INCREASE OF SKIN-CERAMIDE LEVELS IN AGED SUBJECTS FOLLOWING A SHORT-TERM TOPICAL APPLICATION OF BACTERIAL SPHINGOMYELINASE FROM *STREPTOCOCCUS THERMOPHILUS*

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Several studies have demonstrated that ceramides play an essential role in both the barrier and water-holding functions of healthy stratum corneum, suggesting that the dysfunction of the stratum corneum associated with ageing as well that observed in patients with several skin diseases could result from a ceramide deficiency. In a previous study our group reported a significant increase in skin ceramide levels in healthy subjects after treatment *in vivo* with a cream containing a preparation of *Streptococcus thermophilus*. The presence of high levels of neutral sphingomyelinase activity in this organism was responsible for the observed increase of stratum corneum ceramide levels, thus leading to an improvement in barrier function and maintenance of stratum corneum flexibility. The aim of the present work is to investigate the effects of the topical treatment of a *Streptococcus thermophilus*-containing cream on ceramide levels of stratum corneum of healthy elderly women. The ceramide levels, transepidermal water loss and capacitance were evaluated on stratum corneum sheets from the forearms of 20 healthy female subjects treated with a base cream or the same cream containing a sonicated preparation of the lactic acid bacterium *Streptococcus thermophilus*. A 2-week topical application of a sonicated *Streptococcus thermophilus* preparation led to significant and relevant increase of stratum corneum ceramide levels. Moreover, the hydration values of the treated forearm of each subject was significantly higher than control sites. These results suggest that the experimental cream was able to improve the lipid barrier and to increase a resistance against ageing-associated xerosis.

The stratum corneum (SC), a highly specialized structure, is the outermost layer of the skin and functions as an important barrier to maintain biological homeostasis (1). The SC is comprised of both non-viable, protein-enriched corneocytes and a surrounding lipid-enriched extracellular matrix (2). This structure explains such functions as regulation of transcutaneous water loss, stratum corneum moisturization, corneocyte cohesion, and percutaneous drug delivery. The lipid component includes cholesterol, ceramides and fatty acids (FA) typically with ratios in the range of 3:4:2, respectively (3). However, it is the ceramides that are thought to play the essential role in the formation of the bilayer system. The extracellular Cer comprise ~50% of the SC lipids, and represent a heterogeneous family of at

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least eight molecules (4). In some reports changes in total stratum corneum lipid content with increasing age have been described and it is now generally accepted that there is an overall decrease in total stratum corneum lipids (5). Significantly decreased ratios of ceramide 2 to total sphingolipids with age in white females have been described (6). The amount of ceramide in the stratum corneum is regulated by the balance among the ceramides generating enzymes, including serine-palmitoyltransferase (7), sphingomyelinase (SMase) (8-9), and β-glucocerebrosidase (10), and the degradative enzyme ceramidase (11). Both sphingomyelin (SM) and sphingomyelinase are present in the epidermis and are originally contained in lamellar bodies (12). To clarify the role of SM as a potential precursor of bulk or specific skin ceramide Uchida et al (13) demonstrated that two epidermal SM (SM-1 and SM-2) are important precursors of two corresponding ceramides in mammalian SC, i.e. Cer-2 and Cer-5, but other ceramide species can be generated by β-glucocerobrosidase-dependent hydrolysis of epidermal glucosylceramides. In our previous works we reported evidence that the treatment with a sonicated preparation of *Streptococcus salivarius* subspeciem *thermophilus* was able to induce an increasing ceramide level either *in vitro*, on cultured keratinocytes, or *in vivo*, on stratum corneum of healthy subjects and atopic dermatitis patients (14-15). Considering the suggested role of the ceramides in regulating the water-holding capacity and in maintaining skin integrity, the aim of the present work is to investigate the possibility that the topic application of *S. thermophilus*, representing a source of exogenous SMase able to hydrolyze skin SM and consequently to generate ceramides, presumably may lead to reducing dryness, loss of tone, fullness and water loss.

