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A lamellar body mimetic system for the treatment of oxazolone-induced atopic dermatitis in hairless mice

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ABSTRACT

Background: Atopic dermatitis is a common skin disease characterized by a Th2 cell-dominant inflammatory infiltrate, elevated serum IgE levels and impaired epidermal barrier function. It is associated to abnormal epidermal lamellar body secretion, producing alteration in lipid composition and extracellular lamellar membrane organization.

Objectives: The oxazolone-induced atopic dermatitis in hairless mice was used to evaluate *in vivo* the effect of the application of a lipid system that mimics the morphology, structure and composition of epidermal lamellar bodies.

Methods: The skin barrier function was evaluated measuring TEWL and skin hydration *in vivo*. Inflammation was assessed by analysis of serum IgE levels and histological analysis. The microstructure of the intercellular lipid region was also evaluated before and after treatment.

Results: The skin condition was improved after 10 days of treatment indicated by decreased TEWL, decreased serum IgE levels, reduced epidermal thickness and reduced lymphocyte-dominated infiltrate. However, the treatment did not improve skin hydration.

Conclusions: The treatment with this lipid system seems to improve the skin condition by reinforcing the barrier function and reducing the skin inflammation. Therefore, the present study provides evidence that this lipid system combining appropriate lipid composition and morphology could be of interest for the development of future treatments for atopic dermatitis.

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1. Introduction

Atopic dermatitis is a common skin disease characterized by inflammatory, pruritic eczema. It is a multifactorial disease which involves a complex interplay of environmental and genetic factors. Atopic dermatitis shows a Th2 cell-dominant inflammatory infiltrate, elevated serum IgE levels and impaired epidermal barrier function [1], indicated by an increased transepidermal water loss (TEWL) [2] and decreased water-holding capacity [3].

The cutaneous permeability barrier is mediated by extracellular lipids mainly ceramides, free fatty acids and cholesterol, which form extracellular lipid-enriched lamellar membranes between the corneocytes that block the movement of water and electrolytes [4]. These lipids are delivered to the extracellular spaces of the

stratum corneum by the secretion of lamellar bodies. Atopic dermatitis is associated to abnormal lamellar body secretion, producing alteration in lipid composition, especially ceramides and free fatty acids [5,6], and in extracellular lamellar membrane organization, as well as dysfunction in antimicrobial defense. Moreover, in atopic dermatitis alkaline pH of the skin surface is another cause of increased skin susceptibility to bacteria, mainly *Staphylococcus aureus* [7].

One animal model that closely reflects these features is the oxazolone-induced atopic dermatitis in hairless mice [8]. When oxazolone is applied to the skin of hairless mice for a period of 3 weeks, mice develop symptoms characteristic for atopic dermatitis including barrier dysfunction, secretion of IgE, epithelial cell hyperplasia, fibrosis and infiltration of inflammatory cells into the dermis and epidermis and secretion of Th2 cytokines.

Bicosomes are lipid aggregates (200–400 nm) formed by disks encapsulated in lipid vesicles [9]. The interaction of these systems with the skin can modify the permeability of the barrier, reinforce

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the lipid structure and promote targeted delivery of molecules to specific skin layers, among others effects [10–12]. In a previous study, we designed a lipid system based on bicosomes that mimic the morphology, structure and composition of epidermal lamellar bodies [13]. In addition, we performed *in vitro* studies to evaluate the effect of this system on delipidized stratum corneum. We concluded that the treatment with the lamellar body mimetic system (LBms) re-establishes *in vitro* part of the lipid lamellar structure of delipidized stratum corneum, mainly the short periodicity phase described by Bouwstra et al. [14]. In other study, we evaluate *in vivo* the hydration-dehydration kinetics of human skin after treatment with the LBms [15]. We found that skin increased its ability to retain water. Thus, this lipid system seems to reinforce the barrier function.

Our previous results point to a high potential of the LBms to improve skin conditions. Thus, in the present study, we sought to evaluate the effect of the application of the LBms on oxazolone-induced atopic dermatitis mouse model. Accordingly, we evaluate skin hydration and TEWL to study the skin barrier function. Inflammation was assessed by analysis of IgE levels in serum. In addition, skin biopsies were taken the last day of the experiment for histological analysis by optical microscopy and for evaluating the microstructure of the intercellular lipid regions by freeze substitution transmission electron microscopy (FSTEM).

2. Material and methods

2.1. Chemicals

1,2-Dipalmitoyl-sn-glycero-3-phosphocholine (DPPC) and 1,2-dihexanoyl-sn-glycero-3-phosphocholine (DHPC) were from Avanti Polar Lipids (Alabaster, United States). Cholesterol from lanolin (chol), cholesteryl sulphate sodium salt (Schol), cholesteryl palmitate (Echol), stearic acid (SA), oxazolone, hematoxylin solution Gill 2, eosin Y alcoholic solution, lithium carbonate, xylene and DPX mountant for histology were from Sigma-Aldrich (Steinheim, Germany). Ceramide IIIb (cer3) and ceramide VI (cer6) were from Evonik (Essen, Germany). Lipoid S100, whose main component (>94%) is soybean phosphatidylcholine (PC) and Lipoid S LPC 80 whose main component (80%) is soybean lysophosphatidylcholine (lysoPC) were from Lipoid GmbH (Ludwigshafen, Germany). Chloroform, formaldehyde solution 4% and acetic acid were from Merck (Darmstadt, Germany). Acetone and ethanol absolute were from Panreac (Barcelona, Spain). Optimal cutting temperature compound (OCT) was from Sakura Finetek (Torrance, United States).

