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Plant-derived system boosts hydration and lipid barrier

ABSTRACT

In the field of corneobiology, the skin barrier has been pointed out to play a crucial role in skin homeostasis. In the treatment of dry skin, it is important to repair and augment the skin barrier in order to achieve positive and long-lasting results. To adequately describe the hydration state of the human skin, a number of complementary measuring techniques are often employed. Therefore, besides the classic methods of corneometry and the determination of the transepidermal water loss, we tested our moisturising active Hydro-Gain and the two industry standards, glycerol and hyaluronic acid, in a PCR-array and in a study using confocal Raman spectroscopy. In the comparison to glycerol and hyaluronic acid, Hydro-Gain gave the best results regarding skin moisturisation and we also found evidence that Hydro-Gain stimulates strengthening of the skin barrier.

The human skin is a multi-functional organ. It provides protection against numerous environmental factors and, among others, regulates the body temperature and water balance. The outer layer of the skin, the *stratum corneum* (SC), mainly consists of lipids (ceramides, cholesterol, fatty acids), proteins (filaggrin) and differentiated keratinocytes collectively which form a hydrophobic and antimicrobial barrier.[1]

A certain balance between skin surface lipids and the water content in the skin layers below provides for a water concentration gradient which is necessary for a healthy and functional skin. An even distribution of moisture to the SC is necessary to allow enzymatic activity and to provide skin elasticity and flexibility.[2] This distribution of moisture happens from the inner to the outer layers of the skin, which means that the process of skin hydration is not about water being absorbed from the outside but about water being transferred from the inside.[3] The skin barrier therefore provides protection in two directions, one against environmental factors and, in the other direction, against an uncontrolled loss of water.[4] In more than 50 years of research in the field of corneobiology the 'bricks and mortar' model has been established to describe the skin barrier and the water holding capacity of the SC.[5] The three main mechanisms of this model are the cornified envelope, the intercellular lamellar lipid bilayer and the presence of natural moisturising factors (NMF).[6] As the skin is a constantly growing organ, the skin barrier is also under both constant breakdown and renewal. It starts with the development of the cornified envelope in the *stratum spinosum* and ends with the desquamation process in the outermost layer of the skin.

A disruption in any of these processes can cause an impairment of the skin barrier, which will lead to dry skin. Dry skin appears reddened, rough and scaly. People so affected describe their skin to be aching, irritated and itchy. They also experience feelings of tension. The development of dry skin has been described in a cyclical model where a disruption of the skin barrier, whether extrinsically (environmental influences like temperature, humidity or exposure to surfactants) or intrinsically (chronological ageing and genetics) induced, causes a loss of skin moisture and NMF. It will also lead to the inactivation of enzymes involved in the desquamation process. These circumstances trigger an inflammatory hyperproliferative state, which causes the production of poor quality cell material and

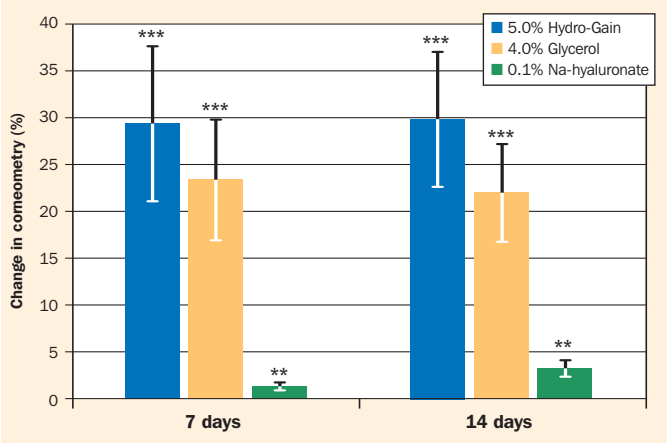

Figure 1: *Average change of corneometry results of volar forearms treated with cream formulations containing 5.0% Hydro-Gain, 4.0% glycerol or 0.1% Na-hyaluronate after 7 days and 14 days. Measurements were taken 24 hours after product application (*: p= 0.01-0.05; **: p<0.01; ***: p<0.001).*