MATERIALS AND METHODS

Preparation of experimental cream

Streptococcus thermophilus (Strain S244) cultivated in 10% skimmed-milk sterilized at 110°C for 30 min and added to 0.1% yeast extract was obtained from VSL Pharmaceuticals (Gaithersburg, MA, USA) in a pure lyophilized form (10^8 colony-forming units [CFU]/g). Stocks of 1.7g lyophilized *S. thermophilus* were resuspended in 5 ml of phosphate buffered solution (PBS) (Sigma-Aldrich, Milan, Italy), sonicated (30 min, alternating 10 s sonication and 10 s pause) with a Vibracell sonicator (Sonic and Materials Inc., Danbury, CT, USA). For the topical applications, the sonicated bacteria (1.7 g/5 ml) were firstly analyzed for the neutral SMase activity and then mixed with 20 ml of a base cream (CD Investments, Rome, Italy) containing the following components: demineralised water, imidazolidynil urea, trietanolamine, transcutol, glycerol, vaselin oil, vitamin E acetate, polyacrylamide, C_{13}-C_{14} iso-paraffin, Laureth-7,methyl gluceth-20, Fenotan, EDTA, methyl-, buthyl-, ethyl-, propyl-paraben-mix, carbomer, Bensil dm 350, perfume.

Study design

The study was conducted on 20 healthy Caucasian females (68 ± 3 years) with healthy skin. At the beginning of the study each volunteer signed the informed consent drawn up by the technicians. The eligibility criteria used to include subjects in this study were the following: Caucasian race; over 60 years old, healthy skin, mentally competent to complete the treatment. Exclusion criteria were: history of intolerance to drugs and/or cosmetic products and mild-moderate atopic or contact dermatitis. For the whole duration of the study the subjects did not have to use different products on the tested areas and had to avoid exposure to UV radiation. The trial was conducted during the autumnal season in order to avoid interseasonal variations of the skin ceramide content. During the period of the trial no other drug, topical or systemic, was allowed. Each subject applied twice daily for 15 consecutive days 0.5 g of 'active' formulation (base cream as vehicle containing *S. thermophilus*) to one forearm (treated site, T) and SMase-free cream to the contralateral forearm. The SMase-free formulation was used as control (control site, C). The area of application was a site ≈25 cm^2 and 10 cm below the antecubital fossa on the volar aspect of the forearms (unclean before sampling).

At the beginning of the study (time T0) and at the end of application period (time Tf) biochemical and instrumental evaluations were performed on each investigated area of 25 cm^2. The study was carried out in a bioclimatic room (24°C; 50% RH) in order to keep the temperature and the humidity constant during the measurements. Each volunteer was required to neither cleanse nor moisturize the forearms for 4 hours prior to the beginning of the test and was acclimatized for 15 min in a conditioned room (24°C, 50% RH) before the biophysical measurements.

Corneometer measurements

Skin hydration was investigated using a Corneometer CM 825 (Courage und Khazaka, Köln, Germany), which was mounted on a Multi Probe Adapter MPA 5

(Courage und Khazaka, Köln, Germany). Capacitance changes depending almost solely upon the water content in the stratum corneum were detected and evaluated. The conductivity of this closed system changes as a function of stratum corneum hydration. Three readings were taken in contiguous points of each testing area on the volar forearm. The results are given in "arbitrary units" (A. U.) (16).

Tewameter measurements

Epidermal barrier function was evaluated by measuring the transepidermal water loss (TEWL) (g/m^{2}h) using the Tewameter TM 210 (Courage und Khazaka, Köln, Germany), in accordance with applicable guidelines (17). TEWL is the total amount of water vapour lost through the skin and appendages under non-sweating conditions. Evaporimetry consists of applying a probe with two twin sensors directly to the skin, with one sensor pair measuring humidity and the other temperature. The acquired data are used by an integrated microcomputer to compute the water vapour partial pressures at the two parallel levels of each sensor pair and, via the partial pressure gradient, the rate of evaporation. To minimize outside interference, the measurements were carried out in an open-top Plexiglas chamber with closed sides.

Sample collection

To assess the skin ceramide levels, stratum corneum sheets were removed from the volar aspect of the forearms (unclean before sampling), 10 cm below the antecubital fossa, by a six stripping with 25 cm^2 of cyanoacrylate resin, before (T0) and 2 weeks after the application (Tf). The tape stripping was made at each site after the biophysical measurements. The cyanoacrylate resin was immersed for 1 h in 25 ml of chloroform:methanol (2:1, v/v), after which cyanoacrylate resin was removed. The samples were filtrated with a 0.45 μm micropore filter (Millipore Corporation Bedford, MA, USA), concentrated in a rotary evaporator (Savant Instruments Inc., Holbrook, NY, USA) and evaporated to dryness in glass tubes under a stream of nitrogen. The residues were dissolved in 1 ml of chloroform:methanol (9:1, v/v) and stored at -20°C until use.