2.2. Preparation and characterization of the system

2.2.1. Discoidal structures preparation

Four types of discoidal structures were prepared: (a) DPPC/DHPC, $q = 3.5$ ($q = \text{DPPC/DHPC molar ratio}$) including 5 mol% cer3 and 5 mol% cer6; (b) DPPC/DHPC, $q = 3.5$ including 5 mol% chol; (c) DPPC/DHPC, $q = 3.5$ including 5 mol% Schol and (d) DPPC/DHPC $q = 3.5$ including 5 mol% Echol.

For each of the discoidal systems, appropriate amounts of lipids were mixed in chloroform and evaporated to dryness using a rotary evaporator. The systems were hydrated with distilled water to obtain a 10% (w/v) total lipid concentration and then subjected to several cycles of sonication, freezing and heating until the samples became transparent.

Next, different volumes of these samples were selected to obtain a mixture in which the molar ratio of cer, chol, Schol and Echol was similar to that found in the skin. To obtain 1 ml, 440 μl of (a), 440 μl of (b), 88 μl of (c) and 32 μl of (d) were mixed. This mixture was used to prepare the LBms.

2.2.2. LBms preparation

A chloroform solution containing 95 mg/ml PC, 5 mg/ml lysoPC and 1.6 mg/ml SA was rota-evaporated to remove the chloroform and thus form a lipid film. The film was hydrated with the previously prepared mixture of discoidal structures.

The total lipid concentration in the final system was 20% (w/v) with a cer:SA:chol:Schol:Echol molar ratio of 1:1:0.5:0.1:0.04.

The LBms was analysed 24 h after preparation by dynamic light scattering (DLS) and cryogenic transmission electron microscopy (Cryo-TEM).

2.2.3. DLS

The hydrodynamic diameter (HD) was determined by DLS with a Zetasizer nano ZS (Malvern Instruments, UK). The DLS technique measures the diffusion coefficient (D) of the particles corresponding to Brownian motion, and converts this to size by using the Stokes-Einstein equation,

$$HD = kT/3\pi\eta D$$

where HD is the hydrodynamic diameter, D is the translational diffusion coefficient (m^2/s), k is the Boltzmann constant ($1.3806503 \times 10^{-23} \text{ m}^2 \text{ kg s}^{-2} \text{ K}^{-1}$), T is the absolute temperature (298 K), and η is the viscosity of the dispersant at 25 °C (water, 0.8872 mPa s). The particles sizes were determined from the scattered light that was detected at an angle of 173° when a laser beam of 633 nm wavelength crossed the sample. Measurements were performed in triplicate.

2.2.4. Cryo-TEM

The morphology of the LBms was evaluated by Cryo-TEM. A thin aqueous film was formed by dipping a glow-discharged holey carbon grid in the suspension and then blotting the grid against a filter paper. The resulting thin sample film spanning the grid holes was vitrified by plunging the grid into ethane, which was maintained at its melting point with liquid nitrogen, by use of a Vitrobot (FEI Company, Eindhoven, The Netherlands). The vitreous sample film was transferred to a Tecnai F20 TEM (FEI Company, Eindhoven, The Netherlands) by use of a cryotransfer holder (Gatan, Barcelona, Spain), and the sample was observed in low-dose mode. Images were acquired at 200 kV at a temperature between –170 °C and –175 °C, under low-dose imaging conditions. Ten overviews and approximately 50 detailed electron micrographs were taken.

2.3. Animals and study protocol

The study protocol was approved by the Animal Experimentation Ethics Committee of the University of Barcelona according to the regulations of the local government (Decree 214/1997, July 30th). Female hairless mice SKH-1 ($n = 8$; Charles River, Germany) of 8–9 weeks old were housed in two cages ($n = 4$) in a room with controlled temperature and humidity. Food and water were provided *ad libitum*. Atopic dermatitis was induced by topical application of oxazolone in acetone. The design of the study is summarized in Fig. 1. The animals were sensitized on dorsal skin with 10 μl of 5% oxazolone in acetone (D0). A week later, the dorsal skin of the mice was treated with 60 μl of 0.1% oxazolone in acetone for 20 days (from D7 to D26). In addition, one group was treated with 30 μl of LBms (treated-dermatitis group) whereas the other was treated with 30 μl of water (dermatitis group) for 10 days (from D17 to D26). Treatment with LBms or water was applied one hour after oxazolone application. Twenty four hours after the last treatment, animals were euthanized and biopsies from the dorsal skin of the mice were taken (D27).

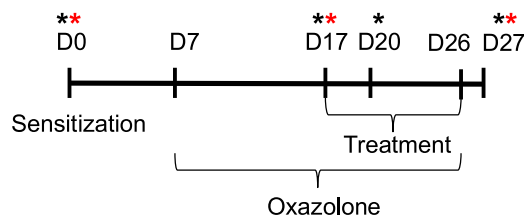


Fig. 1. Study design. The mice were sensitized with 5% oxazolone in acetone on day 0 (D0). A week later, the dorsal skin of the mice was treated with 0.1% oxazolone in acetone for 20 days (from D7 to D26). Treatment with LBms or water was applied during 10 days (from D17 to D26). Black asterisk indicates TEWL and hydration measurements, and red asterisk indicates blood sample collection.

