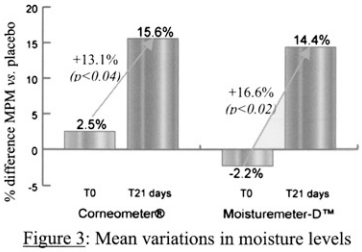

Figure 3: Mean variations in moisture levels

CONCLUSION:

Thus, a body of data precisely connects genetic expression to protein synthesis and protein activity in monolayer keratinocyte culture, 3D epidermal models and on human volunteers: the installation of the corneocyte layer, needs both the construction of the cornified cell envelope and the essential intra-corneocyte lipids, for which the "raw materials" (profillagrin, sphingolipids) are supplied and then metabolized by the appropriate enzymes (fillagrinase, transglutaminase, caspase-14, ceramidase, sphingosine kinase). The MPM analog of S1P clearly mirrors the activity of the endogenous substance.

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Polymers as Stabilizers for O/W Emulsions with Solid Particles: A Comparison between Crosslinked Homopolymers and Copolymers of Acrylic Acid

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Introduction

Emulsions are inherently thermodynamically unstable preparations. The use of emulsifiers and more recently polymers to stabilize emulsions has been well documented and is widely used (1). The addition of solid particles to emulsions is often associated with a decrease in emulsion stability as it limits formulators with their choices of polymeric thickeners and stabilizers(2). Historically, most success in stabilizing such system was obtained from using gums and/or clays (3). Since most gums and clays are not efficient thickeners, formulators relied on other routes to increase the viscosities of these emulsions. The use of crosslinked acrylic-acid (AA) based polymers is very limited if non-existent (4). In the current paper, we are introducing a crosslinked copolymer of acrylic acid and vinyl pyrrolidone (AA/VP) that is shown to enhance the stability and aesthetics of such systems.

Experimental

The ability to incorporate zinc oxide, titanium dioxide, and iron oxides into non-ionic o/w emulsions stabilized with different polymers was studied. The surfactant system used in the emulsions prepared was composed of Glyceryl Stearate, Laureth-23, and Ceteareth-20. The level of surfactants was varied from 0.5% to 4% w/w to accommodate the size of the oil phase in the emulsions prepared which was varied from 16 to 46% w/w. The polymers were incorporated in three different emulsion types, a daily lotion, a sunscreen lotion, and a daily make-up composition. The polymer level was kept at 0.5% w/w in all emulsions and was neutralized with Triethanolamine. Microscopy (Nikon, optical microscope equipped with a polarizer) was used to determine the quality of dispersions and the ability of polymers to lower interfacial tension. Stabilities of all formulations were monitored at 5°C, F/T, RT, and 45°C for three months.

Results

Both AA-based polymers and AA/VP copolymer were able to reduce the interfacial tension on o/w dispersions as demonstrated by the reduction in particle size in the daily lotion. The polymers appeared to be more effective at reducing interfacial tension than non-ionic emulsifiers when used at the same concentration. In sunscreen formulations containing zinc oxide, AA/VP copolymer was found to be superior to AA-based polymers in dispersing the zinc oxide. The emulsions made with AA/VP were more uniform and performed well on stability, whereas the ones obtained with AA showed some separation at 45°C. In the case of make-up formulations containing iron oxides and titanium dioxide, the same trend was observed where AA/VP copolymer performed better than AA polymer in producing a homogeneous dispersion of titanium dioxide and creating emulsions with good stability at elevated temperatures (45°C).

Discussion

The data generated in this paper seems to indicate that AA/VP copolymer is superior to AA-based polymers in stabilizing emulsions with relatively high level of solid particles. The dispersions created were uniform, monodisperse, and stable. This will enable formulators not only to create stable formulations, but also thicken such system effectively, and thus imparting excellent aesthetics to such formulations.

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The Autophagic System: A New Era in Skin Detoxification

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Introduction:

The cellular protection systems, essential for the maintenance of cell integrity in the face of the various internal and external threats, are organized in different systems of defense. Among them, the detoxification system is composed of 2 major cellular mechanisms: proteasome and autophagy.