Analysis of stratum corneum ceramides

For the lipid extraction, 400 μl of methanol, 500 μl of chloroform and 400 μl of water were added to the sample. Samples were stirred for 2 min on a vortex-mixer and centrifuged at 10978x*g* for 10 min. The extraction and centrifugation steps were repeated twice. Lipids, previously dried under nitrogen, were then incubated with *Escherichia coli* diacylglycerol kinase (DAG kinase assay kit and ^{32}P-ATPgamma, spec. act. 3Ci/mmol, Amersham, Buckinghamshire, UK) to determine the levels of ceramide, according to the manufacturer's instructions, and applied to thin layer chromatography (TLC) silica gel plates using a TLC applicator (Camag; Berlin, Germany). Ceramide-1-phosphate was then resolved using chloroform:methanol: acetic acid (65:15:5, v/v) as solvent. Authentic ceramides from bovine brain (Ceramide Type III, non-hydroxy fatty acid ceramides; and Ceramide type IV, hydroxy fatty acid ceramides, Sigma, Milan, Italy) were identified by autoradiography at R_f=0.25 and R_f=0.11, respectively. Specific radioactivity of ceramides-1-phosphate was determined by scintillation counting of corresponding spots scraped off the gel. Quantitative results for ceramide production were obtained by comparing the experimental values with linear curve of the ceramide standards, and are expressed as pmoles of ceramides-1-phosphate/cm^2.

Statistical analysis

The STATPAC Computerized Program was used to perform statistical analysis. Mean values and SEM were calculated for each set of instrumental and biochemical values (basal and final) and for each parameter relating to the tested skin areas. Furthermore, the variation of the parameter (Tf-T0; Tf = mean value at the end of the treatment and T0 = mean value at the beginning of the treatment) was calculated The basal and the final instrumental values of the investigated areas were statistically compared by *t*-test. The variations (Tf vs T0) which occurred in the treated and control sites of the forearms were statistically compared by Wilcoxon test. The Student's *t*-test for paired data was used to analyse the differences between the ceramide levels in the subjects before (T0) and after (Tf) treatment with the experimental cream and base cream. The groups of data were considered significantly different for a probability value $p \leq 0.05$.

RESULTS

Effect of experimental cream on skin hydration and barrier function

To evaluate the efficacy of the experimental cream on healthy human aged skin, biophysical measurements were performed, on a group of aged subjects, before (T0) and after two weeks (Tf) of the treatment. The barrier function was determined by evaluating the transepidermal water loss (Fig. 1). No statistically significant change of TEWL values was found on treated (T0: mean±SEM=10±2.6 g/m^{2}h; Tf: mean±SEM=10.4±4.7 g/m^{2}h) and control sites of the forearms (T0: mean± SEM=10±3.3 g/m^{2}h; Tf: mean±

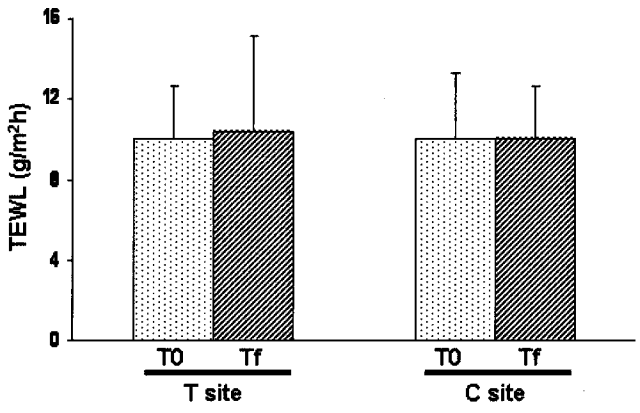

Fig. 1. *TEWL values in control (C) and S. thermophilus extract-containing cream treated (T) subjects (mean±SEM n. 20, p>0.05). The activity of the product on transepidermal water loss on the investigated sites is shown. The TEWL values were determined at the beginning (T0) and at the end (Tf) of a two week treatment.*

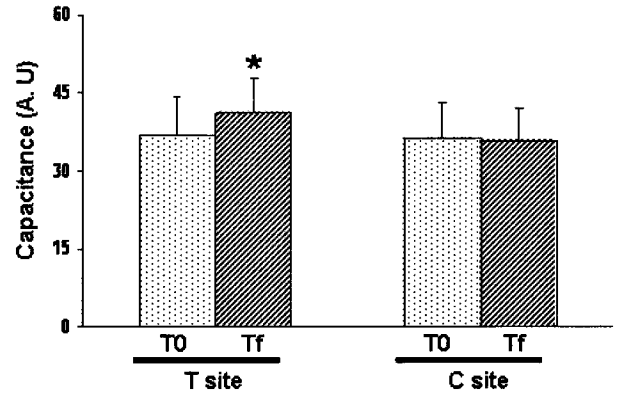

Fig. 2. *Alteration in skin capacitance values in control (C) and S. thermophilus extract-containing cream treated (T) subjects (mean±SEM n. 20 *p=0.001). Capacitance was measured in the investigated areas at the beginning (T0) and at the end (Tf) of a two week treatment.*