TEWL was measured with a tewameter TM 210 (Courage & Khazaka, Germany) to assess the skin barrier disruption. Skin hydration was determined with a corneometer CM 820 (Courage & Khazaka, Germany) which measures skin capacitance in arbitrary units (a.u.). Both TEWL and skin hydration were measured on day 0, 17, 20 and 27 prior to the application of oxazolone and the treatment.

Blood samples were collected from mice tails on day 0, 17 and 27. These samples were centrifuged (3000 rpm, 15 min, 4 °C) and serum was stored at –80 °C. Serum IgE concentration was determined with a mouse IgE ELISA quantification kit from Thermo Scientific (Waltham, United States) following instructions provided by the manufacturer.

2.4. FSTEM

The skin biopsies were cut into small ribbons with a size of approximately 2×1 mm. The ribbons were fixed in 5% (w/v) glutaraldehyde in a 0.1 M sodium cacodylate buffer at pH 7.2. Next, the ribbons were post-fixed in 0.2% (w/v) RuO_4 in sodium cacodylate buffer at pH 6.8 with 0.25% (w/v) potassium ferrocyanide ($\text{K}_4\text{Fe}(\text{CN})_6$). After 1 h, the RuO_4 solution was replaced with fresh RuO_4 to establish optimal fixation. After a rinse in buffer, the tissue samples were cryofixed by rapid freezing on a liquid nitrogen-cooled metal mirror (Cryovacublock, Leica) at –196 °C prior to freeze-substitution, which was performed using an AFS (Automatic Freeze Substitution) system (Leica). The tissue samples were cryosubstituted at –90 °C for 48 h using 100% methanol containing 1.0% (w/v) osmium tetroxide (OsO_4), 0.5% (w/v) uranyl acetate and 3.0% (w/v) glutaraldehyde. After the 48 h substitution period, the temperature was increased to –50 °C, and the samples were washed three times in 100% methanol. Subsequently, the methanol solution was gradually replaced with the embedding medium, Lowicryl HM20 (100%). This resin was replaced after 24 and 48 h with freshly prepared embedding medium. Finally, the samples were transferred to a mould containing Lowicryl and were incubated for 48 h at –50 °C under UVA radiation to facilitate

polymerization. Ultra-thin sections were cut (Ultracut UCT, Leica), transferred to Formvar-coated grids, and examined using a Hitachi 600 transmission electron microscope.

On average, 10 overviews and approximately 30–40 detailed electron micrographs for each freeze-substitution process were taken.

2.5. Histological analysis

First, skin samples were covered with aluminium foil and then with OCT. The aluminium foil was used to avoid the direct contact between the skin and the OCT. Then, skin samples were frozen in liquid N_2 and cut into transverse $6 \mu\text{m}$ thick sections. The cuts were performed using a Cryostat CM3050 S (Leyca Biosystems, Nussloch, Germany). Tissue sections were then stained with hematoxylin-eosin (H&E). Epidermal thickness was measured in ten randomly selected areas from the basement membrane to the base of the stratum corneum.

2.6. Statistical analysis

Means and standard deviations (SD) were calculated. Statistical analysis of the experimental data was performed by Wilcoxon rank-sum test using STATA/SE 12.0 software. Differences were considered to be statistically significant at $p \leq 0.05$.

3. Results

3.1. Characterization of the system

DLS was used to determine the average size of the LBms. The size distribution curve of the system which was obtained by analysis of the scattered light intensity, showed two peaks corresponding to a population of small particles with a diameter of 53 ± 12 nm that scattered $28 \pm 5\%$ of the light and a population of large particles of 267 ± 21 nm that scattered $72 \pm 5\%$ of the light.

Cryo-TEM allows us to directly visualize the sample and to characterize the morphology of the different structures present in the system. Two representative Cryo-TEM images of the LBms are shown in Fig. 2. These images show two types of structures, which is in agreement with the data reported by DLS, corresponding to discoidal structures (white arrows) with diameter of approximately 40 nm and vesicles encapsulating disks (black arrow) with size approximately 300 nm. Moreover, disks are shown in all dispositions, edge-on (white open arrows) and face-on (white close arrow).

3.2. TEWL and hydration

TEWL of animals on D0 was $12.1 \pm 0.7 \text{ g/m}^2 \text{ h}$ ($n=8$). The oxazolone treatment resulted in a marked increase in TEWL value being of $30.8 \pm 4.8 \text{ g/m}^2 \text{ h}$ ($n=8$) on D17 as shown in Fig. 3A. The

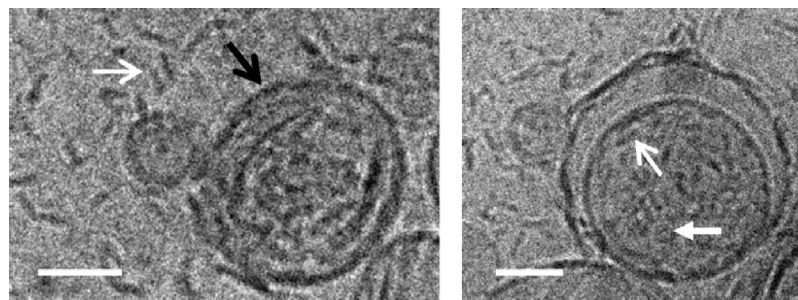


Fig. 2. Cryo-TEM micrographs of the LBms. The system is formed by encapsulated and non-encapsulated disks with diameters of ~ 40 nm, and vesicles encapsulating disks (black arrow) with size of ~ 300 nm. The disks are shown in all projections: edge-on (white open arrows) and face-on (white close arrow). Bar = 100 nm.