At a basal level, these systems are complementary: proteasome degrades short-lived soluble proteins, both functional (small regulatory proteins) and non-functional (misfolded proteins) and autophagy is a dynamic process essentially eliminating long-lived proteins (the majority of cellular proteins) and altered organelles such as mitochondria, which are source of Reactive Oxygen Species (ROS) and DNA mutations. In this way, Autophagy is the "quality control" to the components of the cytoplasm **[1]**.

In the case of more severe stress or moderate but repeated stresses, resulting damages can be dramatic for cells. Being numerous, altered intracellular proteins overload then deplete the degradation capacity of proteasome and thus accumulate. These modified or aggregated proteins, become not only resistant to degradation by the proteasomal pathway, but also inhibit the process **[2]**.

Under these conditions, autophagy takes over from proteasome in order to compensate its failure (**Figure 1**). The autophagic machinery ensures the degradation of soluble altered proteins, and also it guarantees its role in elimination of insoluble proteins, oxidized lipids and damaged organelles **[3]**.

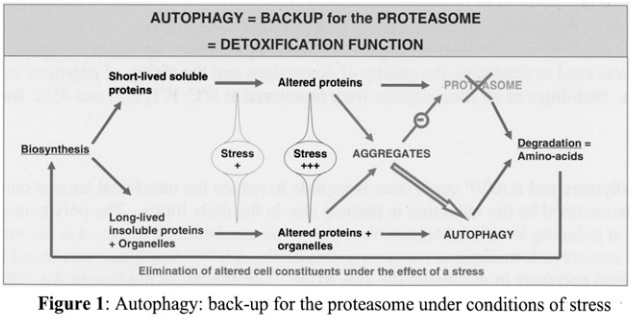

Figure 1: Autophagy: back-up for the proteasome under conditions of stress

Powerfull cellular mechanism, capable of eliminating components that pollute cells, autophagy is an essential survival and adaptation mechanism **[4]**.

The autophagic system has been studied extensively in medical research, and its importance has been underscored, for example in neurodegenerative diseases. In this field of research, autophagy is considered as a "luxury replacement for the proteasome"**[5]**.

In skin cells, damages due to intrinsic and extrinsic stresses seem to be removed by autophagic pathway, although very little studies were achieved in dermo-cosmetic research.

Consequently, the purpose of this presentation is to analyze the autophagic system in a cutaneous cellular model. This new skin cellular system of detoxification may serve as a target for the development of detoxifying active ingredients in cosmetic.

Material and methods:

Cell culture and H_2O_2 exposure of human keratinocytes:

The spontaneously immortalized human keratinocyte HaCaT cell line was maintained as monolayer cultures at 5% CO2 at 37°C in KSFM (Keratinocyte Serum Free Medium, Invitrogen, CergyPontoise, France) supplemented with growth factors (defined Keratinocyte Serum Free Medium Growth supplement) and used at passages 40–60. Normal human keratinocytes (NHK) were isolated from plastic surgery of healthy donors and maintained in Keratinocyte-SFM supplemented and used at passages 2–3.

Dishes were rinsed and cells incubated in K-SFM and treated or not during 3 hours with H_2O_2 (125 µM, 250 µM, 1000 µM). After H_2O_2 exposure cells were directly analyzed (XTT test, JC-1/TOPRO 3 double staining, immunostaining, western blotting…) or were incubated in complemented K-SFM during indicated time in a step named cell "recovery", for further analyzes.

Measurements of cell viability:
Cell survival was assayed by measuring mitochondrial activity with the Cell Proliferation Kit II (XTT, Roche diagnostics, Meylan, France).

JC-1 / TOPRO-3 double staining:
Mitochondrial Transmembrane potential ($\Delta\Psi m$) and plasma membrane integrity were assessed by flow cytometry by using JC-1 and TOPRO™-3 probes respectively. Cytometric data were analysed with Win MDI 2.8 software (developed by Dr. J. Trotter, Scripps Institute, La Jolla, CA, USA).

Gel electrophoresis and immunodetection of carbonyl groups on proteins:
SDS/PAGE was done on a 12% acrylamide (w/v) separating gel. Immunoblot detection of carbonyl groups was performed with the OxyBlot Protein Oxidation Detection Kit (Millipore, France), according to the manufacturer.

