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Anti-microbial and -inflammatory activity and efficacy of phytosphingosine: an *in vitro* and *in vivo* study addressing acne vulgaris

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Synopsis

Lipids are important constituents of the human epidermis. Either free and organized into broad lipid bilayers in the intercorneocytes spaces, or covalently bound to the corneocyte envelope, they play a crucial role in permeability barrier function and are major contributors to cutaneous anti-microbial defense. Free sphingoid bases are a recent addition to this family of active lipids, which emerged from studies of breakdown products from ceramides. Phytosphingosine (PS) is a lipid occurring naturally in the stratum corneum, both in its free form and as a part of the major fraction of ceramides. The biotechnological production of PS patented by Degussa yields to PS with the correct configuration present in the skin. So, application of a PS containing formulation leads to its integration into the natural lipid structures of the skin. In acne, different pathogenetic factors contribute to the inflammation process, defect in keratinization, increased sebaceous gland activity and increased colonization of *Propionibacterium acnes*. The results of *in vitro* and *in vivo* studies confirm the previous reports on strong anti-microbial effectiveness of skin-identical PS produced by Degussa *in vitro* and *in vivo*. In addition, PS shows excellent clinical results in the context of skin care in acne, based on both anti-inflammatory and anti-microbial activity. These results demonstrate the potential of PS to enhance or complement existing acne therapies acting as an active cosmetic ingredient.

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Introduction

The earliest subclinical acne 'lesion' is a microcomedone, hyperproliferation of the follicular epithelium being its characteristic feature. Recently, significant pro-inflammatory factors, such as interleukin-1, have been identified around clinically normal pilosebaceous follicles from uninvolved skin in acne patients prior to hyperproliferation of the follicular epithelium [1]. This contributes to the concept that acne vulgaris should be classified as an inflammatory skin disease.

In many scientific articles, stratum corneum lipids have been recognized as important constituents of the human epidermis [2]. In 1988, Miller *et al.* [3] first showed that epidermal keratinocytes, not sebaceous glands, were responsible for epidermal lipid formation. In this process, polar phospholipids, the major constituents of cell membranes, are transformed by enzymatically catalysed hydrolysis of the polar lipids in the lamellar bodies liberating free fatty acids, glycosphingolipids and ceramides. In the stratum corneum, ceramides are present in an equimolar ratio with cholesterol and free fatty acids, which play a crucial role for the epidermal barrier function [4–6]. Ceramides are complex epidermal sphingolipids, whose basic structural frame-

work consists of a sphingoid base (amino alcohol of varying chain length), which is covalently attached by its amino group to a fatty acid of varying chain length by an *N*-acyl bond. There are four types of sphingoid bases: sphinganine, sphingosine, 6-OH-4-sphingenine and phytosphingosine (PS).

Most of the published research on free sphingoid bases deals with the inhibition of micro-organisms and their 'second messenger' function. Therefore, the free sphingoid bases are known to be very efficient in cutaneous anti-microbial barrier function [7, 8] and natural anti-inflammatory agents. Recently performed *in vitro* and *in vivo* studies showed that PS has anti-microbial properties against *Propionibacterium acnes*, a bacterium linked to inflammatory host response in acne and *Staphylococcus aureus* as well as anti-inflammatory function.

Increased levels of interleukins (cytokines), especially IL-1α, are a prime feature of inflamed skin. Moreover, the activation of the enzyme protein kinase C (PKC) can contribute to an inflammatory response. By inhibiting these two pathways, a relevant control over inflammation can be achieved.

This study addresses in its *in vitro* part the influence of PS on relevant pre-inflammatory factors and in its *in vivo* part the potential of PS to enhance or complement existing acne therapy judging from the number of comedones and papules. In the context a well-known anti-acne preparation, i.e. 4% benzoyl peroxide, the 0.2% hydrochloride salt of PS, the mixture of both and a placebo preparation were applied.

Materials and methods

In vitro studies

Materials

The skin-identical PS is obtained by yeast fermentation with the strain *Pichia ciferii*. Its preparations and cosmetic formulations were provided by Degussa AG.

