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Effects of a Topically Applied Preparation of Epidermal Lipids on the *Stratum Corneum* Barrier of Atopic Dogs

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Summary

Canine atopic dermatitis (AD) is characterized ultrastructurally by disorganization of the lamellar lipids (LLs) in the *stratum corneum* (SC), similar to that seen in the human disease. This study, based on the examination of biopsy samples, was designed to investigate the expression of canine epidermal lipids and to evaluate quantitatively, by means of electron microscopy and ruthenium tetroxide post-fixation, the effect of a new topical skin lipid complex (SLC) on the structural deficit in the skin of five dogs with AD. The non-lesional skin of atopic dogs differed from the skin of healthy dogs in that the LLs were reduced in number and highly disorganized. After repeated applications of SLC to the non-lesional skin of dogs with AD, numerous LLs were observed in the deepest part of the SC, occupying 74% of the inter-corneocyte space, while they accounted for only 31.8% of the inter-corneocyte space in comparable biopsy samples from untreated (control) skin of the same dogs. In contrast, the LLs filled 89.5% of the deepest inter-corneocyte spaces in the SC of healthy dogs. Many keratinosomes were observed at the interface between living epidermis and SC after treatment of non-lesional AD skin. Stacks of short LL discs represented 57.6% of the total LLs found in the newly formed SC *compactum* in the treated atopic dogs. It is suggested that the treatment with SLC stimulated the production and secretion of endogenous SC lipids, contributing to the formation of an improved epidermal barrier.

Keywords: atopic dermatitis; dog; lipid lamellae; skin; *stratum corneum*

Introduction

Atopic dermatitis (AD) is a common chronic inflammatory skin disease which especially affects children and is highly prevalent in well developed industrialized populations (Leung and Bieber, 2003). Genetic, immunological, biochemical and environmental factors play an aetiological role. Genetic background explains the high familial occurrence, but the inheritance pattern is complex, with incomplete gene penetrance and considerable phenotypic heterogeneity (Morar *et al.*, 2006).

A characteristic feature of AD, and a major causative factor, is impairment of the epidermal skin barrier, with enhanced transepidermal water loss and reduced hydration of the skin (Leung *et al.*, 2004). This results in inadequate protection against environmental insults and leads to frequent inflammatory responses, in which T lymphocytes and IgE-mediated mechanisms play a role. Skin inflammation further impairs epidermal differentiation and, as a consequence, barrier formation. This constitutional impairment of the epidermal skin barrier may be due to several different mechanisms. Sandilands *et al.* (2006) showed that two loss-of-function mutations in the filaggrin gene (*FLG*) are strong predisposing factors for human AD. Insufficient extrusion of lamellar bodies (LBs, also called keratinosomes) by epidermal keratinocytes and defective lipid processing within these organelles has also been demonstrated (Fartasch *et al.*, 1992; Pilgram *et al.*, 2001).

The *stratum corneum* (SC), the outermost layer of the epidermis, plays an essential role in the skin barrier function against environmental insults and transepidermal

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water loss. It is composed of terminally differentiated epithelial cells, corneocytes, and extracellular lamellar lipids (LLs) that tightly fill the inter-corneocyte spaces. The extracellular SC lipids originate from LBs, initially appearing in the upper *stratum spinosum* and abundantly produced in the *stratum granulosum*. LBs migrate to the apical membrane of the cells in the granular layer, merge with it, and then release their contents into the intercellular space. The excreted lipids are enzymatically processed from a relatively polar lipid mixture into more hydrophobic species and self-organize into arrays of superimposed lipid bi-layers. In man, these lipid domains are composed mainly of ceramides, cholesterol and free fatty acids in equimolar proportions (Holleran *et al.*, 1993; Mao-Qiang *et al.*, 1995b).

The clinical features of canine AD, first described by Halliwell and Schwartzman (1971), are comparable with those of the disease in man. AD is defined as a genetically determined, allergic, inflammatory, pruritic skin disorder, with characteristic clinical features, and is commonly associated with IgE antibodies to environmental allergens (Hillier and Olivry, 2004). Canine AD affects up to 10% of dogs and has a strong breed predilection (Sousa and Marsella, 2001); its familial occurrence indicates that, as in human AD, genetic factors play a role (Hillier and Griffin, 2001). Furthermore, clinical and immunological studies suggest that canine AD is probably the most appropriate of the existing animal models for the human disease (Taïeb *et al.*, 2005).