Immunodetection of LC3 by fluorescence confocal microscopy:
Stressed or control cells were cultured in specific support Lab-Tek™. Chamber Slides or cover glass (14mm diameter, VWR, France), then were fixed in Paraformaldehyde 4% and incubated with a primary antibody anti-LC3 and a secondary fluorescent antibody (provided from Molecular Probes). Immunostaining was visualized by fluorescence confocal microscopy (ZEISS LSM510).

Immunodetection of LC3 by Western Blotting:
Stressed or control cells were collected and proteins were extracted. Proteins were separated by SDS PAGE (15%) and transferred to Immobilon P membrane (IPVH, Millipore). Membranes were incubated with a primary mouse monoclonal anti-actin antibody, a primary rabbit polyclonal anti-LC3 antibody, and a secondary mouse anti-IgG antibody coupled to HRP, and a secondary rabbit anti-IgG antibody coupled to HRP. Blots were developed with Immobilon Western chemiluminescent HRP substrate from Millipore. The strips were semi-quantified by densitometry using an LAS-4000 instrument (FUJIFILM) followed by image analysis using Multigauge software (FUJIFILM).

Results & Discussion:

Nowadays, the cellular autophagic mechanism is intensively studied in neurons, hepatocytes, cardiomyocytes or yeasts. This is an inducible mechanism in these cell models following exposure to different stress. Hydrogen peroxide is widely used in these studies to induce oxidative stress which alters cells components and activates detoxification systems to unblock cells **[6]**. Firstly, we determined optimal experimental conditions of H_2O_2 exposure without inducing cell death. Secondly, we noticed that H_2O_2 treatment of keratinocytes induced a significant increase in the level of oxidized proteins with a dose-dependent manner. Then, a significant reduction of the rate of oxidized proteins was observed after a step of recovery. It seems that the step of recovery from oxidative stress was used by keratinocytes to repair or to recycle alterations due to oxidative stress. This step of detoxification could involve the autophagic system, we thus studied the LC3-II marker. This marker, expressed during all the autophagic process, is considered by specialists as "autophagic tracer". We have shown that 250 µM H_2O_2 induced a significant increase of synthesis of this protein. These data were confirmed by fluorescence immunocytology: in control cells, no significant green fluorescence was visualized, while in H_2O_2 treated keratinocytes, the synthesis increased in a dose dependent manner (**Figure 2**). Consequently, it seems that skin cells use the autophagic machinery to eliminate altered cellular components.

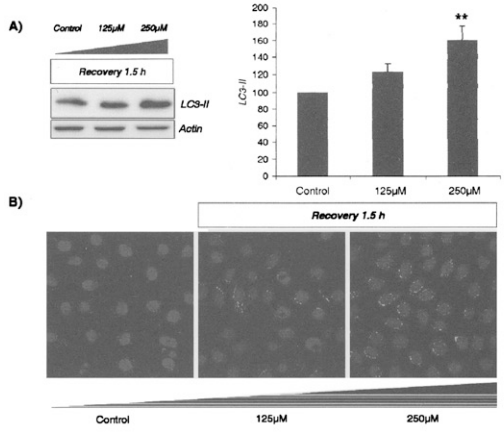

Figure 2: Study of LC3-II synthesis in keratinocytes during the step of cell recovery from oxidative stress. A / Results by Western Blotting. B / Results by Immunocytology.

Conclusion:

This study provides an insight into a new cellular mechanism, the autophagic system by which the skin cell deals with oxidized components and altered organelles. This first approach opens a new era in skin cell detoxification and autophagy may be used as a valid target for increasing skin cellular resistance to oxidative stress. This new cellular mechanism may serve as a basis for further developments of innovative natural active ingredients for the cosmetic industry.

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The Corneovacumeter: A New Device for Evaluation of Biomechanical Properties of the Skin

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Introduction:
The evaluation of the biomechanical properties of the skin with aging and/or photo-aging is one of the main targets of cosmetic and dermatological research.
For this, many different non invasive devices using alternative measuring approaches such as stretching, torsion, indentation, and suction have been developed. The measurements of skin deformation after suction or torsion are the most widely used techniques in cosmetic research [1].