Methods

Anti-microbial activity. Using a methodology similar to that previously described [8], the inhibitory effect of PS on growth of different micro-organisms was tested. Tween 80 instead of ethanol was used to solubilize PS, which enabled the reduction of the ethanol concentration to non-inhibitory levels. The following solutions were prepared:

PS stock solution: variously concentrated solutions of PS (Degussa AG) in ethanol (96%).

Tween 80 stock solution: fivefold dilution of Tween 80 in demineralized water.

Phytosphingosine test solutions were prepared by mixing one volume of an appropriate strength PS stock solution with nine volumes Tween 80 stock solution and 110 volumes Bacto-peptone Broth (Difco, Detroit, MI, USA).

The following micro-organisms were tested: Gram-positive bacteria (*Micrococcus luteus*, *S. aureus*, *Corynebacterium xerosis*, *P. acnes*), Gram-negative bacteria (*Pseudomonas aeruginosa*, *Escherichia coli*), Yeasts (*Candida albicans*) and Dermatophytes (*Microsporum canis*). Bacterial growth was quantified as the number of colony-forming units (CFUs). Growth inhibition was investigated on a linear scale, using different concentrations of PS. A 10-fold reduction would be scored as 'no growth'. The concentrations of PS required for growth inhibition of different micro-organisms within 1 h were determined.

Release of interleukin-1α by UVB-irradiated human skin on culture. The effect of UVB was investigated by using human skin explants in culture as a model. Phytosphingosine (0.2% and 1.0%) and dexamethasone (10^{-6} M) (a potent anti-inflammatory reference drug) were applied to human skin explants in culture to test their anti-inflammatory potential. The products were applied 1 h before and immediately after irradiation (20 min of UVB 2 J cm^{-2}, maximum at 312 nm, Vilbert-Lourmat-Lamp). Just before irradiation, the products were rinsed off the skin to prevent a possible filtering effect during irradiation.

The interleukin-1α secretion was measured by using an ELISA kit (Human IL-1α immunoassay, R&D Systems GmbH, Wiesbaden, Germany) at 24 h. Results were expressed as pg mL^{-1} of IL-1α. The baseline was defined as the amount of IL-1α in the non-treated non-exposed skin, the maximal IL-1α production as the amount in the non-treated UVB-exposed skin. The inhibitory effect of two concentrations of PS and dexamethasone was statistically compared with the non-treated UVB-exposed control according to a one-way ANOVA followed by Dunnett's *t*-test ($P < 0,05$).

Activity of PKC. Pure PKC was incubated with substrate (histone) and radiolabelled adenosine-3-phosphate (ATP), in the presence and absence of

the reference product staurosporine (10 μM) and varying concentrations of PS (range 0.001–0.2%). Phytosphingosine was used as its hydrochloride.

The incorporated radioactivity in the radiolabelled reaction product was measured by liquid scintillation. The more active the enzyme is, the more phosphorylated histone is produced. The degree of inhibition by staurosporine and PS–HCl compared with the control was calculated.

Effect on the artificial human epidermis after irritation with SDS. The efficacy of PS on a 3-D artificial skin model (SkinEthic™; SkinEthic Laboratories, Nice, France) was investigated after damage with the irritant surfactant sodium laurylsulphate (SDS).

After thawing of the artificial human epidermis followed by controlling their viability, a 0.25% SDS solution (dissolved in PBS) has been added to the skin models for 40 min to induce chemical stress.

Afterwards the skin slides were washed and a cosmetic O/W formulation (vehicle, formulation containing 0.15% PS) was applied.

After 24 h, different parameters as cell death represented by lactate dehydrogenase (LDH), viability according to the XTT assay, inflammatory response judging from interleukin-1α (IL-1α) expression were determined.

The LDH release was measured by using a colorimetric test kit from Roche Diagnostics (Mannheim, Germany).

The XTT test is based on the ability of the mitochondrial enzyme succinate dehydrogenase to reduce the dye sodium 3,3′-1-[(phenylamino)carbonyl]-3,4-tetrazolium-*bis* (4-methoxy-6-nitro) benzene sulphonic acid hydrate (XTT) into a water-soluble formazan salt detectable via photometry. Thus, the metabolic activity and viability of the cells can directly be measured.