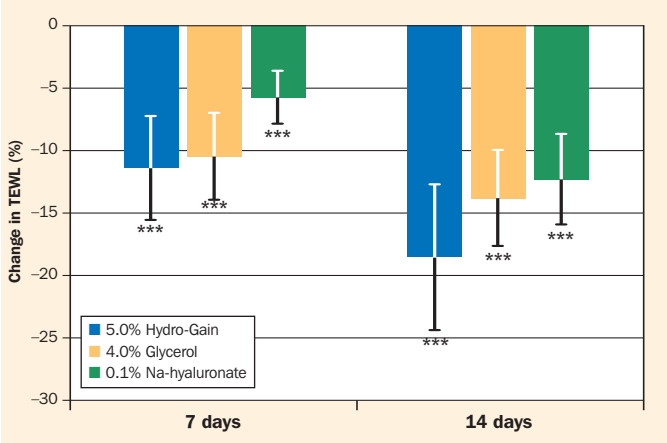

Figure 2: *Average change of the transepidermal water loss (TEWL) of volar forearms treated with cream formulations containing 5.0% Hydro-Gain, 4.0% glycerol or 0.1% Na-hyaluronate after 7 days and 14 days. Measurements were taken 24 hours after product application (*: p= 0.01-0.05; **: p< 0.01; ***: p< 0.001).*

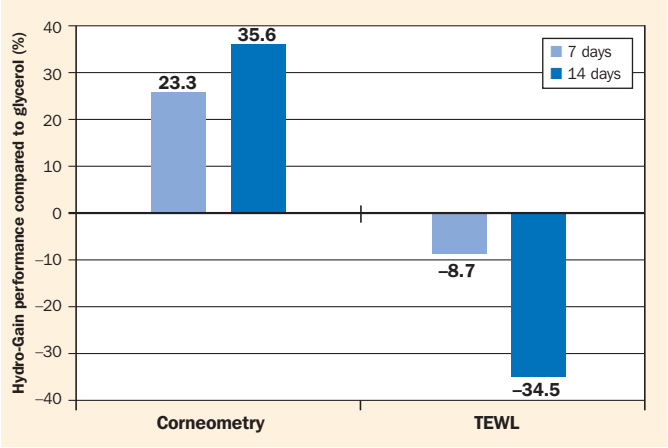

Figure 3: *Hydro-Gain* vs. *Glycerol. Comparison of Hydro-Gain cream formulation and glycerol cream formulation performances in corneometry and TEWL measurement after 7 days and 14 days. Measurements were taken 24 hours after product application, results are expressed as change [%] related to the results of the glycerol treatment.*

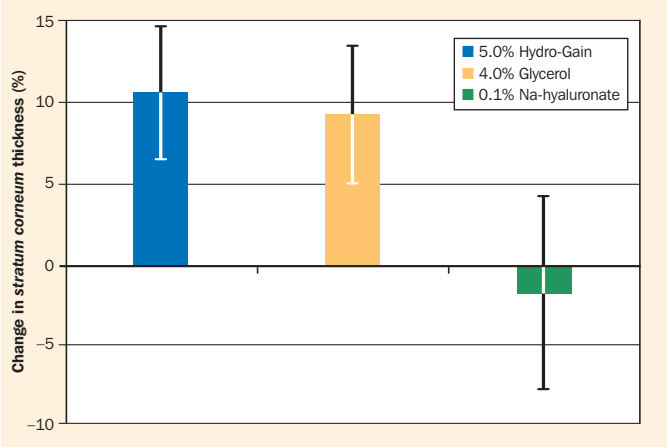

Figure 4: *Confocal Raman spectroscopy: Change in* stratum corneum *thickness of volar forearms after treatment with cream formulations containing 5.0% Hydro-Gain, 4.0% Glycerol or 0.1% Na-hyaluronate for 7 days. Measurements were taken 24 hours after product application.*

structures and ultimately a further weakening of the skin lipid barrier and additional loss of moisture.