Objective of the study:
Demonstrate the suitability and validity of the newly developed device, Corneovacumeter, based on the principle of multiple point measurements for evaluation of skin biomechanical properties, and compare its performance to a standard non invasive method of suction.

Methodology:
A total of 113 female volunteers participated in the study after giving written informed consent. The population was divided into four groups: Group A: 28 subjects [18-30 years old]; Group B: 28 subjects [31-44 years old]; Group C: 28 subjects [45-60 years old] and Group D: 29 subjects [61-73 years old]. All participants were Caucasians with no signs of apparent skin disease. The measurements were carried out on two different anatomical regions: inner forearms and face.

The biomechanical properties were measured with a commercially available standard method – Cutometer ® SEM 575 (Courage & Khazaka, Köln, Germany) and with the Corneovacumeter, a new device developed by COGNIS France – Laboratoires Sérobiologiques. For both devices, the evaluation of the mechanical properties of the skin is based on skin deformation induced by suction, but different systems are used for the measurement of displacement: optical measurement for Cutometer® and electric capacitance measurement between the skin and the metallic plate for Corneovacumeter. The main difference between these 2 instruments is the number of apertures on the probe head. The skin deformation can be measured and calculated simultaneously on 12 apertures with the Corneovacumeter against only one aperture for the Cutometer®.
The operating strain mode used was as follows: from one to five skin deformations with 2 seconds suction period and 2 seconds relaxation period, with a measurement time to 20 seconds, for each device.

Cutometer® and Corneovacumeter data were analyzed using 2 different techniques:
- Standard curve analysis with classical parameters: deformation parameters (Ue, Uf, Ur), residual deformation (End), the ratio parameters: elastic deformation recovery (Ur/Ue), elastic recovery rate (Ur/Uf).
- Inflection point and area analysis with following parameters: elastic recovery (Re), overall elasticity ([Re+Rv]/R0), elastic component (Re/R0) [2].

Results:
Table 1: Correlations between Cutometer® / Corneovacumeter parameters and age (n=113) on the measurements realized on the inner forearms.

	Cutometer®		Corneovacumeter	
	R	P	R	P
Ur/Ue	-0.565	<0.0001	-0.725	<0.0001
Ur/Uf	-0.698	<0.0001	-0.796	<0.0001
Ur	-0.649	<0.0001	-0.543	<0.0001
End	0.549	<0.0001	0.770	<0.0001
[Re+Rv]/R0	-0.744	<0.0001	-0.793	<0.0001
Re/R0	-0.768	<0.0001	-0.793	<0.0001
Re	-0.649	<0.0001	-0.558	<0.0001

Both devices have confirmed a significant evolution of skin biomechanical properties with aging: loss of skin firmness and elasticity, increase of the residual deformation.
These correlation coefficients are higher for the measurements realized with the Corneovacumeter than for those obtained with the Cutometer.

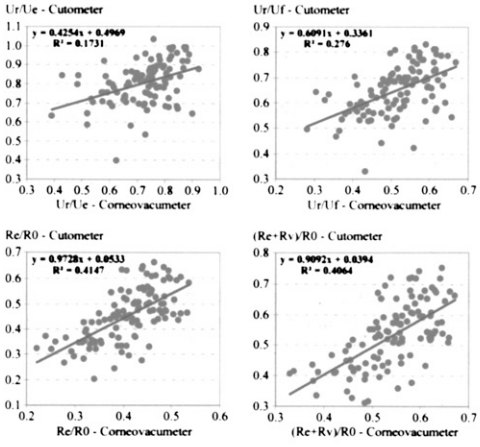

Figure 1. Regression curves for the skin biomechanical parameters between Cutometer® and Corneovacumeter on the inner forearms.

A good correlation of the skin biomechanical properties evaluated with Corneovacumeter and Cutometer® was found on the inner forearms, mainly observed on the firmness and elasticity ratio.