In vivo studies

Topical in vivo study on anti-microbial efficacy

In this study, the anti-microbial efficacy of topical PS within an emulsion-based formulation was determined in an *in vivo* test. Both PS and its salt PS–HCl were compared with a control formulation, and a frequently used anti-microbial, triclosan, as a positive control. The formulations were tested on the unwashed hands of 12 subjects based on the bacterial counts. The total microbial count was determined on the skin at $t = 0$, after 1 h and after 4 h. Percentages from baseline were calculated, indicating the reduction in microbial count. After ranking the data, the binomial distribution was used to test if at least one of the products was better than the control. The percentage microbial count reduction per active product relative to the control was analysed by using Dunnett's one-tailed *t*-test.

Clinical study on acne skin

The randomized, half-face trial was performed in three separate dermatology centres based in France.

The study was divided into two different parts: in the first part of the study, the effectiveness of PS with benzoyl peroxide was compared with that of benzoyl peroxide alone, in the second part PS was tested vs. placebo.

Subjects

In the first part of the trial, 15 non-pregnant women and 15 men with an average age of 20 years and moderate inflamed acne were enrolled after written informed consent. Inclusion criteria were moderate inflammatory acne and age of 10–50 years. Patients with known hypersensitivity to benzoyl peroxide were excluded from participation. All volunteers were asked to use only the purifying gel ZENIAC® (LED, Paris, France) as a cleansing base and not to expose to the sun. A wash-out period of 1 month was realised prior to the study.

In the second part of the trial, 10 volunteers aged between 10 and 50 years participated.

The subjects were randomly allocated to the treatment groups. They were instructed to cleanse their faces twice a day with the purifying gel applied either with cotton pad, compresses or paper wipes.

The first group received after face cleansing in the morning and evening on the right face side benzoyl peroxide (Brevoxyl®, Stiefel, Rueil-Malmaison, France), and on the left face side a combination of PS–HCl and benzoyl peroxide from the SYMBIO® dispenser (Airspray, NL). In the second group, the right side of the face was treated with the placebo preparation (benzoyl peroxide like formulation without benzoyl peroxide) and the left side with PS–HCl, combined with benzoyl peroxide like formulation without benzoyl peroxide in dispenser,

also twice a day. A precise explanation for the use of preparation was given to each participant, which included a demonstration in the office of the dermatologist. The products were applied for the duration of the whole treatment period of 60 days.

All subjects taking part in the study were submitted to a search test for allergy and irritation potential to benzoyl peroxide. A pastille with 0.5% benzoyl peroxide, diluted with vaselin, was applied to the backs after previous degreasing with alcohol. The results were evaluated 72 h later.

Materials

Benzoyl peroxide was used as an anti-acne preparation available on the market. It contains 4% benzoyl peroxide and was applied as an established acne treatment approach. The hydrochloride of PS was tested in a concentration of 0.2% vs. benzoyl peroxide.

Benzoyl peroxide and PS–HCl are incompatible products. Therefore, PS–HCl and benzoyl peroxide were combined in the dispenser system.

The second group received PS–HCl combined with a vehicle based on the components of the benzoyl peroxide formula without the active ingredient benzoyl peroxide. The vehicle formulation contained neither benzoyl peroxide nor PS–HCl.

All patients were instructed to use the purifying gel as a cleansing base.

List of ingredients of Zeniac Purifying Gel – High Tolerance® (LED, Paris, France) is given hereunder:

Water, cocamidopropyl betaine, decyl glucoside, glycerine, PEG-40 glyceryl cocoate, PEG-120 methyl glucose dioleate, propylene glycol, PEG-7 glyceryl cocoate, sodium, coceth sulphate, arctium majus (root extract), sodium methyl cocoyl taurate, imidazolidinyl urea, fragrance, tocopheryl acetate, zinc PCA, PEG-54 hydrogenated acstor oil, PEG-15 hydroxystearate, phenoxyethanol, biotin, citric acid, sodium hydroxide, CI 42090 (blue 1), limonene, linalool, hydroxycitronellal, citral, benzyl salicylate, alpha-isomethyl ionone, hexyl cinnamal and citronellol.