The biochemical composition of LLs in dogs has not been investigated. However, the canine epidermis is thinner than that of other mammalian species, and its content of intercellular lipids is less (Olivry and Hill, 2001). It has been suggested that canine AD, like human AD, is characterized by an impaired epidermal barrier. A preliminary electron microscopical study showed that both continuity and thickness of SC lipid lamellae were significantly decreased in the lesional skin of dogs with AD (Inman *et al.*, 2001).

Atopic dogs could be considered as a suitable model for studies on the physiopathology, prevention and treatment of human AD. The purpose of the present study was (1) to investigate the expression of canine epidermal lipids, and (2) to examine the effect of a topically applied preparation of epidermal lipids on the defective SC lipid barrier in atopic dogs.

Materials and Methods

Animals

Five dogs with atopic dermatitis (AD), three males and two females from different breeds, as well as five non-atopic dogs (five beagles, all females), were selected for this study. The diagnosis was made on the basis of the clinical criteria proposed by Willemse (1986) and modified by Prélaud *et al.* (1998). Intradermal tests performed on the atopic dogs gave positive results for *Dermatophagoides farinae* (five animals), *Dermatophagoides pteronyssinus* (two animals) and ragweed pollen (one animal). The clinical severity was scored by means of a modified canine atopic dermatitis extent and severity index (CADESI) (Olivry *et al.*, 2002) based on the assessment of erythema, lichenification, and excoriations (normal = 0, mild = 1, moderate = 2, and severe = 3) at 40 body sites, to give a total score of 0–360. The CADESI scores in the five atopic dogs were 25, 35, 50, 55 and 67. The study was approved by the ethics committee of the National Veterinary School of Lyon.

Experimental Procedure

The atopic dogs were treated on six occasions at 3-day intervals with topical applications of a preparation containing ceramides, free fatty acids and cholesterol (skin lipid complex [SLC], a non-registered experimental formulation [Virbac Laboratory, France]). SLC (50 µl) was smeared on an area of 4 cm^2 situated on the lateral aspect of the thorax and gently massaged into the skin for 15 s. The lipid mixture was applied to non-lesional skin on one side only, the symmetrically opposite non-lesional side (untreated) serving to supply control samples. The five non-atopic dogs, which received no treatment, were used to supply reference normal control samples. One day after the final application of SLC, punch skin biopsy samples (6 mm) were taken from the lateral thorax (treated and untreated sides) of the atopic dogs after anaesthetization by the subcutaneous injection of xylocaine. Comparable samples were also taken from the non-atopic dogs.

Electron Microscopy (EM)

The specimens were cut into five or six pieces, fixed overnight in phosphate-buffered saline (PBS) containing paraformaldehyde 4% and glutaraldehyde 1%, and then post-fixed either with osmium tetroxide 1% in 0.4 M cacodylate buffer (2 h) or with ruthenium tetroxide 0.25% in PBS (1 h) in a dark chamber at room temperature. The blocks were then dehydrated in graded alcohol, impregnated with propylene oxide and embedded in Epon. The tissue blocks from each dog were sectioned perpendicularly to the skin surface. Ultrathin (86 nm) sections were collected on Formvar-coated copper grids and stained with uranyl acetate 7% in methanol, and lead citrate. The samples were examined by transmission EM and photographs were taken with a digital camera MegaView III (Olympus, Soft Imaging Systems,

Hamburg, Germany) at magnifications of 28 000×, 60 000×, 125 000× and 260 000×. Non-lesional atopic skin (both treated and non-treated) and healthy skin were compared ultrastructurally. Cross-sectional surfaces of the intercellular spaces and the visible LL structures within them after ruthenium tetroxide staining were measured and analysed with the AnalySIS system (Olympus Soft Imaging Solutions, Hamburg, Germany). Briefly, the digitalized pictures were projected on a computer screen and the surfaces were manually defined with a pointer. The system measured the areas indicated, expressed them in units related to the picture's magnification, and listed the results on an Excell (Microsoft) data sheet ready for a statistical analysis. At least five pictures taken from various sections obtained from each biopsy sample were used for such measurements. The quantitative results were expressed as percentages and compared by the non-parametric Mann–Whitney test, $P \leq 0.05$ being accepted as significant.