To regain skin moisture, it is important not to only treat the symptoms of dry skin, but to repair and augment the skin barrier in order to break the dry skin cycle.[6] Moisturising creams and lotions fight dry skin by creating occlusive oil, or polysaccharide layers, or delivering NMF such as urea, glycerol or other polyols. Following water, glycerol is the second most widely used raw material in the cosmetic industry. It shows excellent solubility in polar solvents, has numerous skin care benefits, and due to its GRAS status, is very safe to use.[7] Endogenous glycerol maintains the hydration properties of the SC and thus the barrier function and it has been proven that topically applied glycerol is a very potent humidifying agent.[8] This is why it is reasonable to include glycerol as a benchmark in testing the efficacy of newly developed moisturising actives.

In the following study, we examined our recently developed moisturising active Hydro-Gain in extensive testing regarding product safety and humidifying effects in human skin and compared it to the two established industry standards glycerol and hyaluronic acid.

Methods

Formulation

Hydro-Gain is the result of raw material evaluation, screening assays and formulation experiments. It is meant to be the first product of a customisable line of combinations of hydrophilic and lipophilic active ingredients in the form of an oil in water emulsion-like system, emulsified by hydrogenated lecithin. Due to their promising effects described in literature[1,9–11] barbary fig (*Opuntia ficus-indica*) seed oil and birch (*Betula alba*) bark extract were chosen to be incorporated in our first product. Both plants are resistant to extreme environmental conditions. Birch bark contains a variety of compounds protecting the tree against cold and dryness. One of those compounds is the biologically active triterpene betulin, which has been found to improve moisturisation,[1] skin regeneration and wound healing.[9] The oil of the barbary fig, a species of cactus mostly native to hot and dry regions, is exceptionally rich in vitamin E and contains a variety of biologically active ingredients (eg: phytosterols, omega-3 and omega-6 essential fatty acids) with anti-oxidative and anti-inflammatory properties.[10,11] Hydrogenated lecithin, also described as gel-state phosphatidylcholine, has proved to be a valuable additive to skin moisturising formulations.[12] It elevates the hydration of the SC and makes the lipid barrier less rigid.[13]

Safety assessment

To secure the safe use of Hydro-Gain (now referred to as 'the moisturising active') and its corresponding cream formulation, several safety tests were carried out. The moisturising active was tested for phototoxicity (according to OECD Guideline No. 432), mutagenicity (Ames test according to OECD Guideline No. 471) and eye irritation (HET-CAM). Skin irritation was examined using a cream formulation containing 5.0% of the moisturising active in a single patch test (SPT) and an allergy certificate was issued in accordance with Directive 2003/15/EC Annex III.

Effect on selected key genes involved in epidermal biology

A PCR-array study was carried out in order to investigate possible effects of the moisturising active on 96 genes involved in key processes of epidermal barrier homeostasis, using an *in vitro* reconstituted human epidermis. The moisturising active was applied to a fully differentiated reconstituted human epidermis at a concentration of 1% (w/w) in the culture medium. After 24 hours of contact, RNA was extracted, and qRTPCR was performed for 96 target genes using a 384-well TaqMan array. Vitamin D3 was used as an assay control.[14,15]

Application formulations

For the *in vivo* tests regarding product safety and efficacy, a formulation containing 5.0% of the moisturising active (Table 1) was created. The components of phase A except Keltrol were mixed and heated to 75˚C. Then Keltrol was added whilst stirring thoroughly. Phase B was heated to 75˚C and added to phase A. The mixture was then gently stirred and cooled down to 35˚C. In the last step, the moisturising active was added and the mixture was stirred while cooling down to room temperature. The stability of the cream formulation was tested at 4˚C, room temperature and 40˚C for 6 months.

For the comparison of the moisturising active to industry standards, glycerol and hyaluronic acid, two additional cream formulations based on the formulation shown in Table 1 were created. For the cream formulation containing glycerol and hyaluronic acid, phase C was left out and 4% glycerol (99.5%) or 0.1% Na-hyaluronate (HyaCare; Evonik; 800kDA hyaluronic acid) respectively had been added in phase A. The amount of glycerol was chosen in order to match the amount of glycerol contained in the moisturising active. The use level of Na-hyaluronate was chosen according to the use level in the manufacturer's efficacy studies.

