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ORIGINAL ARTICLE

Modulation of sebum oxidation and interleukin-1α levels associates with clinical improvement of mild comedonal acne

B. Capitanio,[1] V. Lora,[2] M. Ludovici,[2] J.-L. Sinagra,[1] M. Ottaviani,[2] A. Mastrofrancesco,[2] M. Ardigò,[3] E. Camera[2,*]

[1]Pediatric Dermatology, San Gallicano Dermatological Institute (IRCCS), Rome, Italy
[2]Laboratory of Cutaneous Physiopathology, San Gallicano Dermatological Institute (IRCCS), Rome, Italy
[3]Department of Clinical Dermatology, San Gallicano Dermatological Institute (IRCCS), Rome, Italy
*Correspondence: E. Camera. E-mail: camera@ifo.it

Abstract

Background Sebum plays a key role in the initiation of the acne lesions. Oxidized sebum lipids cause keratynocytes hyperproliferation and inflammatory cytokines release. Association between sebum oxidation and comedogenesis has been little investigated in comedonal acne.

Objectives Evaluation of sebum oxidation parameters and levels of inflammatory cytokines (IL-1α) in patients with mild comedonal acne (MCA) before and after the treatment with a mixed RetinSphere®- vitamin E formulation.

Methods Sebum excretion rate (SER), squalene concentration, and oxidation degree of sebum were measured in 18 MCA patients and 10 controls. IL-1α levels in the stratum corneum were measured in both lesional and non-lesional facial areas of MCA patients. Sebum parameters and IL-1α were measured at week 4 of topical treatment. Reflectance confocal microscopy (RCM) was performed in a subset of four patients at the baseline and at week 4 and all patients were assessed clinically before and following the 8 week-treatment.

Results Sebum excretion rate and squalene concentration were comparable between MCA patients and healthy controls. Lipid peroxidation (LPO) and the percentage of oxidized squalene (SQOX) were significantly elevated in the sebum of MCA patients. The concentration of the proinflammatory cytokine IL-1α in stratum corneum was significantly higher in the lesional area compared with non-lesional area of the MCA patients at the baseline. At week 4, while SER and squalene concentration did not vary significantly, the LPO levels and the SQOX percentage resulted decreased at a significant extent. Following the treatment, IL-1α concentration in the lesional area reached values comparable to those of unaffected areas. Consistent with the biochemical data, RCM showed the reduction of hyperkeratinization and of inflammatory cells infiltration of the adnexal structures epithelium, significant clinical improvement was recorded at week 8.

Conclusion The data further support the involvement of lipid oxidation and particularly by-products of squalene oxidation in comedogenesis.

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Conflicts of interest

None declared.

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Introduction

Acne pathogenesis is centred on the interplay of multiple factors. Dysregulation of sebum production and alterations of the sebum composition play a major role in the abnormal follicular proliferation and development of inflammation leading to the comedogenesis.[1] Nevertheless, conclusions on the exact sequence of events that occur in comedogenesis have not been reached as yet. Some evidence support the hypothesis that the inflammatory response is secondary to hyperproliferation and hyperkeratinization of the follicular duct.[2,3] More recent data indicate that inflammation can primarily start comedogenesis.[4,5] Among the 211 and 18 genes, found upregulated and downregulated, respectively, in acne lesions a significant proportion of them were involved in inflammatory pathways.[6] Consistent with it, increased expression at the mRNA and protein levels of IL-1α, IL-6 and IL-8 are common findings in

lesional skin of acne patients.[3,7] Interestingly, IL-1α, which induces hyperproliferation and abnormal keratinisation of keratynocytes[8,9] has been detected in comedones and around uninvolved follicles, prior to hyperproliferation.[3,10,11] However, the correlation between comedo- and sebo-genesis remains to be fully clarified. Hyperseborrhea and the more recently defined 'dys-seborrhea' are important aetiopathogenetic factors in acne.[5] Indeed, the alteration of sebum lipid composition and the increased oxidised lipids have showed to play a key role in the induction of inflammation and comedogenesis.[1,12] In particular, squalene peroxide, beyond the comedogenic effects exhibited in experimental animals, is able to induce an inflammatory response in keratinocytes in culture through lipoxygenase activation and the increase of the proinflammatory cytokine IL-6 production.[13–15]