Results

EM of the non-lesional untreated skin of atopic dogs revealed long, thin corneocytes (Fig. 1a–c). The inter-corneocyte spaces appeared almost empty, even when examined after ruthenium tetroxide staining, and corneodesmosomes persisted only laterally on the periphery of the corneocyte discs. Consequently, the whole thickness of the horny layer resembled the SC *disjunctum*. In healthy dogs the SC cells were also very long and flat, but in the lower half of the horny layer they coalesced (Fig. 1d, e). In this location, the LLs were well-organized in compact sheets and almost entirely filled the spaces between the SC *compactum* cells. Numerous LBs were observed in the *stratum granulosum* keratinocytes (not shown), persisting focally after extrusion into the first inter-corneocyte spaces.

Comparison of the treated and untreated non-lesional skin in atopic dogs indicated that the SC *compactum* re-appeared after application of the lipid preparation (Fig. 2). Without treatment (Fig. 2a, c), the intercellular spaces were wide and empty; LLs were sparse, poorly organized and had a wavy "fragile" appearance. On the other hand, in treated skin voluminous LBs were abundant at the apical parts of the *stratum granulosum* keratinocytes (Fig. 2b). Some had already merged with the cell membranes, forming extracellular conglomerates of short multidirectional LLs (Fig. 2d). In the deeper inter-corneocyte spaces, these short lamellar discs coalesced until well-organized sheets of LLs could be observed (Fig. 2d, e). Such LLs were long and sharp, and almost entirely filled the remaining extracellular space in the newly reconstituted SC *compactum*.

Quantitative evaluation demonstrated significant differences ($P \leq 0.05$) in LL expression between the treated and control sides (Fig. 3). In the lower SC of healthy dogs, the LLs occupied 89.5% ± 6.8 (mean ± SD) of the total intercellular space. This figure fell to 31.8% ± 16.7 (mean ± SD) in the untreated non-lesional skin of dogs with AD. By contrast, in the SLC-treated non-lesional atopic skin, the LLs represented 74% ± 10.9 (mean ± SD) of the inter-corneocyte space, a value not significantly different from the normal control value. Furthermore, 57.6% ± 21.2 (mean ± SD) of the space occupied by the newly formed LLs between the granular layer and SC of treated skin sites was constituted by LB-derived short lipid discs – a figure not significantly different from the 51.7% found in the same area of the SC in normal dogs.

Discussion

The SC, composed of corneocytes and intercellular lipids, provides an effective epidermal barrier in mammals, protecting against harmful physical, chemical and microbial insults. A lipid monolayer composed of ω-hydroxyceramides, replacing the plasma membrane of viable cells, constitutes a scaffold for the intercellular lipid lamellae (Jensen *et al.*, 2006). Such a structure is characteristic of the normal SC of all terrestrial mammals, although the thickness of the SC and the organization of cell layers may vary between species (Kwochka, 1993; Wertz, 2000). Few studies have focused on the characteristics of the epidermal barrier in dogs. Low-temperature scanning EM of skin samples showed that the intercellular lipid content of the canine SC was less than that observed in other mammalian species (Olivry and Hill, 2001). Inman *et al.* (2001) demonstrated by transmission EM that the intercellular lipid lamellar structures of atopic dogs were disorganized. This accords with the present study, in which the SC *compactum* of healthy canine skin was found to contain a regularly arranged – albeit relatively thin – layer of lipid lamellae, while the SC of non-lesional skin of atopic dogs exhibited an abnormal and incomplete lamellar lipid structure. Differences from the findings of Inman *et al.* (2001) may have been due to the more precise quantitative approach used in the present study, or to individual or inter-breed variation.