Test design *in vivo* studies

The skin hydrating effects of the moisturising active on human skin were examined in two independent, double-blind, randomised, placebo-controlled *in vivo* studies. Parameters examined were skin moisture, transepidermal water loss, as well as the thickness and the composition of the SC.

The preconditioning and treatment procedures in both studies were similar. In the pre-conditioning phase, participants had to wash their volar forearms with a conventional shower gel every evening for five days. After the pre-conditioning phase, volunteers applied a placebo formulation as well as three cream formulations (prepared according to Table 1) containing either 5.0% moisturising active, 4.0% glycerol or 0.1% Na-hyaluronate to the volar forearms, two times a day. While both studies employed 20- to 65-year-old Caucasian female volunteers with dry skin, there were differences in the number of volunteers and days of application. The study examining SC thickness and composition was conducted with 10 volunteers who applied the test formulations over seven consecutive days. In the second study, which focused on skin moisture and transepidermal water loss, the number of participants and study-duration were doubled to 23 volunteers and 14 days of application. In both studies, measurements were performed 24 hours after the last application, in order to examine the long term effect of the test formulations.

Table 1: Cream formulation containing 5% Hydro-Gain™.

Phase	Ingredient	INCI	%
A	Water	Water	64.70
	Keltrol CG SFT[1]	Xanthan Gum	0.60
	NaOH 10%	Water, Sodium Hydroxide	1.20
B	Tegin[2]	Glyceryl Stearate SE	1.50
	Crodafos CES[3]	Cetearyl Alcohol, Dicetyl Phosphate, Ceteth-10 Phosphate	6.50
	Myritol 318[4]	Caprylic/Capric Triglyceride	10.00
	Cetiol RLF[4]	Caprylyl Caprylate/Caprate	5.00
	Silicon Oil	Dimethicone	0.50
	Cetiol C5[4]	Coco-Caprylate	5.00
	Preservative		qs
C	Hydro-Gain[5]	Glycerin, Water, Canola Oil, Hydrogenated Lecithin, Opuntia Ficus-Indica Seed Oil, Betula Alba Bark Extract, Citric Acid	5.00

Suppliers: 1 CP Kelco 2 Evonik 3 Croda 4 BASF 5 Lipoid Kosmetik AG

Skin moisture and transepidermal water loss

Skin moisture was determined by capacitance measurement using a Corneometer CM 825 (Courage and Khazaka). Transepidermal water loss (TEWL) was measured using a Tewameter TM300 (Courage and Khazaka), which performs a density gradient measurement of the water evaporated from the skin. Measurements were taken prior to the first application of test products (t_0) and 24 hours after the last application of the 14 days treatment (t_1). Results were calculated relative to untreated skin at t_0 and the corresponding placebo value was subtracted.

Confocal Raman spectroscopy

The thickness and composition of the SC was examined using a Raman Skin Composition Analyzer 3510/HPRM 2500d (River Diagnostics). This technology relies on light's ability to transfer energy to and from molecules. An alteration of the energy level can be detected as a change in the light's wavelength. If light hits an optical-transmissive substance, it is either absorbed, scattered or passes the substance unaltered. The difference between the incoming light's original wavelength and the wavelength of the scattered light is defined as the Raman shift which is expressed in relative wavenumbers (cm^{-1}). The distinct vibrational energy levels of each molecule result in a Raman spectrum which can be used as a specific fingerprint to detect and quantify substances in complex mixtures. Raman spectroscopy has been proven to be a non-invasive and effective *in vivo* method to characterise human skin in terms of skin thickness, water content or penetration of certain molecules applied to the skin.

Results

The objective of the PCR-array study was to investigate possible effects of the moisturising active, on 96 genes involved in key processes of the epidermal barrier homeostasis. The moisturising active was applied to a fully differentiated reconstituted human epidermis at a concentration of 1% (w/w) in the culture medium.