Comedonal acne (CA) is suitable for understanding the role played by the major triggering factors described to be involved in the comedogenesis. In CA open and closed comedones present no visible inflammation or infective signs. In contrast, in moderate to severe acne the occurrence of numerous papules and/or pustules in association with inflammatory process makes it difficult to correct by secondary events that superimpose to the primary triggering factors. In addition, due to the incomplete understanding of the pathogenesis, CA of mild degree (mild comedonal acne, MCA) suffers of scarcity of first choice treatments more than the other grades of acne.[16] To investigate the relevance of lipid oxidation in the acne development, we measured this parameter in sebum of MCA patients and controls. Concentrations of the inflammatory cytokine IL-1α were measured in comedonal and non-lesional stratum corneum of the MCA subjects. To evaluate the influence of anticomedonal agents on the above pathogenic factors we revaluated the same parameters at 4 weeks of treatment with a commercially available topical formulation containing natural retinoid (RetinSphere®) mixed with vitamin E (α-tocopherol), a major lipid-soluble antioxidant vitamin. In a subset of four patients, *in vivo* reflectance confocal microscopy (RCM) was conducted at week 4, whereas the clinical evaluation of outcomes were completed at week 8 in all the CA patients.[17,18]

Materials and methods

Study participants

Eighteen teenage patients with MCA were recruited from the Acne ambulatory. MCA was defined as such when the clinical severity score ranged between 1 and 3 according to the Leeds revisited acne grading system.[19] Patients under antiacne treatments were excluded from the study. The control group consisted of 10 healthy subjects, age and gender matched, who attended the Division of Dermatology for nevi screening. All the MCA subjects underwent treatment with the commercially available anti-acne product described below. The institutional Ethical Committee approved the study and informed consent was obtained from each participant before the start of the study. Sebum excretion rate (SER) and sebum oxidation indexes were evaluated in patients, at the baseline and at week 4, and in control subjects. The concentration of IL-1α was measured in the stratum corneum sampled from lesional and non-lesional areas of the face in the CA patients at the baseline and at week 4 in the same areas. Morphology and inflammatory signs at the level of the comedones were assessed by RCM, as described,[17,18] in a subgroup of four patients at the baseline and at week 4. To assess clinical outcomes of the anti-acne treatment, patients were evaluated at week 8 of treatment. Correspondingly, the patient satisfaction was recorded and rated on an 11-point analogue scale, ranging from 0 (not satisfied) to 10 (very satisfied). Patients used this scale to assign a rating to their acne treatment at baseline and at week 8. Satisfaction with treatment was determined by assessing the difference between final and initial score.[20] For the treatment, patients were instructed to apply an amount of product sufficient to cover the entire facial surface once a day, either in the morning or at night, depending on their personal habits. The formulation contained combined RetinSphere®-vitamin E and was kindly supplied by Industrial Farmaceutical Cantabria, S.A.

Sampling

Sebum samples were collected on the foreheads of the MCA patients and control volunteers using Sebutapes™ (Cuderm, Dallas, TX, USA) weighed before hands as previously described.[21] Briefly, skin was cleaned gently with 70% ethanol and two tapes were applied onto the foreheads and hold for 30 min. The tapes were weighted after sampling to assess the amount of sebum collected. Sebum excretion rate was calculated and expressed as $\mu g/cm^2/min$. Stratum corneum was sampled from lesional and non-lesional areas by tape stripping with D-squame™ (Cuderm, Dallas, TX, USA), as previously described.[22]

Determination of lipid hydroperoxides, squalene and squalene oxidation products

Sebum lipids were extracted from the tapes with ethanol (Merck, Darmstadt, Germany) containing 0.025% of butylhydroxytoluene (BHT; Sigma–Aldrich, Milan, Italy) to prevent oxidation, and further cleaned-up by liquid-liquid extraction with ethyl acetate, as previously described.[21] The extract was dissolved in acetone/methanol/isopropanol (40/40/20 v/v) mixture at the 5 mg/mL concentration and stored at −80°C until analysis. Lipid peroxides (LPO) levels were determined by the ferric-xylenol orange peroxide assay (PCA-FOX Assay) as described.[23] LPO results were expressed as optical density measured at 560 nm (OD 560) with a spectrophotometer. Concentrations of squalene were determined by a gas-chromatography-mass spectrometry (GC-MS) method, and reported as μg/mg of sebum.[24,25] Oxidation of squalene was evaluated

by HPLC-DAD at 210 nm as previously described.[13,26] Extent of squalene peroxidation was evaluated as the percentage of the peak area of oxidated SQ (SQOX) with respect to the peak area of SQ, i.e. SQOX percentage.