A number of diseases in which impaired epidermal barrier function plays a role result from genetic defects in structural proteins or in the enzymes that contribute to the synthesis or processing of other structural elements, including SC lipids (Nemes and Steinert, 1999). Human patients with AD exhibit delayed and incomplete extrusion of LBs, which leads to abnormalities in the lipid composition of the SC

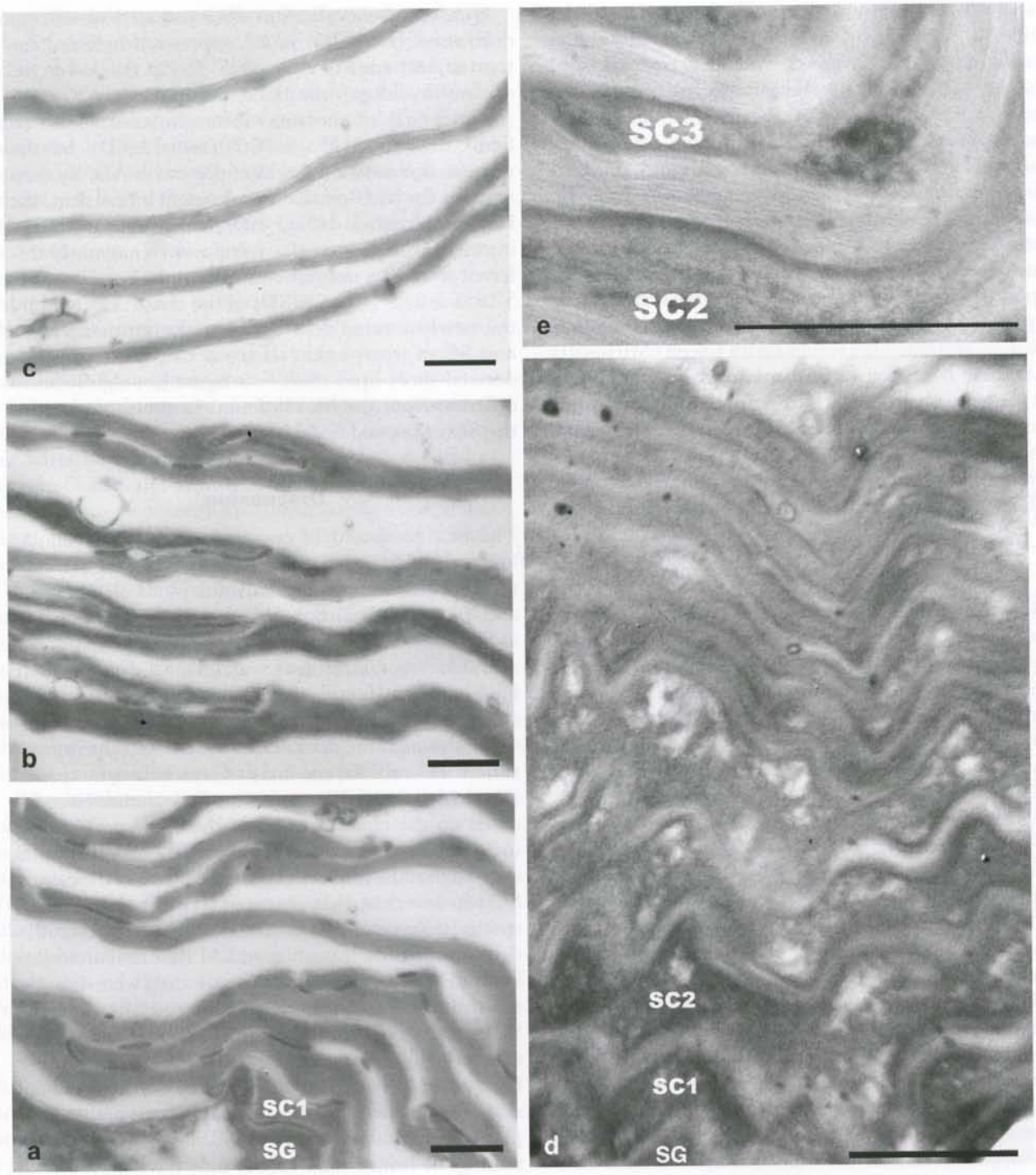

Fig. 1a–e. The non-lesional epidermis in atopic dogs shows virtually no *stratum corneum* (SC) *compactum* and little vertical cohesion between the corneocyte strata. Ruthenium tetroxide staining for transmission electron microscopy indicates the absence of structured inter-corneocyte lipid lamellae in the atopic non-lesional SC (a–c), in contrast to the SC *compactum* of normal dogs (d, e). Precocious separation of corneocytes is apparent in atopic dermatitis, in the inferior SC (a), intermediate SC (b), and superior SC (c). SG = *stratum granulosum* keratinocyte; SC1, SC2, SC3 = the first, second and third *stratum corneum* corneocytes, respectively. EM. Bars in a–c, e = 500 nm and in d = 1.5 μm.