Table 2 shows an excerpt from the results obtained in the qRT PCR performed after 24 hours incubation time. The relative quantification (RQ) value represents the factor by which the corresponding gene has been up- or down-regulated, compared to the reference gene B2M (Beta-2-microglobulin). The p-value gives information about the statistical significance of the corresponding result. All five genes listed in Table 2 play a role in skin moisturising processes and have been up-regulated after the 24 hours treatment with the moisturising active.

After the successful passing of safety- and stability-tests, three cream formulations (Table 1) containing either 5.0% moisturising active, 4.0% glycerol or 0.1% Na-hyaluronate were used in two *in vivo* studies as to compare their skin hydrating properties.

In the larger of the two studies, 23 volunteers applied the test formulations for 14 days. After 7 days and 14 days, skin water content and the transepidermal water loss were determined 24 hours after the last application.

Figure 1 shows the average changes in corneometry results for the three test formulations, obtained at the volar forearms after 7 days and 14 days of application. After 7 days, the cream formulation containing 5.0% moisturising active caused an improvement in skin moisture of 29.2%; after 14 days the results slightly rose to 29.8%. The glycerol cream formulation gave an improvement of 23.3% after 7 days and 21.9% after 14 days. The cream formulation containing 0.1% Na-hyaluronic acid only slightly raised skin moisture by 1.2% after 7 days and 3.1% after 14 days of appliance.

Table 2: Effects of 1% Hydro-Gain™ on genes involved in key processes of epidermal barrier homeostasis.

	Gene name	RQ	*p*-value
SPRR1A	Cornifin-A (Small proline-rich protein IA)	2,2496	0.0031
HBEGF	Heparin-binding EGF-like growth factor	1,9239	0.0289
KLK7	Kallikrein-7	1,6466	0.0047
LOR	Loricrin 1	1,5189	0.0280
CASP14	Caspase-14	1,4727	0.0118

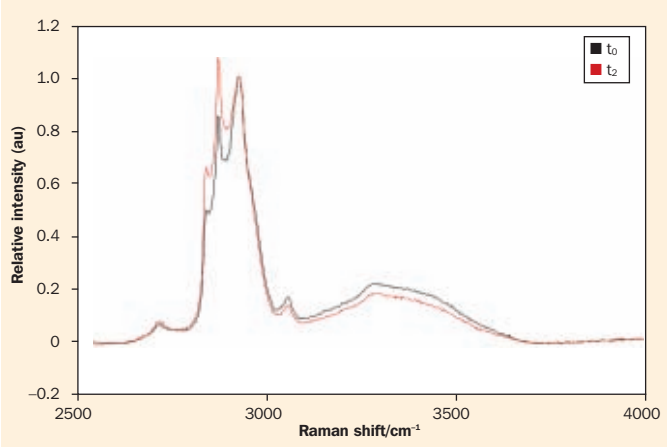

Figure 5: *Confocal Raman spectroscopy: Composition of SC in 2 µm depth after Hydro-Gain treatment. Comparison of Raman spectra of the SC before (t0; black spectrum) and after (t2; red spectrum) the treatment with a cream formulation containing 5.0% Hydro-Gain for 7 days. Measurements were taken 24 hours after product application.*

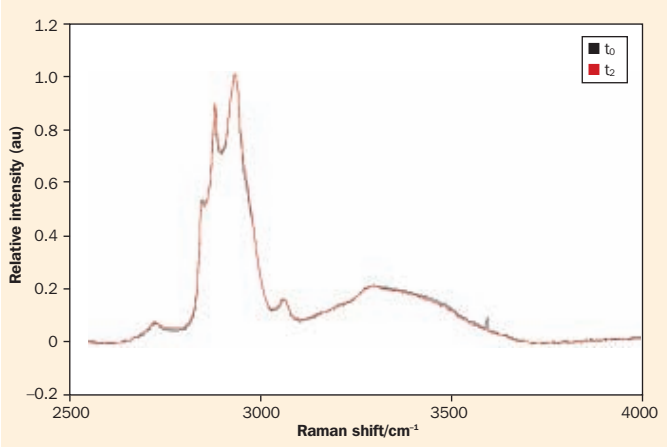

Figure 6: *Confocal Raman spectroscopy: Composition of SC in 2 µm depth after glycerol treatment. Comparison of Raman spectra of the SC before (t0; black spectrum) and after (t2; red spectrum) the treatment with a cream formulation containing 4.0% glycerol for 7 days. Measurements were taken 24 hours after product application.*