Determination of interleukin-1α protein concentration

Concentration of IL1-α was assessed by specific enzyme-linked immunosorbent assay (ELISA; Life Technologies, Invitrogen, Milan, Italy) in the stratum corneum specimens. Soluble proteins, including lL-1α, were extracted as described.[22] Concentration of IL-1α was normalized by the total amount of soluble protein determined with the Bradford assay (Bio-Rad, Milan, Italy). Levels of IL-1α was reported as pg/μg protein.

Statistical analysis

Differences between MCA patients and controls were analysed by unpaired Student's t test, whereas changes of parameters at the baseline and week 4 of treatment were evaluated by paired t test. Two-sided P values < 0.05 were considered significant.

Results

Sebum excretion rate and squalene concentrations were comparable between MCA patients and unaffected controls (Table 1). In contrast, LPO and the percentage of oxidated squalene occurred at elevated extent in sebum from MCA compared to healthy controls ($P < 0.05$, and $P < 0.01$, respectively, Table 1). The cytokine IL-1α was found at significantly higher concentration in the stratum corneum from the comedones areas compared to apparently unaffected skin detected in the individual patients ($P < 0.05$; Fig. 1). The average concentrations of IL-1α in the lesional and non-lesional stratum corneum were 8.3 ± 5.7 and 5.7 ± 4.9 pg/μg protein, respectively. No significant correlation was found between the indexes of lipid peroxidation, namely LPO and percentage of SQOX, in sebum and the concentration of IL-1α in the stratum corneum, collected from both the lesional and non-lesional areas. Patients received the RetinSphere®-vitamin E treatment and all completed the 8-week study. After 4 weeks of treatment, SER did not change significantly. Squalene tended to increase after the treatment, although not at a significant extent. By contrast, the parameters of lipid peroxidation, such as LPO and the percentage of SQOX, decreased significantly ($P < 0.05$ and $P < 0.001$, respectively, Table 1) at week 4 of treatment. Comparably, concentration of IL-α measured at 4 weeks of treatment was decreased at a significant extent in the area identified as lesional at the baseline ($P < 0.001$, Fig. 1). The main RCM features observed in four patients at week 4 of treatment were the reduction of hyperkeratinization of adnexal ostia (that could be considered as inhibition of comedones formation) and the reduction of the inflammatory cells infiltration of adnexal structures epithelium. No changes on the dilated vessel were observed (Fig. 2). At week 8 significant improvements in the clinical assessment of the acne grading and in patient satisfaction score were recorded (Table 2). Tolerability of the topical was excellent and no treatment-related adverse events occurred.

Discussion

Sebum is a complex mixture of lipids continuously produced by the sebaceous gland and excreted on the skin surface.[25] Modifications of the lipids components, associated or not to increased sebum production are important pathogenetic factors in acne. The accumulation of lipoperoxides (LPO) in the comedones correlates with the severity of the lesions and the occurrence of inflammation.[27] By-products of sebum components susceptible to lipid oxidation including squalene, induce both inflammation and hyperkeratinization in cultured keratynocytes.[25,28,29] Besides squalene, targets of lipid peroxidation in sebum are polyunsaturated fatty acids such as linoleic acid and sebaleic acid.[30,31] Fatty acid derived hydroperoxides exhibit comedogenic activity similarly to byproducts of squalene oxidation.[14] The study focused on cases of MCA with no clinical signs of inflammation, in order to exclude the influence of superimposed inflammatory triggers of the parameters addressed. We found that lipid oxidation occurred at increased extent in sebum of patients with MCA compared to control sebum. The used sorbent tapes allowed for

Table 1 Comparison of sebum excretion rate (SER), squalene, peroxidated lipids and percentage of oxidized squalene (SQOX) between controls and acne patients at the baseline and after 4 weeks of therapy

	Controls (n = 10)	Patients with mild comedonal acne (n = 18)		P-value*	P-value**
		Baseline	At week 4		
SER, μg/cm^2/min	2.74 ± 1.82	3.45 ± 1.58	3.55 ± 1.7	ns	ns
Squalene, μg/mg sebum	79.1 ± 43.2	78.7 ± 28.8	93.1 ± 49.9	ns	ns
Peroxidated lipids, OD 560	0.36 ± 0.16	0.71 ± 0.50	0.51 ± 0.21	$P < 0.05$	$P < 0.05$
SQOX percentage	0.62 ± 0.36	1.11 ± 0.39	0.49 ± 0.32	$P < 0.001$	$P < 0.001$

ns, not significant.

*Differences between controls and MCA patients.