The correlation coefficients between both devices calculated from the measurements on the face are less significant. This may be due to the difference of the measurement site, temples for Cutometer® and upper cheeks for Corneovacumeter.

The variability of the measurement was checked on 17 volunteers for both devices and both measurement sites, forearms and face (figure 2).

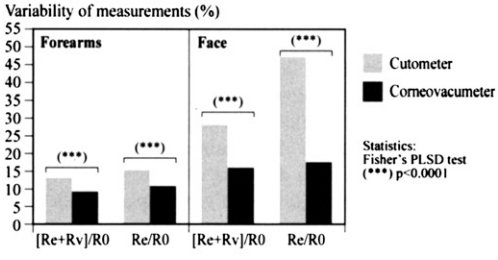

Figure 2. Study of the variability of biomechanical properties measurements with Corneovacumeter versus Cutometer®, on different sites of the skin

The results obtained with Corneovacumeter have shown a significantly lower variability ($p<0.0001$) in comparison with Cutometer®, on the different sites of the skin.

This lower variability is probably due to 12 simultaneous measurements of skin deformation comparatively to a single deformation for Cutometer®.

Conclusion:
The newly developed device is suitable for the evaluation of the biomechanical properties of the skin. The Corneovacumeter can be used as an additional tool for cosmetic research due to a good repeatability of the measurements, a low variability of the obtained results and a good responsiveness to quantify the modification of skin properties with aging.

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Powered Devices for Facial Cleansing: Should They Occupy Space in the Facial Cleansing Device Toolbox?

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Introduction

There is an ever increasing number of beauty and grooming devices being promoted and marketed for at-home use. However, there are few reports describing clinical data demonstrating the therapeutic benefits often claimed. For powered facial skin cleansing devices, there are little clinical data proving the advantages device-aided cleansing has over conventional cleansing[1]. Commercially available powered facial cleansing brushes include rotating, oscillating, vibrating, and stationary brush heads. Using standardized methods, we have tested two such devices, a rotating and an oscillating brush, and compared them to conventional manual skin cleansing for make-up removal, stratum corneum exfoliation, barrier function, effects on anaerobic bacteria populations, and stratum corneum hydration when cleansing is followed with topical moisturizer.

Materials and Methods

Two commercially available powered cleansing devices were tested. The DDF® Revolve 400X Micro-Polishing System rotating brush (Procter & Gamble, Cincinnati, OH) uses a 3.5-cm diameter rotating brush head operating at 300 rpm. This brush was used with Olay® Professional Pro-X Exfoliating Renewal Cleanser (ERC) for all tests. The Clarisonic Pro Skincare System® (Pacific Bioscience Laboratories, Inc. Bellevue, WA) employs an oscillating/vibrating brush and was used with Nourishing Care Cleanser® (NCC).

Standard Cleansing Protocol: Normal healthy women ages 25-65 were enrolled in these studies. Cleanser or water (0.25 mL) was applied by syringe to a 3x4-cm skin site. The site was then washed with the device or by finger cot for 15 sec, rinsed for 10 sec with warm tap water and allowed to dry. The cleansing treatments referred to below were (A) ERC with rotating brush, (B) NCC with oscillating brush, (C) ERC with finger cot or (D) water with finger cot.

Make-Up Removal: Sixteen subjects were enrolled. Four test sites on the volar forearms, one each for treatment A, B, C, or D, were treated with an anhydrous makeup covering the entire site evenly (Elizabeth Arden® Warm Beige 08 Flawless Finish) and cleansed per the standard cleansing protocol. Make up remaining on the skin was then collected with a white cotton pad soaked with an alcohol-based make-up remover (Lancôme® Bi-Facil). The pad was analyzed for ΔE values with a spectrophotometer (Minolta® CM2600d). The higher the ΔE value, the poorer the cleansing effectiveness.