SEM=10±2.6 g/m²h). The statistical comparison of the variations obtained in the treated and the control sites did not show any significant difference (T site: % Variation = +4 g/m²h; C site: % Variation = 0 g/m²h, p>0.05), thus suggesting that changes in transepidermal water loss are too small to be taken into consideration. On the other hand, significant difference was obtained in skin capacitance (Fig. 2). During the application period, the "active" formulation increased the mean skin hydration from 37.0±7.3 A.U. (mean±SEM) to 41.2±6.7 A.U. (mean±SEM), whereas the control sites remained unchanged (T0: mean±SEM=36.3±6.7 A.U.; Tf: mean±SEM=35.7±6.2 A.U.). A statistically significant increase of hydration values was found on the treated site (T site: % Variation = +11.3 A.U.; p<0.05), while no significant change was observed on the control site (C site: % Variation = -1.6 A.U.). The statistical comparison between the variations obtained in the treated and the control sites showed a highly significant difference (p=0.001).

Ceramide levels in the healthy aged subjects

In order to determine whether the treatment with *S. thermophilus*-containing cream was able to affect skin ceramide levels of healthy aged subjects, we analyzed the amounts of ceramide from lipid extracts from the forearms of 20 aged women, before (T0) and after two weeks (Tf) of the treatment either with the base cream or the experimental cream. In Fig. 3 the ceramide levels at the beginning (T0) and at the end (Tf) of the two week treatment are shown. The skin ceramide levels of healthy aged subjects showed strong basal subjective variability (range: 0.2-50 pmol total ceramides/cm²; mean±SEM=8.95±2.83 pmol total ceramides/cm²). Considering intraindividual differences, the unquestionable increase in skin ceramides levels can be observed in almost all subjects, following topical application of base cream (range: 1.9-75 pmol total ceramides/cm²; mean±SEM=19.84±5.06 pmol total ceramides/cm²; Tf versus T0, p=0.068), and "active" formulation (range: 2-460.60 pmol total ceramides/cm²; mean±SEM=61.65±25.9 pmol total ceramides/cm²; Tf versus T0, p=0.050). Fig. 3B shows some representative autoradiographies of SC ceramide levels obtained before (T0) and after 2 weeks of the application (Tf) of base (C site) or experimental cream (T site). These results suggest that observed increase in skin ceramides was caused primarily by the presence of *S. thermophilus* SMase in active cream, thus confirming previously reported findings (14-15).