**Differences in MCA patients before and after treatment.

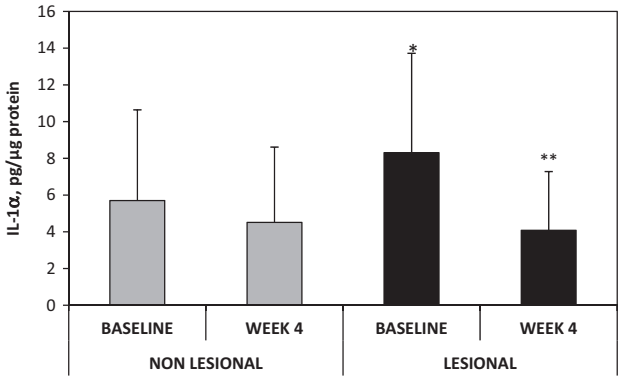

Figure 1 Concentration if interleukin-1α (IL-1α, pg/μg protein) in the stratum corneum of non-lesional and lesional skin of mild comedonal acne patients, at the baseline and at week 4. *$P < 0.05$ (between IL-1α concentrations in the non-lesional and lesional areas at the baseline); **$P < 0.001$ (between IL-1α concentrations in the lesional area at baseline and week 4 of treatment).

Table 2 Acne severity and patient satisfaction at the baseline and at week 8 of treatment. Data were reported as mean ± SD

	Baseline	8 weeks	*P*-value
Severity (Leeds revised grade)	2.0 ± 0.9	1.4 ± 0.9	0.001
Patient satisfaction	2.2 ± 1.2	6.9 ± 1.9	<0.001

the collection of sebum excreted by skin surface-open follicles, therefore, it is likely that the degree of lipid oxidation was not necessarily associated with the presence of comedones. Thus, it can be excluded that the lipid oxidation occurred at the level of the comedone content. This observation suggests that the sebum oxidation likely occurs before comedones become clogged with sebum and keratins. Parallel to LPO, squalene peroxides were upregulated in the sebum from patients with MCA compared to controls. Squalene is a symmetrical 30-carbon isoprenoid compound with six double bonds, and it is the last acyclic intermediate metabolite preceding cholesterol in its biosynthetic pathway. The limited conversion of squalene to cholesterol in the sebaceous gland results into the high concentrations of squalene in sebum.[1] Squalene takes part into the antioxidant skin defence system and, as a quencher of singlet oxygen, it prevents human skin surface from extensive lipid peroxidation. Due to the high abundance of squalene, its oxidation is likely to cause consistent reduction of the oxygen tension in the human pilo-sebaceous unit.[28] On the other hand, upon oxidation, squalene deploys proinflammatory actions.[29] Oxidized squalene has been shown to induce hyperplasia and hyperkeratosis of the epithelium in follicular infundibulum, sebaceous gland hyperplasia in hamster and rabbit ears[13,14] and, *in vitro*, it mediates release of IL-6 from keratinocytes.[13] Cigarette smoke is accounted among the triggering factors of squalene oxidation on the skin surface. A particular type of comedonal acne, defined as 'smoker's acne', has been observed in female smokers with hypersoberrhea.[32] On the other hand, inflammatory acne is associated with increased LPO. Pharmacological inhibition of 5-lipoxigenase activity has been demonstrated to contrast the overall LPO detectable at high extent in sebum collected from inflammatory acne cases and to be associated with clinical improvement.[33] Cytokine expression by ductal keratinocytes is closely associated with the comedogenesis[27] and content of IL-1α in comedones occurs at a biologically pathologically relevant extent.[11] Notably, the release of IL-1α can induce abnormal keratinization as demonstrated by the accrual of keratin 6, keratin 16 and filaggrin expression in follicular keratinocytes.[10,11] In our study, the levels of the inflammatory cytokine IL-1α were elevated in the stratum corneum

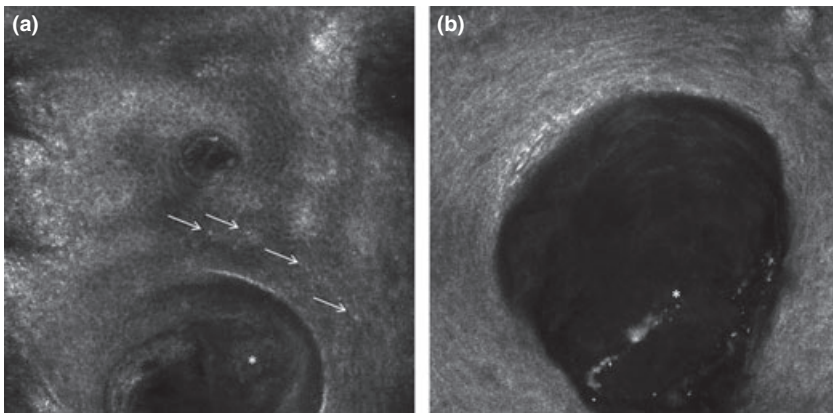

Figure 2 Reflectance confocal microscopy images (0.5 by 0.5 mm) of a dilated comedone before (a) and after treatment (b). Presence of inflammatory cells (arrows) around the dilated follicular ostium-, presence of refractile matherial filling the adnexal ostium (*). After treatment, absence of inflammatory cells involving the adnexal epithelium and significant reduction of the refractile matherial in the adnexal ostium (b).