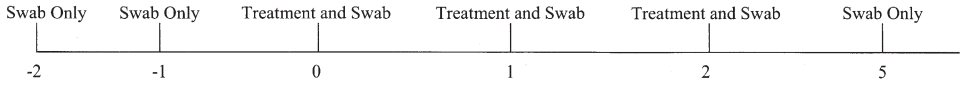
Stratum Corneum Exfoliation: Nineteen subjects were enrolled. Four test sites on the volar forearms , one each for treatment A, B, C, or D, were treated with dihydroxyacetone (8% in o/w emulsion) for 24 hours to evenly stain the stratum corneum. Prior to the first wash, b-value (yellowness) was measured at each site (Minolta® CM2600d Spectrophotometer). The sites were cleansed per the standard cleansing protocol once per day for a total of 4 days with b-value measured 2 hours after each treatment.

Stratum Corneum Hydration: Sixteen subjects were enrolled. Three test sites on the outer calf, one each for treatment A, C, or D, were treated per the standard cleansing protocol. Immediately after cleansing, each site was treated with 36 µL moisturizer (Olay® Professional Pro-X Wrinkle Smoothing Cream). One hour later, the site was analyzed with a Corneometer (Courage & Khazaka Corneometer® 820) for skin capacitance.

Stratum Corneum Barrier Function: Sixteen subjects were enrolled. Two tests sites on the volar forearms, one each for treatment A or C were cleansed per the standard cleansing protocol. A third site was used as an untreated control. Five minutes after cleansing, transepidermal water loss (TEWL) was measured at each site using the Servomed Evaporimeter®.

Anaerobic Bacteria Loads: Twelve female subjects, without clinical acne, were enrolled into this randomized split-face protocol. One side of the face was designated for treatment A, the other for treatment D. The schedule for bacterial swabs and facial cleansing is shown in Table 1. Facial skin areas demarcated by a 2-cm diameter cup scrub chamber were swabbed for 10 seconds. Bacteria were harvested from the facial skin using sterile polyester swabs saturated with sterile 1X phosphate buffered saline + 0.1% Triton X-100. Cheek swab sites were rotated each day such that no one site was repeatedly sampled throughout the course of the study. Swabs were stored at 4°C until extraction of bacteria the same day. Bacteria were enumerated via growth on enriched tryptic soy agar (Anaerobe Systems) plates. Viable anaerobic CFUs/cm^2 of skin were calculated after plating.

Table 1: Swabbing and treatment schedule in days relative to initial treatment for bacteria removal study. Treatments were test legs A and D.



Results and Conclusions

Both powered facial cleansing devices provided significantly greater anhydrous make-up removal and DHA stain removal compared to manual cleansing (Table 2 and Figure 1). Moisturizer applied to skin after cleansing with the rotating device was significantly more efficacious than when applied to skin that had been cleansed by conventional manual methods (Table 2). There was no significant difference in barrier function of skin cleansed by the rotating device compared to conventional cleansing.

Table 2: Cleansing, exfoliation, hydration and barrier function for the powered devices and manual cleansing. Numbers with a different letter are significantly different ($p<0.05$).

	Make-up Removal (ΔE)	**Hydration** (Corneometer)	**Barrier Function** (TEWL, g/m^2/h)
Rotating Device + ERC (**A**)	18.088 *a*	82.47 *a*	4.29 *a*
Oscillating Device + NCC (**B**)	20.602 *b*	----	----
ERC Only (**C**)	29.188 *c*	79.29 *b*	4.08 *a*
Water Only (**D**)	32.061 *d*	60.74 *c*	4.45 *a*

