Pierre-Yves Morvan, Romuald Vallée – Codif International, France

Instant anti-wrinkle effect of a marine exopolysaccharide

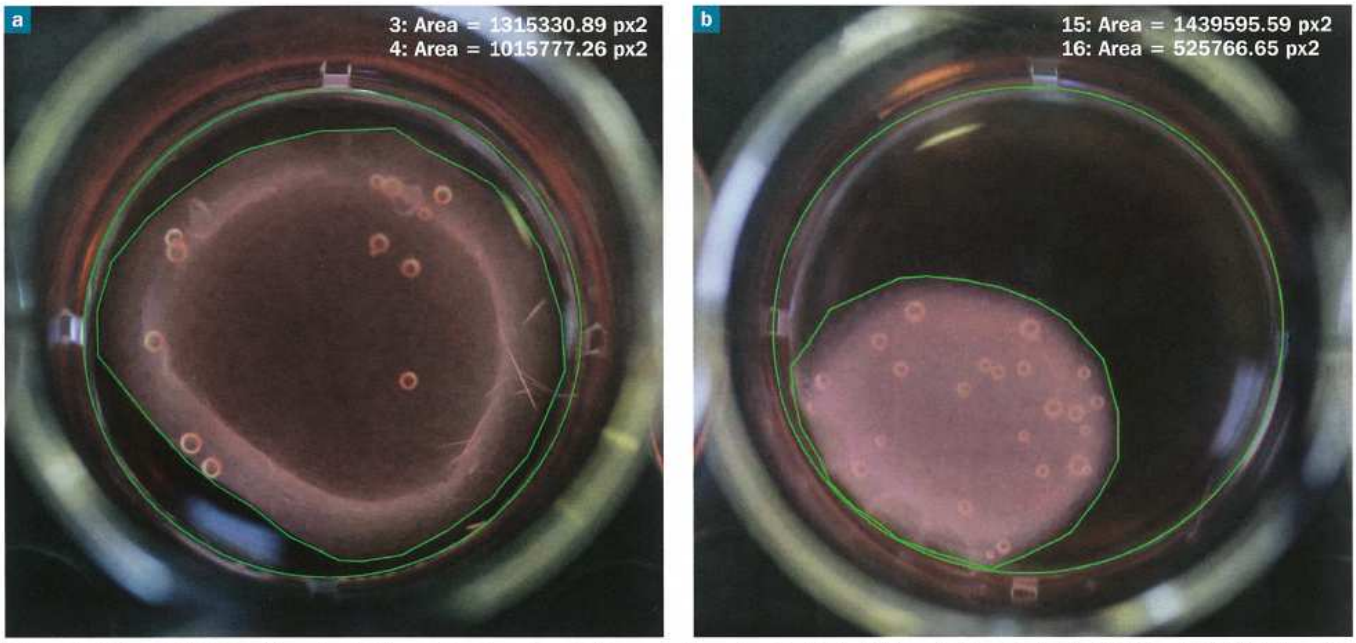


Figure 1: *Effect of EPS Seafill on the lattice contraction after 52 hours* – **a)** *non treated (1% FCS) and* **b)** *treated with 1% FCS +0.2% EPS Seafill.*

Skin ageing is a complex phenomenon involving two simultaneously occurring processes: intrinsic ageing, known as chronological ageing, which is genetically determined, and extrinsic ageing which is due to environmental factors such as chronic sun exposure, known as photoageing. Chronologically aged and photoaged skin share important molecular features including decreased proliferative capacity of skin-derived cells, decreased matrix synthesis in the dermis associated with an increased expression of enzymes that degrade the collagenous matrix, increased inflammatory process and oxidative stress.[1,2,3]

With time, the cellular renewal rate decreases, as does the effectiveness of protection systems. Collagen and elastin synthesis diminishes markedly with age: ageing skin contains fewer collagen and elastic fibres. The epidermis and dermis become atrophic and thin.[4]

As the skin has a direct interface with the environment, it is exposed to many environmental aggressions. It cannot avoid the phenomenon of ageing, and is subject to inevitable changes.

The aim of the present study was to evaluate the anti-ageing properties of a solution containing 1% of a purified and completely sequenced marine exopolysaccharide (named EPS Seafill), using *in vitro* tests on normal human dermal fibroblasts (NHDF) in monolayer (2D cellular model) and in collagen lattices (3D cellular model) by immuno-fluorescence and *in vivo* tests using fringe projection.