The average changes in tewametry results are shown in Figure 2. After 7 days of treatment, the moisturising active formulation led to a reduction of transepidermal water loss by 11.5%. After 14 days, the reduction was increased to 18.4%. The formulation containing 4.0% glycerol reduced the TEWL by 10.6% after 7 days and 13.7% after 14 days. Na-hyaluronate caused a reduction of 5.9% after 7 days, which rose to 12.37% after 14 days. The results shown in Figures 1 and 2 have been recalculated to a comparison of the moisturising active versus glycerol, which is shown in Figure 3.

The results of the glycerol treatments were set as the baseline, so the comparison to the moisturising active treatments is expressed as difference in per cent. After 7 days of application, the increase in skin moisture (measured by corneometry) caused by the cream formulation containing 5.0% moisturising active was 25.3% higher than the effect caused by the cream formulation containing 4.0% glycerol. The difference rose to 35.6% after 14 days of treatment. The comparison of TEWL results showed that the transepidermal water loss in the moisturising active treated skin was 8.7% less than in glycerol treated skin. After 14 days the moisturising active cream formulation showed a TEWL reduction which was 34.5% better than the TEWL reduction caused by the glycerol cream formulation.

Confocal Raman spectroscopy was used in the second *in vivo* study, in which 10 volunteers applied the aforementioned cream formulations for 7 days. Measurements were conducted 24 hours after the last application. Figure 4 shows the changes in SC thickness after a 7 days treatment with cream formulations containing 5.0% moisturising active, 4.0% glycerol or 0.1% Na-hyaluronate. The moisturising active cream increased SC thickness by 8.9%, the glycerol cream by 7.7% and the Na-hyaluronate formulation caused a decrease of 1.3%.

Besides the measurement of SC thickness, confocal Raman spectroscopy was used to specify the composition of the SC at a certain depth. Figures 5 and 6 show the overlaid Raman spectra of skin of the volar forearms at a depth of 2 µm, before and after the 7 days treatment with the cream formulations containing 5.0% moisturising active or 4.0% glycerol. Peaks of interest are the CH_2 and CH_3 vibrations caused by lipids and proteins at approx. 2875 cm^{-1} and 2910-2965 cm^{-1}, as well as the OH vibration of water at 3350-3550 cm.[16,17] By comparison of the Raman spectra of t_0 (before the treatment) and t_2 (24 hours after the 7 days treatment) in Figure 5, one can observe that the moisturising active treatment caused an increase in lipids while the water content decreased. The glycerol treatment shown in Figure 6 did not cause any changes in the lipid or water contents.

Discussion

In 2008, Crowther *et al*. stated that the hydration state of the skin cannot be adequately be described by a single technique but, rather, a number of complementary techniques are required for a complete description.[18] We therefore chose several techniques to investigate the skin hydrating properties of the moisturising active and compared it to the industry standards glycerol and Na-hyaluronate.

The results of the *in vitro* PCR-array study (Table 2) show that the moisturising active enhances the expression of genes coding for SPRR1A (small proline-rich protein 1A or cornifin A), LOR (loricrin), KLK7 (human kallikrein-related peptidase 7), CASP14 (caspase-14) and HBEGF (heparin-binding EGF-like growth factor). These genes are involved in major processes responsible for skin renewal and the formation of the skin barrier.