DISCUSSION

The presented study is aimed at investigating

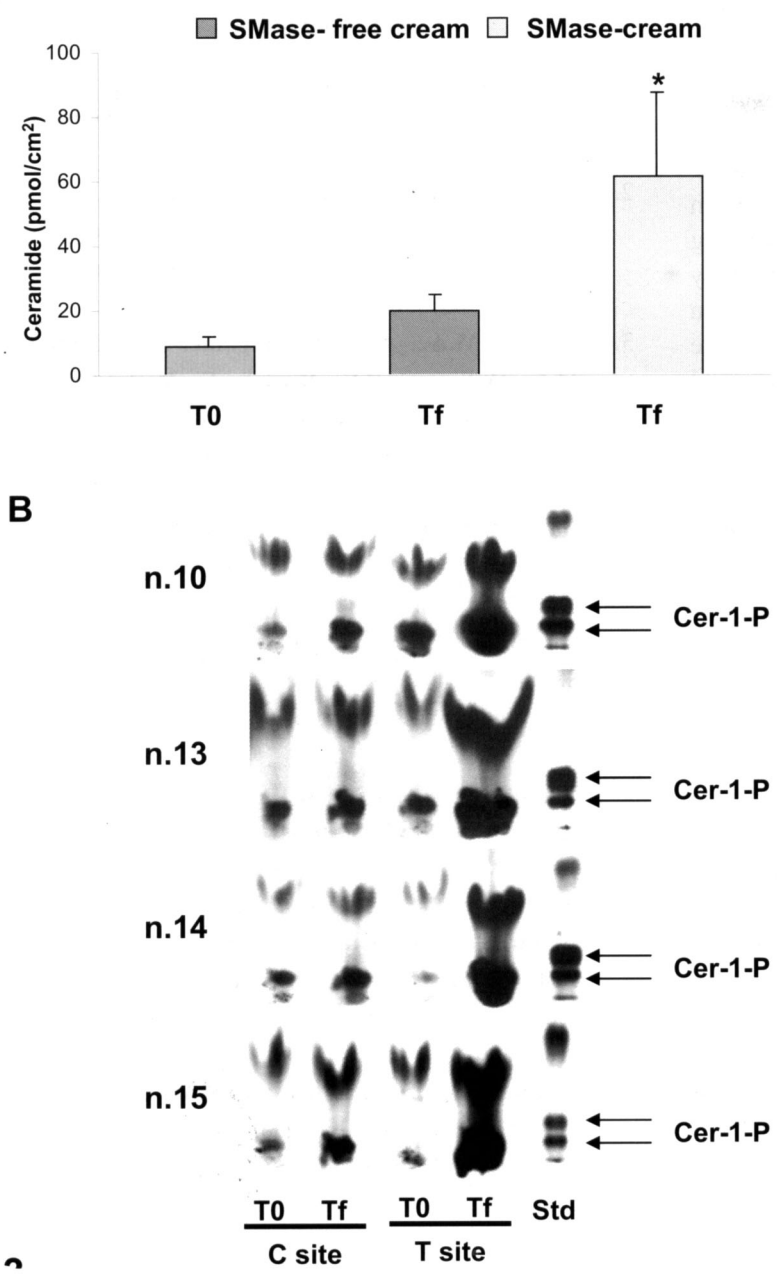

Fig. 3. ***(A)*** *Effects of S. thermophilus extract-containing cream on stratum corneum ceramide levels in 20 healthy aged subjects. Forearm skin lipid extracts before (T0) and after (Tf) a two week treatment, with SMase-free cream and SMase-cream were analyzed for ceramide level determination by diacylglycerol (DAG) kinase assay. *p=0.05 when compared to T0.* ***(B)*** *Representative authoradiographies of TLC of ceramides-1-phosphate (cer-1-P) extracted from foream stratum corneum of four individual (relative number are indicated) before (T0) and after the treatment (Tf) for two weeks with SMase-free formulation (**C site**) or with the active formulation containing sonicated S. thermophilus (**T site**). Std = authentic ceramides*

whether *S. thermophilus* extracts, which showed to express high levels of neutral SMase activity (14) could exert a beneficial effect on the properties and characteristics of aged skin by measuring its effect on the TEWL, capacitance and ceramide levels.

Our results show that no significant modification of transepidermal water loss was observed after the topical application of *S. thermophilus*-containing cream. This result suggests that normal permeability barrier may exist in our experimental conditions because the biophysical parameter was determined T0 and Tf before the tape stripping. In fact, Ghadially et al (5) found that the barrier function after several modes of barrier insult in the younger subjects typically was recovered by 4 d, whereas in the aged this recovery was seen by 7 d. Thus, it seemed likely to us that the seemingly normal levels of TEWL displayed by intrinsically aged epidermis could be due to time points of experimental analysis.

The skin barrier and the water-holding capacity are the other most important functions of the SC and these functions are related to the composition and

structure of SC intercellular lipids (6, 18), including cholesterol, ceramides and fatty acid. Therefore, we determined capacitance and ceramide levels as markers of epidermal hydration. Our findings indicate that the barrier improvement, resulting in a prompt increase in the water-holding capacity, was observed when the aged subjects applied *S. thermophilus*-containing cream. In fact, at the end of treatment a statistically significant increase in hydration values was shown when compared with the values observed at the beginning. An amelioration in skin hydration could be attributed to the increase of the stratum corneum ceramide levels. Our results, obtained with the topical application of a sonicated *Streptococcus thermophilus* preparation, led to increased non-hydroxy fatty acid ceramides and hydroxy fatty acid ceramide levels in stratum corneum. These results could be explained with the presence of high levels of neutral SMase in *S. thermophilus,* as previously reported (14-15). Altogether, our findings suggest that there are two eventual possibilities by which topical application of a sonicated *Streptococcus thermophilus* preparation may contribute to the improvement of lipid barrier and a more effective resistance against ageing-associated skin xerosis. One possibility would be that the presence of high levels of neutral SMase in *S. thermophilus* hydrolyses skin SM thus generating ceramides, with structural function in the stratum corneum lipid bilayers. The other eventuality is that *S. thermophilus* SMase-produced ceramides are involved in epidermal differentiation and proliferation signalling pathway as important second messenger, as previously described (19). Thus, although the mechanism of action of topical application of a sonicated *Streptococcus thermophilus* preparation is not investigated, the results obtained with this experimental cream consist in a relevant increase of skin ceramide levels which was associated to a more effective resistance against ageing-associated skin xerosis.

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