(Fartasch *et al.*, 1992). In addition to a mixture of relatively polar lipids, the LBs also release a family of lipid catabolic enzymes which transform newly excreted lipids into more hydrophobic products that spontaneously form lamellar structures (Holleran *et al.*, 1993). Indeed, an impaired lateral packing of the inter-corneocyte LLs was observed in human AD (Pilgram *et al.*, 2001), indicating that problems

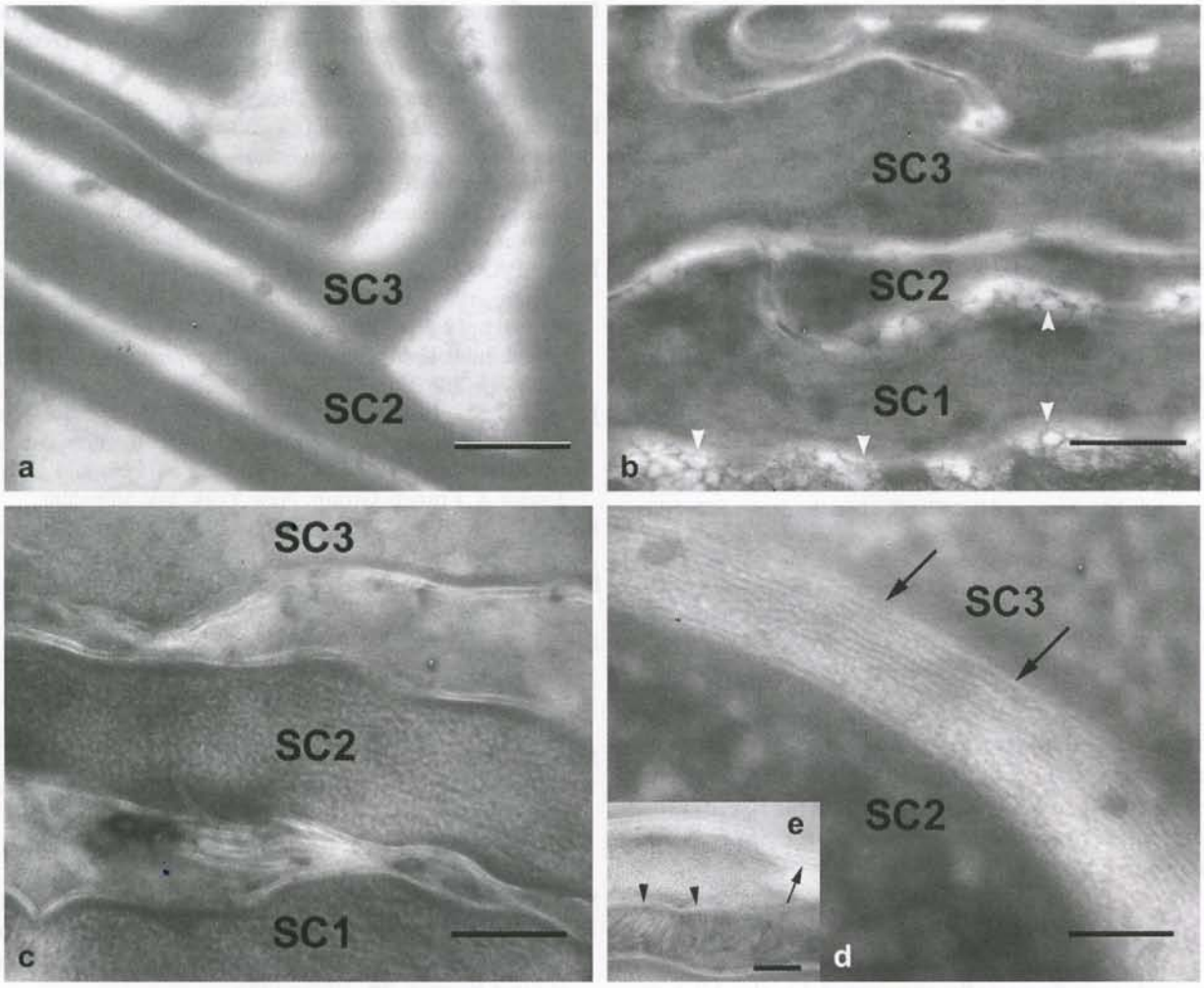

Fig. 2a–e. Topical treatment with skin lipid complex results in replenishment of the SC in the non-lesional skin of atopic dogs. The inferior part of the SC of an atopic dog, stained with osmium tetroxide (a, b) or ruthenium tetroxide (c–e), shows clear-cut changes in the composition of the deeper inter-corneocyte spaces. In untreated skin (a, c), there are infrequent and disorganized lipid lamellae. In treated skin (b, d, e), enhanced extrusion of lamellar bodies (b, e; arrowheads) and lipid lamellae formation (d, e; arrows) are apparent. Abundant short lipid discs originating from the lamellar bodies fill the first intercellular spaces of re-formed SC *compactum*. SC1, SC2, SC3 = the first, second and third *stratum corneum* corneocytes, respectively. EM. Bars in a–c = 500 nm and in d, e = 100 nm.

with lipid structure formation may underlie the pathogenesis of this disease. These findings are compatible with our observations on canine non-lesional atopic skin. However, the abnormal extrusion of LBs reported in man was not found in canine atopic skin in the present study or that of Inman *et al.* (2001).