Measurements

The dermatologists evaluated the results on day 0, day 30 and day 60. The objective criteria were the number of comedones as well as papules and pustules on the left and right side of the face. The intensity of the acne was repeatedly determined by using the scale value based on the scale Echelle d'Evaluation Clinique des Lesions d'Acne (ECLA). ECLA grading appears to be a useful tool in dermatology for the follow-up of acne patients [9]. Photographs were also taken. Any side effects and subjective observations like ease of application, condition of the skin after use and odour of the product were immediately notified by the volunteers.

Results

In vitro studies

Anti-microbial activity

Phytosphingosine inhibited – even at very low concentrations – the growth of Gram-positive and -negative bacteria, yeasts and moulds. The lowest concentration required for growth inhibition within 1 h was seen for *C. albicans* (0.0012%) and the highest for *E. coli* (0.040%). For *P. acnes*, the concentration of PS required for growth inhibition within 1 h was 0.020%. The graph below presents the anti-microbial properties of PS with respect to *P. acnes* by measuring the number of CFUs (Fig. 1). It became obvious that the outgrowth of bacteria, which occurred at longer inhibition times, is prevented by using higher concentrations of PS.

Release of interleukin-1α by UVB-irradiated human skin explants

In the non-treated UV-B exposed skin, the IL-1α release was increased by a factor of 4.2 compared with the non-irradiated skin. Compared with the non-treated UV-B exposed skin, dexamethasone, 0.2% and 1% PS inhibited the release of IL-1α

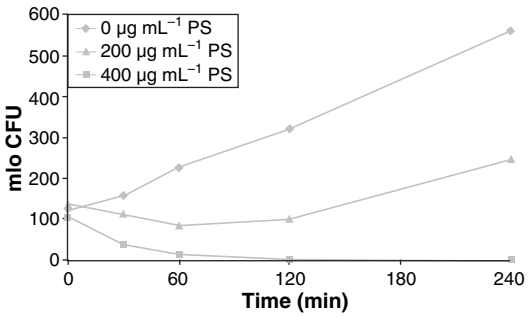

Figure 1 Growth inhibition of *Propionibacterium acnes*.

markedly, figures reading 56%, 78% and 72%, respectively ($P < 0{,}05$). The anti-inflammatory properties of PS were almost the same for the 0.2% and 1% concentration.

Activity of PKC

The reference product staurosporine completely inhibited PKC activity. At a concentration of 0.001%, PS did not show any detectably inhibitory effect on the enzyme. At a concentration of 0.01%, 0.1% and 0.2%, however, PS inhibited approximately 90% of the PKC activity (Fig. 2).

Effect of PS on artificial human epidermis after irritation with SDS

The LDH release of the skin slides treated with the vehicle formulation increased to an amount of approximately 19 U L^{-1} whereas the slides treated with the PS containing formulation only showed a concentration of about 10 U L^{-1}.

It has been found that PS even maintains full viability (data not shown).

The amount of the cytokine IL-1α increased from <15 pg mL^{-1} medium after irritation with SDS to approximately 35 pg mL^{-1} (Figs 3 and 4).

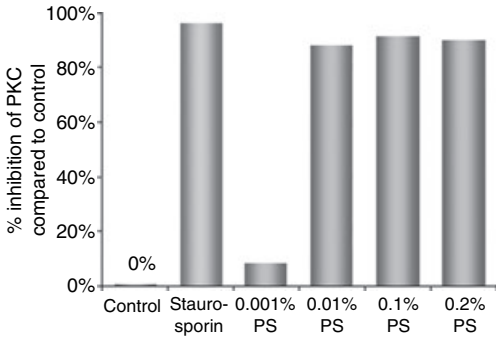

Figure 2 Effect of phytosphingosine on protein kinase C activity.

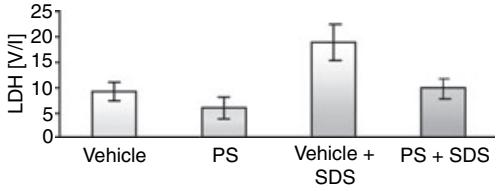

Figure 3 Effect of phytosphingosine on release of lactate dehydrogenase after irritation with sodium laurylsulphate.