Small proline-rich protein 1A (SPRR1A) and loricrin (LOR) are expressed in terminally differentiating human keratinocytes and make up the predominant part, namely 80%, of the cornified envelope (CE).[19–21] SPRR also plays a role in detoxification and the quenching of reactive oxygen species (ROS), adding to the CE's antioxidant defence.[22] Human kallikrein-related peptidase 7 (KLK7) is a protease involved in the desquamation process. In order to maintain epidermal barrier function and homeostasis, old corneocytes need to be removed promptly by shedding. To accomplish this, cell-cell adhesions provided by corneodesmosomes are dissolved by a proteolytic cascade of human kallikrein-related peptidases (KLKs). As KLK7 is a part of this cascade and was overexpressed after application of the moisturising active, the active might favour a quicker shedding of corneocytes and a shorter turnover of the SC. KLK7 activates the progenitor form of caspase-14 (CASP14),[23] a protease which was also up-regulated after the moisturising active treatment. CASP14 plays a role in the generation of natural moisturising factors (NMF), since it is involved in the processing and degradation of filaggrin into free hygroscopic amino acids and urocanic acid.[6] The heparin-binding EGF-like growth factor (HBEGF), a member of the epidermal growth factor (EGF) family produced in the epidermis, is also involved in skin renewal by promoting keratinocyte migration.[24]

In summary, the results of the PCR-array study show that the moisturising active

promotes several genes, which are, among others, contributing to major mechanisms in the treatment of dry skin, namely the formation of the skin barrier, skin renewal and the production of NMF.

The first part of the *in vivo* examinations focused on two classic indicators in skin hydration. The transepidermal water loss and the skin moisture measured by corneometry. In order to evaluate the long term efficacy of the test products, the measurements were taken 24 hours after the preceding treatment with cream formulations containing 5.0% moisturising active, 4.0% glycerol or 0.1% Na-hyaluronate. Figures 1 and 2 show the changes in TEWL and corneometry results after 7 and 14 days. While the results in corneometry only slightly improve after 14 days of treatment, there is a significant difference between the TEWL results obtained after 7 and 14 days. All three treatments reduce the TEWL and the effect improves with the time of treatment. In both measurements, the moisturising active treatment exceeds the results of the treatments with industry standards glycerol and Na-hyaluronate. The comparison of the moisturising active versus glycerol (Fig. 3) points out the very promising moisturising properties of the moisturising active. The increase in skin moisture and decrease in TEWL over time indicate the build-up of a long term moisturising effect of the product.

In the second part of *in vivo* examinations, confocal Raman spectroscopy was used to examine the effects of the test formulations on the skin barrier. Figure 4 shows that only the formulations containing 5.0% moisturising active and 4.0% glycerol could affect SC thickness. While the increase in SC thickness caused by the moisturising active (8.9%) and glycerol (7.7%) are rather similar, the Raman spectra in 2 µm skin depth before and after the 7 days treatments (Figs. 5 & 6) show two very different results. The moisturising active treatment causes an increase in proteins and lipids, recognisable in the signal changes around 2875 cm^{-1} and at the same time a decrease in water content (3350-3550 cm^{-1}). The glycerol treatment does not have an effect on either the lipid and protein or the water content. These circumstances give us reason to conclude that the moisturising active treatment caused an improvement of the skin barrier. An increase of lipids can be related to the film forming properties of hydrogenated lecithin[12,25,26] and the increase in protein is most likely the result of the up-regulation of certain genes contributing to the formation of the cornified envelop. The decrease in water does not have to be described as a hydration loss, but as a result of the changes in the composition of the SC. To further understand the changes we observed in the SC, future studies should focus solely on the SC and should employ techniques like Raman spectroscopy or multi-photon spectroscopy.

Conclusion

Summing up our study results, we observed changes in the thickness and composition of the SC, an up-regulation of genes responsible for the formation of the cornified envelope and also a time- dependent improvement of the transepidermal water loss and skin moisture. We conclude that the treatment with the cream formulation containing 5.0% Hydro-Gain positively changed and enhanced the skin barrier, led to a reduced transepidermal water loss and improved skin moisture. PC

Acknowledgement

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