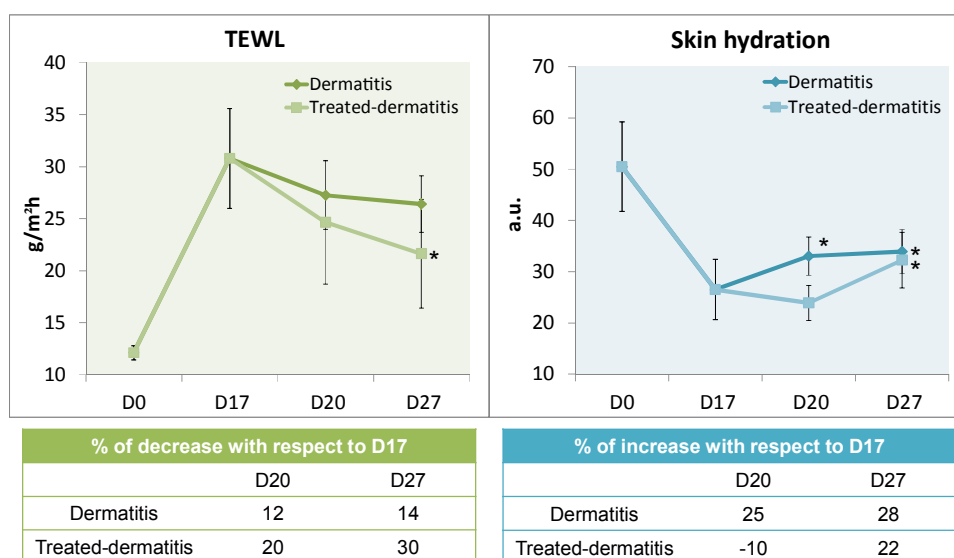


Fig. 3. Values of TEWL and skin hydration in both dermatitis and treated-dermatitis group along the experiment. The tables show the percentage of difference with respect to the day before starting treatment with the LBms or water (D17). *Statistically significant differences ($p \leq 0.05$) compared with D17.

dermatitis group ($n = 4$) presented a TEWL of $27.3 \pm 3.3 \text{ g/m}^2\text{-h}$ (D20) and $26.4 \pm 2.7 \text{ g/m}^2\text{-h}$ (D27) whereas the treated-dermatitis group ($n = 4$) presented a TEWL of $24.7 \pm 6.0 \text{ g/m}^2\text{-h}$ (D20) and $21.6 \pm 5.2 \text{ g/m}^2\text{-h}$ (D27). After 10 days of treatment (D27), TEWL was reduced significantly, approximately 30% compared to D17. This difference is not found in the untreated group. The reduction in TEWL value is associated with an improvement of skin barrier function.

Regarding hydration values (Fig. 3B), the mice presented a capacitance of $50.5 \pm 8.7 \text{ a.u}$ ($n = 8$) on D0. After 10 oxazolone challenges (D17), the capacitance was reduced being $26.5 \pm 5.9 \text{ a.u}$ ($n = 8$). On D20, the dermatitis group ($n = 4$) presented a capacitance of $33.0 \pm 3.7 \text{ a.u}$ whereas in the treated-dermatitis group ($n = 4$) was $23.9 \pm 3.4 \text{ a.u}$. This small difference between treated and untreated group could be related with the initial interaction of the LBms with the skin involving a re-arrangement of the skin lipids by effect of the LBms lipids. However, the last day of the experiment (D27) the capacitance of both groups was similar being of $33.9 \pm 4.3 \text{ a.u}$ (dermatitis group) and $32.3 \pm 5.4 \text{ a.u}$ (treated-dermatitis group) and increased significantly 22–28% compared to D17. Given that skin capacitance is directly related to skin hydration, these results indicate that the treatment did not affect the hydration with respect the normal behaviour of the skin.

Therefore, 10 oxazolone challenges induce atopic dermatitis generating impairment of the epidermal barrier function indicated by markedly increased TEWL and declined hydration. The treatment with the LBms (10 days) reduced TEWL but did not affect the hydration.

3.3. Serum IgE measurements

Basal serum IgE level ($n = 8$) was $0.22 \pm 0.06 \mu\text{g/ml}$. After 10 oxazolone challenges (D17) the serum IgE increased markedly being of $140 \pm 77 \mu\text{g/ml}$ ($n = 8$). The last day of the experiment (D27), serum IgE decrease 19% in the treated-dermatitis group being of $114 \pm 71 \mu\text{g/ml}$ ($n = 4$) whereas was similar, $140 \pm 32 \mu\text{g/ml}$ ($n = 4$), in the dermatitis group compared to the IgE value on D17.