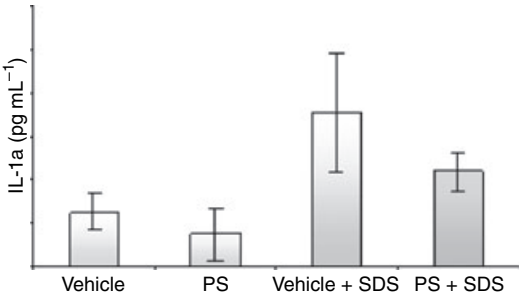

Figure 4 Effect of phytosphingosine on release of IL-1α after irritation with sodium laurylsulphate.

Application of PS reduced the IL-1α release finally to 22 pg mL^{-1}, indicating the anti-inflammatory effect of PS.

In vivo studies

Topical in vivo study on anti-microbial efficacy

A statistically significant difference of $P = 0.078$ at 1 h and $P = 0.020$ at 4 h was detected between the control and the other groups. Phytosphingosine, PS–HCl and triclosan have reduced the amount of bacteria on unwashed hands by 68%, 87% and 79%, respectively, 1 h post-product application compared with the effect of the control (0%). Phytosphingosine, PS–HCl and triclosan compared with the effect of the control have reduced the amount of bacteria on unwashed hands by 42%, 68% and 60%, respectively, 4 h after product application. These data are shown in the graph below. Dunnett's test showed that these effects were significantly different with at least 90% one-sided confidence for PS, PS–HCl and triclosan at 1 h and only PS–HCl at 4 h after application (Fig. 5).

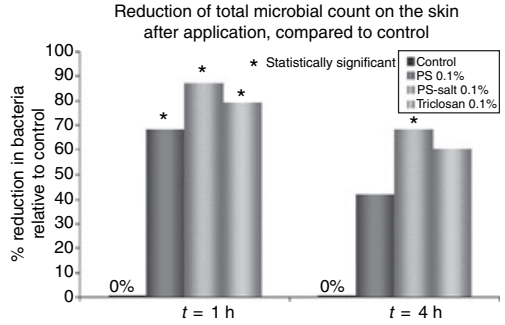

Figure 5 *In vivo* anti-microbial efficacy of phytosphingosine (PS), PS–HCl and triclosan.

Clinical study on acne

Group 1: benzoyl peroxide vs. benzoyl peroxide + PS–HCl. Only 1 volunteer dropped out of the study after 30 days. All the other volunteers were evaluated at day 30 and day 60 of the study. No case of cutaneous intolerance was indicated by any subject.

At day 30, benzoyl peroxide reduced the number of comedones by 15%, the combination product by 43%. At day 60, the reduction of 22% respectively 72% was achieved (Figs 6 and 7).

The effect on papules and pustules was even more impressive. Benzoyl peroxide alone reduced their number by 10% at day 30, respectively, by