of comedonal areas more than non-involved regions. These findings indicated that while lipid oxidative modifications were detectable in sebum excreted in unrestrained manner onto skin areas where comedones and unaffected follicles coexisted, elevation of proinflammatory cytokines was localized at the level of the comedone lesions. Tochio *et al.*[27] found higher levels of LPO and IL-1α within the comedones, leading to the conclusion that sebum oxidation is secondary to the obstruction of the infundibulum. Conversely, our data pointed out that sebum oxidation can develop independently upon the presence of clogged follicles, and peroxidated lipids (particularly squalene), accumulated at significantly high amount, can trigger inflammatory responses. To limit the harmful effects of peroxidated squalene, the skin is equipped with endogenous defence systems. Vitamin E is a lipophilic antioxidant supplied to the skin surface through the sebum outflow. In skin areas with elevated sebaceous glands density a continuous secretion of vitamin E is observed, which is in tight correlation with the levels of co-secreted squalene, highlighting a physiological antioxidant strategy put in place to counteract the generation of squalene oxidation products.[25] In line with these evidence exogenous supply of vitamin E can be associated with improvement of the oxidative status of the sebum lipids, including squalene. Topical retinoids are indicated in the treatment of acne and MCA responds to topical treatments with retinoid at low-doses.[34] However, due to the multifactorial nature of the acne pathogenesis, a combined treatment approach was investigated to address how modifications of acne inducing factors are associated with the clinical outcome. The commercially available non-prescription formulation includes several ingredients shown to target factors underlying the pathogenesis of acne vulgaris, such as natural retinoic acid-like compound (RetinSphere®), vitamin E and ancillary components aimed at improving skin hydration and integrity. Combining retinoic acid like compounds with vitamin E warrants simultaneous targeting of multiple pathogenetic factors implicated in MCA. Upon 4 weeks treatment, the total peroxidated lipids and the degree of squalene oxidation were significantly decreased compared to the baseline levels. By contrast, the sebum outflow collectively remained unchanged, supporting the hypothesis that qualitative modifications rather than secretion are likely to play a major role in the pathogenesis of acne. Consistently with the changes of sebum oxidation parameters, the concentration of cytokine IL-1α in the lesional area decreased significantly at week 4. Reduction of hyperkeratinization and inflammatory cells infiltration of the adnexal structures epithelium have been observed by RCM in 4 studied patients, predicting and anticipating clinical ameliorations registered at week 8. RCM has found several applications for the dermatological research and has proved to be a valuable tool in the clinical management of skin disorders.[35] At the best of our knowledge there are no previous reports that correlate the RCM images with the expression of inflammatory mediators on the skin surface in acne. Nevertheless, the improvement of RCM features and the decrease of IL-1α, LPO, ans squalene oxidation by-products were observed at the same time in the studied patients, suggesting that the treatment exerted antihyperproliferative and anti-inflammatory effects at the lesional area that were detectable with RCM. The measurement of sebum composition by Sebutape™ sampling and the quantification of cytokines in stratum corneum sampled with by D-squame™ tapes demonstrated to be reliable and advantageous for the evaluation *in vivo* of acne etiopathogenetic factors and to investigate response to anti-acne treatments. The clinical amelioration as well as the reduction of peroxidated products and IL-1α levels we observed in this study demonstrated that the RetinSphere®-vitamin E combination is effective in equilibrating the imbalance of the oxidant/antioxidant skin environment in patients with comedonal acne and in reducing comedogenesis. Finally, the diffuse 'dys-seborrhea' we found in patients with MCA confirms that the entire facial skin is at potential risk of developing comedones and needs to be treated to prevent the progression of the disease. In conclusion, our data provide evidence that the oxidative alterations of sebum occur at a detectable extent even in mild acne grades. Oxidation of sebum lipids, in particular squalene, might be critical in the progression of mild acne towards inflammatory lesions. A multitarget therapeutic approach can be effective in improving the early acne manifestations.

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