3.4. FSTEM

The microstructure of the skin was observed by FSTEM. Representative micrographs of healthy skin, dermatitis skin and

treated-dermatitis skin are shown in Fig. 4. The micrographs of healthy skin (Fig. 4a–c) show normal intercorneocyte lipid lamellar structure (white close arrows). The lamellar bodies are well-organized in compact sheets and secrete their contents at the stratum granulosum-stratum corneum interface (Fig. 4c, black arrow). The intercorneocyte spaces appeared wide and almost empty in the dermatitis skin (Fig. 4d,e) and showed disorganized lipid structures (white open arrow). Also, dermatitis skin showed entombed lamellar body contents within the corneocytes cytosol (white asterisks) indicating incomplete lamellar body secretion [16]. Therefore, these contents would be unavailable to form lamellar membranes. The treated-dermatitis skin (Fig. 4f,g) shows a stratum corneum more compact than the dermatitis skin indicating that the extracellular lamellar lipids almost entirely filled the intercorneocyte spaces. However, entombed lamellar body contents within the corneocytes cytosol were also shown after treating the dermatitis skin with the LBms. In addition, abundant vesicular structures similar to lamellar bodies were visualized within the corneocytes (black asterisks). Given that these structures were not observed in the dermatitis group we assume that this effect was consequence of the treatment with the lipid system.

3.5. Histological analysis

Histologic analysis of skin sections (healthy, dermatitis and treated-dermatitis) are shown in Fig. 5 in two different magnitudes ($20\times$ and $60\times$). Skin sections stained with H&E show marked thickening of the epidermis in dermatitis skin ($66.4 \mu\text{m} \pm 6.6 \mu\text{m}$) compared to healthy skin ($17.1 \mu\text{m} \pm 3.1 \mu\text{m}$). However, the epidermal thickness was reduced significantly ($34.7 \mu\text{m} \pm 3.4 \mu\text{m}$) after treating the dermatitis skin with the LBms. These results are represented in the graph of Fig. 6. In addition, dermatitis skin exhibits lymphocyte-dominated infiltrate (indicated by an asterisk in Fig. 5b). However, a reduced lymphocyte infiltration was observed after treating the dermatitis skin with the LBms. Despite this, both dermatitis and treated-dermatitis skin show parakeratosis (retention of keratinocyte nuclei in the stratum corneum, red arrows) and spongiosis (intercellular edema, white arrows).

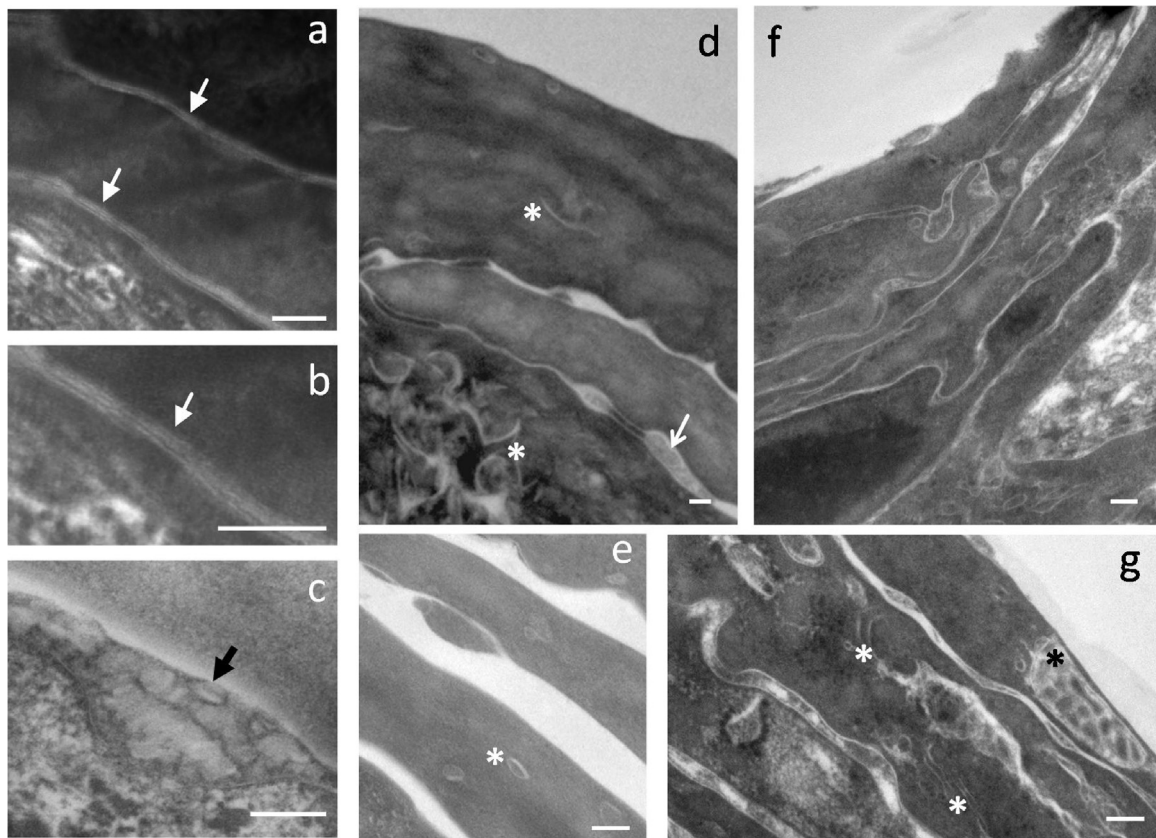


Fig. 4. Microstructure of the skin observed by FSTEM. The micrographs of healthy skin (a–c) show normal intercorneocyte lipid lamellar structure (a,b, close white arrows) and normal lamellar bodies secreting their content at the SG-SC interface (c, black arrow). Dermatitis skin (d,e) shows wide and almost empty intercorneocyte spaces, disorganized lipid structures (d, open white arrow) and entombed lamellar body contents within the corneocytes cytosol (white asterisks). The treated-dermatitis skin (f,g) shows extracellular lamellar lipids almost entirely filling the intercorneocytes spaces and, abundant vesicular structures similar to lamellar bodies (black asterisks). Entombed lamellar body contents are also observed (white asterisks). (Bar = 100 nm).