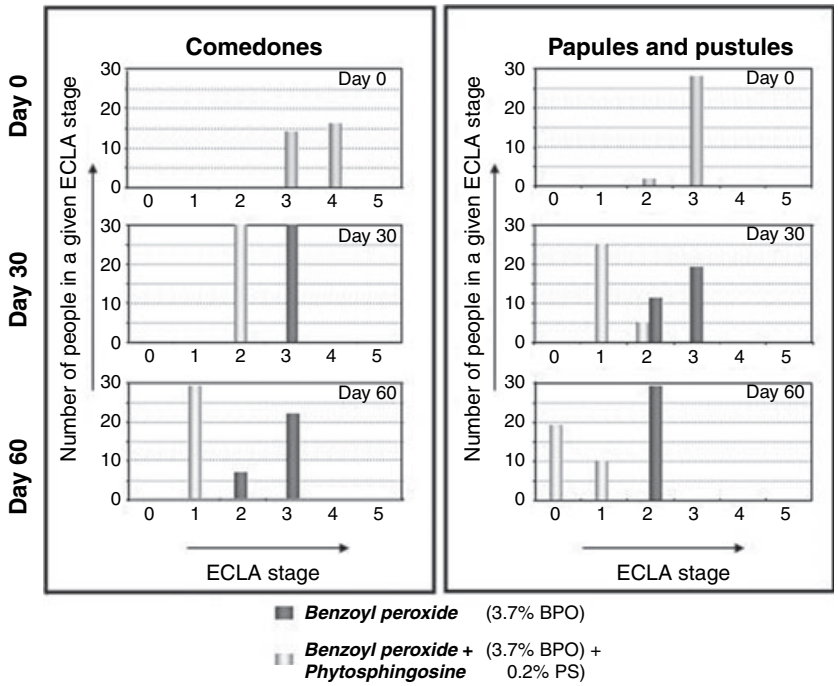

Figure 6 The effect of benzoyl peroxide vs. benzoyl peroxide and phytosphingosine according to the average ECLA stage at day 0, 30 and 60, and consequently comparing that with the average ECLA stage at day 0.

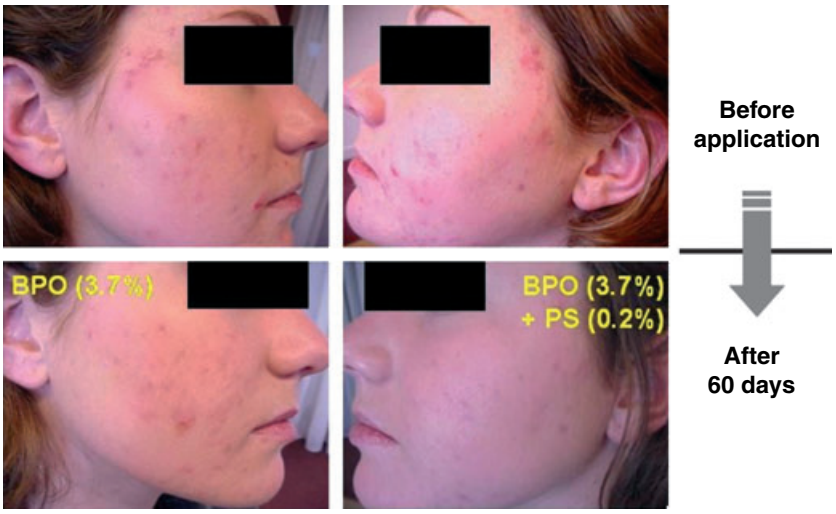

Figure 7 Clinical photographs taken on day 0 and after 60 days in the first treatment group: benzoyl peroxide vs. benzoyl peroxide plus phytosphingosine.

32% at day 60. The combination with PS had a much stronger effect, 60% and even 88% reduction at day 60 being found (Figs 6 and 7).

Group 2: PS–HCl vs. placebo. No volunteer dropped out of the study. Application of placebo increased the number of comedones by 43% according to the data obtained on day 30 and 60. Phytosphingosine–HCl was able almost to control the development of new comedones. Only an increase of 6% was seen.

Papules and pustules were not influenced by the placebo. The preparation containing PS almost reduced the number of papules and pustules at day 60 to zero, exactly by 89% (Figs 8 and 9).