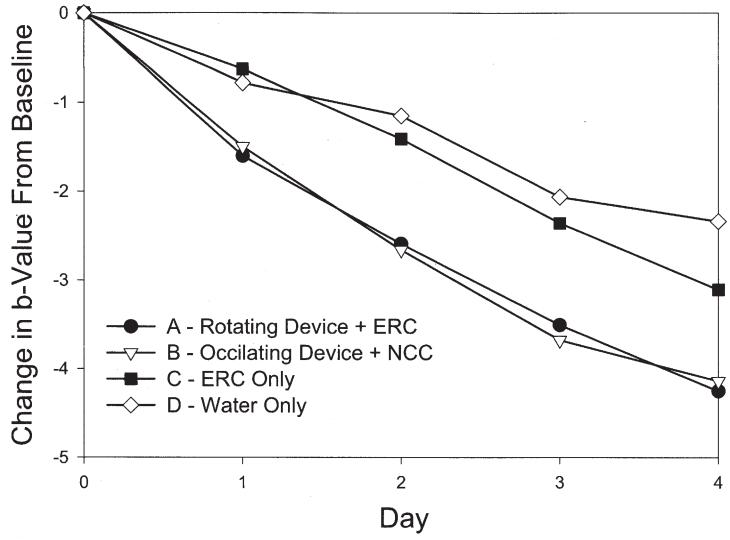


Figure 1: Statum corneum exfoliation. Treatments A and B were significantly different than C and D ($p<0.05$).

Anaerobic bacteria loads are shown in Fig 2. Viable anaerobic loads were consistent across the two baseline data points (-2d and -1d), indicating a stability within the group in this timeframe. A significant decrease in viable anaerobic bacteria loads was observed on the treated side vs. baseline and vs. the untreated control after completion of the cleansing protocol. Finally, after suspension of treatment on day 2, anaerobic bacteria loads on the treated side returned toward baseline levels by day 5.

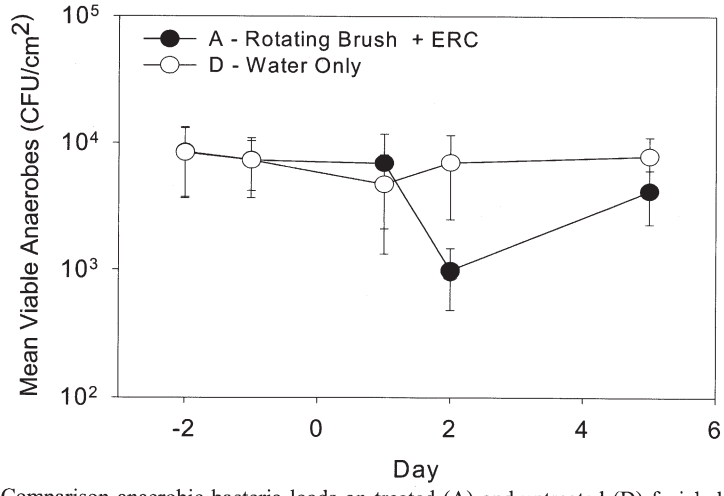


Figure 2: Comparison anaerobic bacteria loads on treated (A) and untreated (D) facial skin. Error bars: standard error of the mean.

Discussion

We have used several standardized test methods to compare two powered facial cleansing devices to traditional manual cleansing. Both devices showed similar advantages for cleansing and exfoliation despite using quite different mechanisms of brush head movement (i.e. rotating or oscillating). While the rotating brush moves in only the counter clockwise direction (relative to the device itself), during normal use the bristles will sweep across the skin in essentially all directions as the device is moved forward and backward and side to side. In this way, the loose skin surface squames will contact bristles from all sides thereby improving the cleansing and exfoliating efficacy. The TEWL and Corneometer data show that powered cleansing devices can gently treat the skin without damaging the stratum corneum barrier and leave it more able to absorb topical humectants. Finally, a significant change in viable anaerobic bacteria populations on the face was detected only after successive uses of the powered rotating device. This change in bacterial populations may result from inherent changes in the skin itself (e.g., structure, metabolism or response) which manifest only after repeated treatments, and not strictly by direct or immediate mechanical removal of these bacteria.

We plan further work including a study on the efficacy of powered cleansing devices to provide deep pore cleansing using UV-induced porphyrin fluorescence as a visual marker. Additionally, further study is needed to determine if cleansing/exfoliation modulates anaerobic bacteria loads indirectly via modulation of skin architecture, metabolism, and/or host response. It remains to be determined if the magnitude of effect on this bacteria population can be increased with more frequent/longer treatment periods. We also plan work to study the effect of powered cleansing devices on skin permeability using both in vitro and in vivo skin models.

In summary, given that the advantages of improved cleansing, exfoliation, and hydration can be achieved while avoiding skin barrier damage, plus the potential beneficial effect seen on anaerobic bacteria populations, we propose that powered cleansing devices should have a defined space in the skin devices toolbox.

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