After topical treatment with SLC, no significant morphological changes were observed in the superior SC, which remained largely dissociated. However, during the treatment, the SC *compactum* was newly formed and contained abundant LLs. These findings accord with those of a study by Chamlin *et al.* (2001), in which application of a ceramide-dominant barrier moisturizer radically reduced the severity of childhood AD and improved SC integrity. Furthermore, repeated application of SLC resulted in a massive production of LBs and their extrusion into the first extracellular spaces of the SC. It has been proposed that lipids from preparations containing ceramides, cholesterol and free fatty acids can penetrate impaired SC and infiltrate the nucleated cell layers. Once absorbed by keratinocytes, the extrinsic lipids may be used by the cells and integrated into nascent lamellar bi-layers (Mao-Qiang *et al.*, 1995a). It is suggested that treatment with SLC stimulates the production and secretion of endogenous SC lipids, thereby rapidly contributing to the formation of an improved epidermal barrier.

Although canine AD still has to be defined through a careful biochemical analysis, the morphological

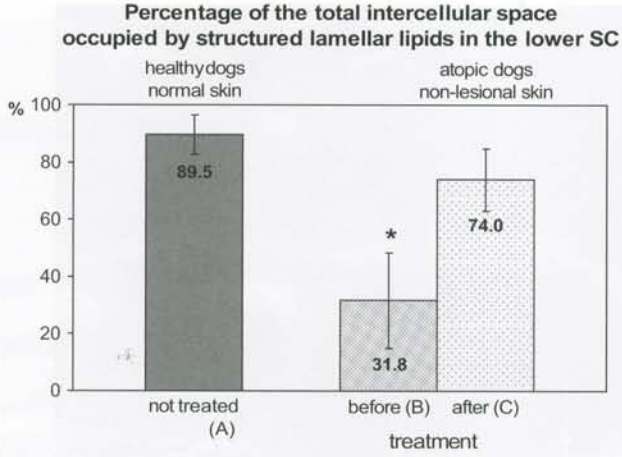

Fig. 3. Quantitative evaluation of the total intercellular space occupied by structured lamellar lipids in the deeper SC of the epidermis of normal and atopic dogs. Values (mean ± SD) for normal skin from healthy untreated dogs (A) and from skin lipid complex-treated non-lesional skin of atopic dogs (C) did not differ significantly. However, both values differed significantly from that of untreated non-lesional skin from atopic dogs (B) ($*P < 0.05$).

results obtained indicate striking similarities to the human disease. The present study suggests that canine AD may prove helpful as a model for the investigation of new preventive and therapeutic approaches in veterinary and human medicine.

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