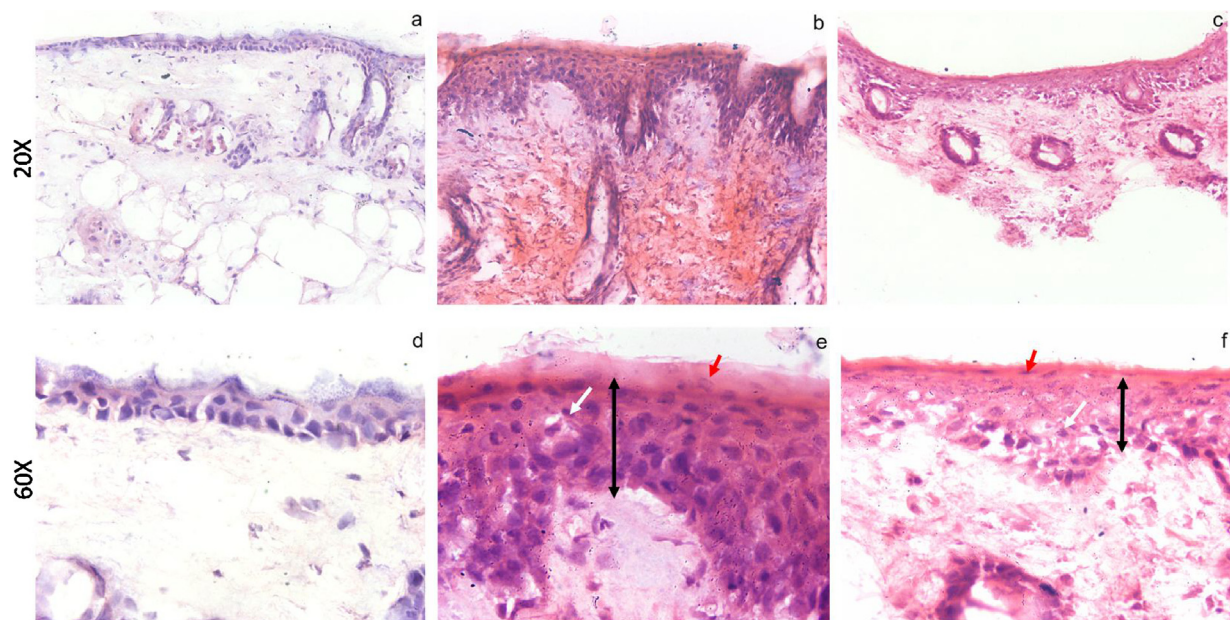


Fig. 5. Histologic analysis of the skin sections stained with hematoxylin-eosin (20× and 60×): healthy skin (a,d), dermatitis skin (b,e) and treated-dermatitis skin (c,f). Measurement of epidermal thickness is indicated by black arrows (d,e,f). The lymphocyte-dominated infiltrate is indicated by an asterisk (b). Parakeratosis and edema are indicated by red and white arrows, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

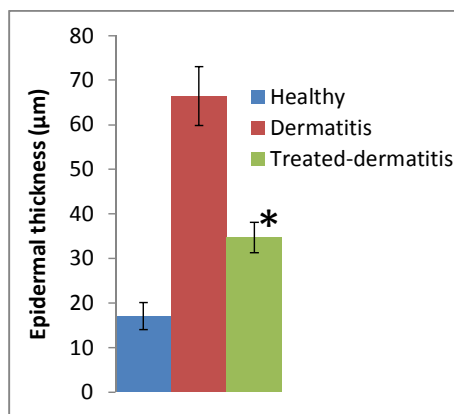


Fig. 6. Epidermal thickness of healthy, dermatitis and treated-dermatitis skin. Mean of ten randomly measurements. *Statistically significant differences ($p \leq 0.05$) compared with dermatitis group.

4. Discussion

4.1. The LBms

The formation of the skin barrier depends on deposition of lipids into the extracellular space between the viable and cornified epidermis [17]. Prior deposition, the lipids are located to, and delivered by, membrane-delimited organelles called epidermal lamellar bodies [18]. The morphology and structure of the LBms observed in our Cryo-TEM images (Fig. 2) are quite similar to those of the epidermal lamellar bodies observed by other authors in electron micrographs [19–21]. The LBms, similarly to epidermal lamellar bodies, is organized in a lipid bilayer that contains discoidal lamellar structures and also is similar in structure to bicosomes previously described in other studies [9,11]. Bicosomes formed exclusively by phospholipids have shown to constitute a good vehicle for incorporating different molecules into the skin, owing to the specific interaction of these structures with the stratum corneum [10,12].

The LBms contains lipids that approximate the stratum corneum lipid composition unlike epidermal lamellar bodies, which contain the precursors of stratum corneum intercellular lipids [22]. In addition, the LBms includes phospholipids, among them LysoPC, which has bactericidal activity [23]. This fact makes LBms appropriate for skin treatment, it is known that patients with atopic dermatitis frequently suffer from bacterial infections [24].