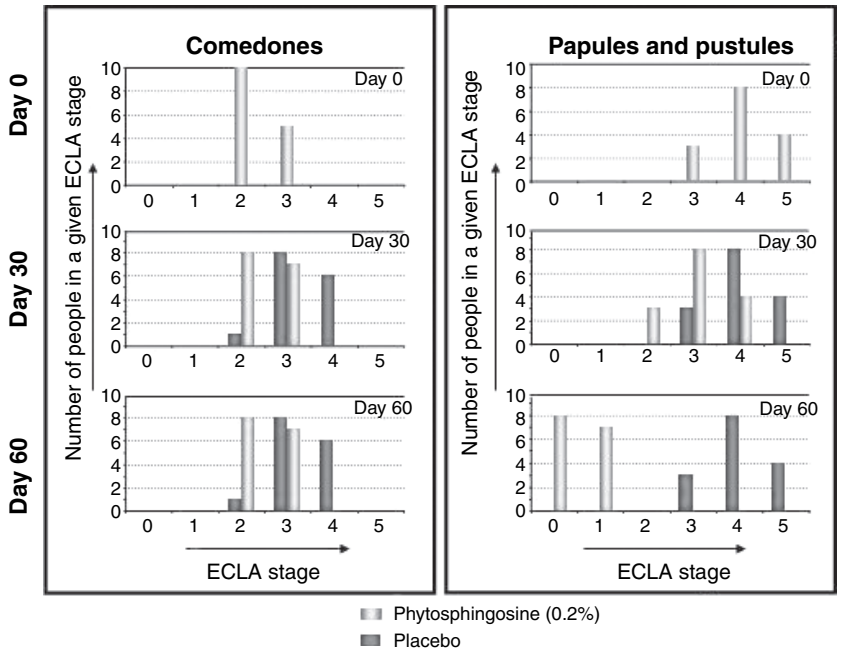

Figure 8 Effect of phytosphingosine vs. placebo by determining the average ECLA stage at day 0, 30 and 60, and consequently comparing that with the average ECLA stage at day 0.

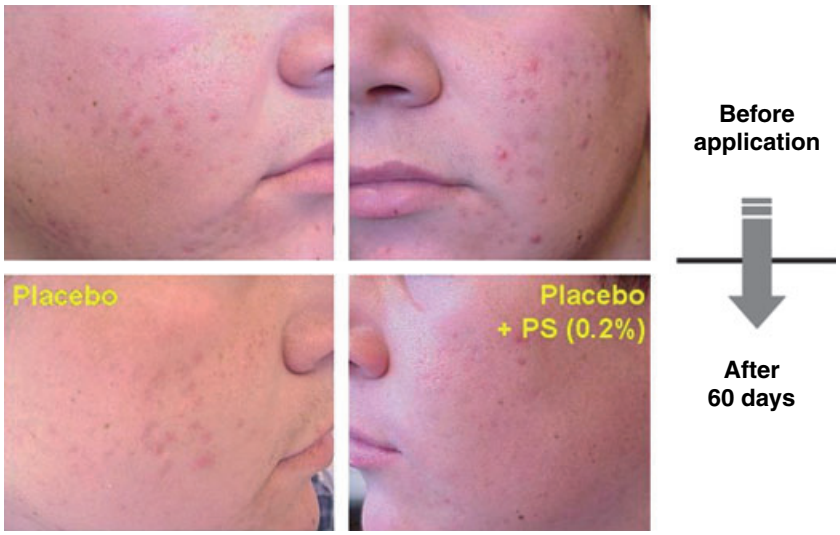

Figure 9 Clinical photographs taken on day 0 and after 60 days in the second treatment group: placebo vs. phytosphingosine.

Secondary effects and subjective observations

The combination of benzoyl peroxide with PS–HCl as well as the pure PS–HCl preparation was considered easy to use, with a good cosmetic acceptability and quality. There was no cutaneous intolerance. The preparations were also considered not to leave any greasy residue.

Discussion

Sphingoid bases in eukaryotic cells are recognized as the defining precursors of complex sphingolipids such as ceramides, sphingomyelins, cerebrosides and others. Phytosphingosine is a lipid occurring naturally in the stratum corneum, both in its free form and as part of the major fraction of ceramides.

The data presented in this study provide novel evidence for the anti-inflammatory and anti-microbial activity of PS. These two anti-acne properties work synergistically resulting in a good effect on the clinical status of acne-prone individuals. This, in particular, applies to the inflammatory type of manifestation. The growth of a wide spectrum of relevant micro-organisms, such as Gram-positive and -negative bacteria, yeasts and moulds, is effectively inhibited by PS already at very low concentrations between 0.0012% and 0.040%. Already in former studies it has been shown that the sphingoid bases naturally occurring in the stratum corneum demonstrate a potent anti-microbial effect and that a decrease in sphingoid base levels results in diminished anti-microbial defense [3, 10]. Bibel *et al.* [8] show that various sphingoid base species cause a highly significant reduction (4.5 log) in CFUs of *S. aureus* at a concentration of only 6.5 μg mL^{-1}. The anti-microbial mechanism of sphingoid bases has not been sufficiently elucidated as yet. There are different explanations based on damaging the bacterial cell wall [11], inhibition of bacterial protein kinase, bactericidal effect on the cell membrane and reduction of the adherence of bacteria to epithelial cells [8]. The preventive antiseptic effect of sphinganine has already been demonstrated in patients with healthy skin: applying 200 μg cm^{-2} sphinganine to the skin can cause a significant, two-log reduction in the growth of *S. aureus* and *C. albicans* [12]. In the pathogenesis of acne – and in particular its inflammatory variant – especially *P. acnes* plays a pivotal role. As shown in this study, PS is able to inhibit the growth of this micro-organism with the minimum inhibitory concentration of 0.020%.This should clearly contribute to clinical efficacy.