The LBms was designed as described in our previous study [13]. The disks would contain the lipids that form the stratum corneum lamellar structure, and the external vesicle would include the compounds that should remain on the skin surface. Following the mechanism of bicosome-skin interaction previously described [12,25], when external vesicles contact the skin, the bilayer of the vesicle bursts, and the encapsulated disks are released from the inside. These disks exhibit size small enough to penetrate into the stratum corneum and once incorporated, the discoidal structures increase in size as a result of the hydration gradient of the skin, and lipids from the LBms mix with the stratum corneum lipids reinforcing the skin barrier [15].

4.2. Characterization of induced atopic dermatitis condition

Atopic dermatitis is a skin disease associated with disturbances of skin barrier function as evidenced by an increase in TEWL, a decrease in water-binding properties, a reduction in skin lipids, specifically levels of ceramides, and impaired lamellar body

secretion [1,18]. Furthermore, it is associated with abnormal immune response including increased serum IgE levels.

Various mouse models have been used in the literature to study the mechanism of atopic dermatitis and the usefulness of specific treatments. For instance, the dry skin mouse model is produced by repetitive exposure of the skin to acetone, diethyl ether and water. Consequently, the mice show marked increase in TEWL, reduced hydration, increased induction of scratching behaviour, epidermal hyperplasia and parakeratosis, but dry skin model does not show infiltration of inflammatory cells [26]. On the other hand, the oxazolone-induced atopic dermatitis mouse model used in this work is produced by treating the skin with an allergen (oxazolone). Oxazolone is an hapten, a small reactive molecule that covalently modifies proteins in the skin, thereby triggering allergic responses [27]. Multiple oxazolone challenges to normal murine skin produce a chronic Th2-like hypersensitivity reaction with multiple features of human atopic dermatitis [8], including barrier dysfunction and lipid abnormalities in addition to variations in biophysical parameters as TEWL and hydration. Therefore, we decided to evaluate the effect of the LBms in the oxazolone model because this model reproduces very well human atopic dermatitis features in clinical, histological and also immunological aspect.

This mouse model is common to assess a therapy for atopic dermatitis [28–30]. Some authors applied the oxazolone on the dorsal skin of the mice [29] and others on the ears [28,30]. In the present study, 10 oxazolone-challenges were applied on the dorsal skin of hairless mice which lead to profound changes in skin barrier function similar as other studies [8,31,32]. The oxazolone-challenges were applied also every day during the 10 days of treatment with the LBms in order to maintain the induced dermatitis during all the treatment period.

The skin barrier function was assessed by measuring TEWL which gives information about water loss across the stratum corneum. It is described that atopic dermatitis patients display a 2–5-fold increase in basal TEWL, in both lesional and non-lesional skin [33]. In the present study, basal TEWL was about 12 g/m² h, and after atopic dermatitis induction TEWL increased to 30 g/m² h. This fact may be explained by disruption of the stratum corneum lamellar structure due to deficiency in certain lipids. Man et al. [8] found in mice that after 10 oxazolone-challenges, total free fatty acid content declined, cholesterol increased and ceramide 3 declined although the total amount of ceramides did not changed. Lipid analysis of human skin affected by atopic dermatitis has also shown similar results [34]. This fact allows to associate dermatitis condition with stratum corneum lipids disorders.

Another parameter useful to determine skin barrier function is skin capacitance, which reflects water content of stratum corneum. The corneometer measures the change in the dielectric constant due to skin surface hydration changing the capacitance of a precision capacitor. Basal hydration of the stratum corneum was about 50 a.u., and after atopic dermatitis induction the hydration was significantly reduced to 27 a.u. The reduced water content in the stratum corneum after 10 oxazolone-challenges may be explained by perturbations in stratum corneum proteins that could affect the natural moisturizing factor. In addition, perturbations in stratum corneum lipids could also affect due to lipids serve a water-holding function through the formation of lamellar structures within the stratum corneum [35]. Studies of electron microscopy from previous authors show abnormalities in the stratum corneum lamellar structure in atopic dermatitis [36,37]. Our results of FSTEM show similar results. The microstructure of the induced-dermatitis skin showed disorganized and decreased number of lamellar bilayers compared to healthy skin. Besides, the partial removal of the stratum corneum lipids by action of the acetone applied with the oxazolone should not be excluded.

In addition, as we discussed before 10 oxazolone-challenges in hairless mice produced a chronic Th2 dominated inflammatory response that is similar to human atopic dermatitis [8]. In the present study, the inflammation was characterized by epidermal thickness, parakeratosis, spongiosis and lymphocyte-dominated infiltrate as shown the histological analysis and also by highly elevated serum IgE levels. Our SKH-1 hairless mice exhibited basal IgE levels of $0.22 \pm 0.06 \mu\text{g/ml}$ and reached values of $140 \pm 77 \mu\text{g/ml}$ after 10 oxazolone-challenges.

4.3. Effect of the LBms on the induced atopic dermatitis condition

The dermatitis-induced mice were used to evaluate the effect of the LBms. The skin condition was improved after 10 days of treatment as shown the results of TEWL, FSTEM, histological analysis and serum IgE levels.