Furthermore, this study demonstrates that PS effectively reduces the release of IL-1α after UVB irradiation, thereby preventing skin inflammation.

Interleukin-1α, the well-known marker of inflammation, is a peptide expressed by many cells including epidermal keratinocytes. In acne lesions, the upregulation of IL-1 around uninvolved follicles could be an initiating factor and be responsible for the inflammatory events observed [1]. The increased expression and release of IL-1 might be a consequence of the overall inflammation present. Another possible explanation might be that a perturbation of the barrier function [4] because of an essential fatty acid (linoleic acid) deficiency would induce the release of pro-inflammatory cytokines, stimulating an inflammatory cascade [13]. An increase in IL-1 activity occurs prior to that of hyperproliferation coincidencing with the 'keratinocyte activation cycle' proposed by Freedberg *et al.* [14], in which keratinocytes can be activated via the release of IL-1. These data support the argument that acne vulgaris should be classified as an inflammatory skin disease. Effectively inhibiting, the activity of IL-1α PS reveals significant protective properties that are relevant for skin-oriented anti-inflammatory claims. In another study, clinically stationary acne patients showed a higher increase in cytokine production compared with improved acne patients after irradiation with UVA and UVB [15].

The control of the inflammatory processes through PS was also shown by inhibiting the activity of PKC, which is a central enzyme eliciting a wide range of cellular responses related to many cell activities. Even at the concentration of 0.01%, PS was able to inhibit the activity of this enzyme by approximately 90%.

The anti-inflammatory effect of PS was demonstrated in the artificial human 3-D skin model (SkinEthic™) after irritation with SDS, too. In former studies, different models of human reconstituted skin were described to be useful to study *in vitro* the onset of cutaneous reactions according to various physical and chemical factors [16–18]. The results were mostly comparable with those found in *in vivo* processes. In this study, the application of PS effectively reduced the cell damage and the inflammation as measured by the amount

of released LDH, respectively, IL-1α. Phytosphingosine even increases the viability of the cells as measured by the XTT test.

From the data demonstrated above, it can be concluded that PS is an effective anti-inflammatory agent that can be used for the topical treatment or skin care of different inflammatory skin disorders, acne vulgaris serving as a prime example. Furthermore, the idea of an active ingredient having both anti-microbial and anti-inflammatory activities is exciting, and forms the rationale for the development of the topical PS formulation tested in our *in vivo* study. Especially the signs of inflammation, papules and pustules, could be reduced through the preparation containing PS and benzoylperoxide but even more through PS alone. Effects on comedones with combination treatment were seen to a similar extent, confirming the potential of PS to enhance or complement existing acne therapy.

The anti-microbial and -inflammatory effect of PS could also be used to improve the condition of other skin disorders with the similar microbial and inflammatory microenvironment, such as atopic dermatitis. It has been well established that the skin of people with atopic dermatitis supports populations of *S. aureus* much bigger than found with normal skin and that greater adherence capacity functions as a contributing factor [10]. The importance of the anti-microbial lipids in the pathogenesis of atopic dermatitis is only looming at the horizon as yet. The current state of knowledge with regard to biology and clinical relevance of anti-microbial lipids as well as the results of this study leave no doubt about their potential as potent active ingredients of the body's own defence system as postulated by Melnik [19].

Our clinical results make obvious that topical application of PS leads to a reduction of inflammatory signs of the skin in acne lesions. Furthermore, they demonstrate that targeted substitution therapy of an anti-microbial lipid deficit in patients with disturbed skin barrier is thus a reasonable starting point for treatment to reduce the pathogenic skin flora and associated release of pro-inflammatory cytokines.

The greatest potential for clinical dermatology might be offered by using PS as a constitutively present part of basic defense as an immunomodulator to treat not just the visible lesions of different inflammatory skin disorders alone, but also to be used in the preventive context.

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