The effect of the LBms reducing TEWL is not as big as other common moisturizers as Vaseline. It is known that Vaseline reduces TEWL by more than 98% by an occlusion effect [38]. The Vaseline forms a layer on the surface of the skin and moisturize by retarding the evaporation of water. However, the LBms penetrates the skin and once incorporated in the lipid matrix mix with the stratum corneum lipids (described in section 4.1). The effect that the LBms exerts on the skin possibly is due to a combination of appropriate lipid composition and structure. Our LBms would avoid limiting factors associated with Vaseline and other occlusives like odor, potential allergenicity and acneiform eruptions [39].

The microstructure of the skin after treatment shows the intercorneocyte spaces almost entirely filled with extracellular lamellar lipids in contrast to the untreated dermatitis skin. This finding accords with our previous *in vitro* study in which the LBms was shown to have a specific interaction with the skin [13]. Treatment with this system *in vitro* re-established part of the lipid lamellar structure of delipidized stratum corneum, mainly the short-periodicity phase described by Bouwstra et al. [14]. Therefore, in the present study, the improvement of the skin condition *in vivo* could be related with a similar effect. The incorporation of lipids from the LBms into the stratum corneum lamellar structure would reinforce the skin barrier function indicated by decreased TEWL. In addition, the treatment with the LBms *in vivo* could stimulate the production and secretion of endogenous stratum corneum lipids in a similar way as other studies [37,40]. Mao et al. proposed that lipids from preparations containing ceramides, cholesterol and free fatty acids can penetrate impaired stratum corneum, and once absorbed by keratinocytes, the extrinsic lipids may be used by the cells and integrated into nascent lamellar bilayers [40]. Therefore, we assume that our lipid system could exert similar effect. The presence of abundant vesicular structures similar to lamellar bodies within the corneocytes in our FSTEM images (Fig. 4g, black asterisk) could be related of this. On one hand, the lipids from LBms applied on the skin could penetrate and remain into the tissue (described in Section 4.1), and on the other the system could stimulate the production of new epidermal lamellar bodies, although we found these structures within the corneocytes instead of the upper keratinocytes layers.

The inflammation was reduced after treatment (reduced epidermal thickness, reduced lymphocyte-dominated infiltrate and decreased serum IgE levels), although the treated group still showed parakeratosis and spongiosis, which is associated with inflammation. It is important to note that during the treatment period, the oxazolone was also applied which could explain this result and the fact that although the amount of IgE is reduced after treatment, it is still high compared to the basal value. Studies on atopic dermatitis and barrier repair treatment,

either in animal model or in humans, showed that adequate lipid replacement therapy restores epidermal barrier function [41,42] and reduces inflammation comparable to a mid-potency steroid (fluticasone cream) [43]. In previous studies we demonstrated that the LBms penetrates into the skin and is retained mainly in the stratum corneum and upper layers of the epidermis [13]. Therefore, considering that skin immune cells are mainly located in the epidermis and especially in the dermis, we do not foresee that our system directly affects these cells, since it would remain in more external layers. However, our LBms mimicking the stratum corneum lipid composition is able to re-establish the lipid structure of damaged skin reinforcing the barrier and could indirectly participate in the immune skin response. It has been described that correction of the barrier abnormality is anti-inflammatory by different mechanisms: down-regulates of signaling mechanisms for barrier repairing that are pro-inflammatory and decreases further penetration of haptens that drive Th2 inflammation. Additionally, the inclusion of free fatty acids impart an acidic pH, which further improves barrier function and blocks activation of proinflammatory serine proteases [44].

Moreover, Schwarz et al. have recently suggested that the topical application of short chain fatty acids (SCFAs) may be used to control inflammatory disorders in the skin due to they have a specific role on skin regulatory T cells. These authors demonstrated that the topical application of sodium butyrate, a representative SCFAs, prevents the expression of hapten-specific skin inflammation in previously sensitized mice [45]. These authors also showed that sodium butyrate application was still effective even when applied after elicitation of the CHS response, suggesting a “curative” potential once inflammation has developed.

According to these results, we would expect that stearic acid forming the LBms could act in a similar way. Moreover, ceramides, in addition to structural roles also play an important role in intracellular signaling and regulates a variety of biological processes, including proliferation, differentiation, apoptosis, inflammation and immune responses [46].

Nevertheless, the dermatitis-induced mice exhibited similar skin hydration after treatment with the LBms as the dermatitis group. It is known that moisturizers improve skin hydration using a combination of occlusive, humectant, and emollient ingredients. In our case, the LBms is formed by lipids organized as bilayer structures in water. Therefore, an improvement of skin hydration is not expected due to the lipids themselves have little or no affinity for water molecules [47]. However, the lipids that form the stratum corneum lamellar structure could be specific modulators of water-retaining properties [35,48]. Our previous study using the sorption-desorption test showed that the treatment with the LBms *in vivo* increase the ability of the skin to retain water in both healthy and irritated human skin [15]. For that reason, the formulation of the LBms in an appropriate base cream could potentiate and complement the effect of this system.

5. Conclusions

The oxazolone-induced atopic dermatitis in hairless mice reflects similar characteristics as human atopic dermatitis including barrier dysfunction, elevated serum IgE levels and a Th2 cell dominant inflammatory infiltrate. This animal model is normally used to assess a therapy for atopic dermatitis. The treatment with the LBms improves the dermatitis skin condition *in vivo* by reinforcing the skin barrier function and reducing the skin inflammation. Therefore, the present study provides evidence that this lipid system combining appropriate lipid composition and morphology could be of interest for the development of future treatments for atopic dermatitis.

Conflict of interest

The authors have not conflict of interest to declare.

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