The 3D model is an *in vitro* three-dimensional reconstituted dermis, with a population of fibroblasts synthesising and interacting with a collagen fibre network. Frei V *et al.* demonstrated that the recent development of two three-dimensional culture systems, in which the cells develop within a porous structure reproducing the extracellular matrix of the human dermis, is a way of reproducing *in vivo* conditions and demonstrating the biological effects of anti-ageing compounds.[3] It is used to assess the effect of an active cosmetic ingredient on the collagen network more realistically than with cellular cultures.[5]

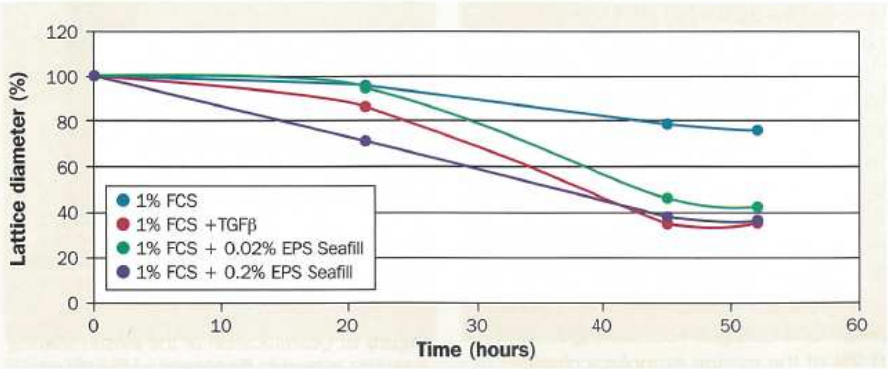


Figure 2: *Evolution of the lattice contraction over time.*

To study the effects of the exopolysaccharide on dermal fibroblasts, we compared retracting collagen lattices, in which collagen fibres are remodelled to a floating disc and the tension is very low and distributed isotropically, to stressed lattices that cannot be retracted due to adherence of the collagen lattice to a nylon thread placed at the inner perimeter of the dish.[6]

Within collagen lattices, fibroblasts utilise $\alpha 2\beta 1$ integrin receptors to mediate cell-collagen contact.[7,8] By their transmembrane nature, $\alpha 2\beta 1$ receptors physically connect external collagen fibres with the actin filament network and thereby transmit forces and information required for contraction into the cells.[9]

In relaxed lattices, fibroblasts appear stellar with short processes; in stretched lattices, fibroblasts are elongated, bipolar, and oriented along the lines of tension according to observations made by Lambert and Bellows.[6,10]

Effect on lattice contraction

Bell reported that the incorporation of fibroblasts in a collagen gel induces a progressive contraction of the gel, resulting in the formation of a dense collagen disc, called retracted lattice. Contraction is determined by measurements of gel diameter over time. Retraction of the dermis equivalent is proportional to the number of fibroblasts incorporated and to the collagen concentration.[5]

In our experiment, the relaxed lattices are made with normal human dermal fibroblasts (NHDF) in passage 6 and collagen I containing or not 0.02% or 0.2% of EPS Seafill (now referred to as 'the marine exopolysaccharide'). Then the obtained lattices were deposited on multi-well plates. They were seeded and placed in an incubator for 52 hours. After 52 hours, the lattices were photographed and the area occupied by the lattice in the culture well was measured.

The treatment with 0.02% and 0.2% of the marine exopolysaccharide improves the contraction of the lattice in the Petri dishes (see Fig. 1). The marine exopolysaccharide generates a tightening effect on the collagen fibre network of the relaxed lattice.

This macroscopic contraction is related to the force induced by fibroblasts on collagen fibrils. Models have been developed to associate with this macroscopic phenomenon, microscopic phenomena taking place within the collagen gel.[11]

Effect on stretched lattice

The stretched lattices were made with NHDF and collagen I containing or not 0.2% of the marine exopolysaccharide. Then the obtained lattices were deposited on multi-well plates, at the bottom of each is deposited a nylon ring. They were seeded and placed in an incubator for five days. After five days the lattices were photographed, the medium was then vacuumed out, the lattices rinsed and then fixed.

Microscopic observation was performed with a Nikon TE300 Eclipse microscope equipped with a Nikon DS DS-2Mv camera head. One photograph in three dimensions per well was taken.

The control lattice shows fibroblasts with a relaxed form and a flexible collagen matrix (see arrow on the control lattice in Figure 3).

In the presence of 0.2% of the marine exopolysaccharide, the shape of the fibroblasts is even more stretched, the collagen network is under tension, and a tensile force is generated over the entire lattice.

The marine exopolysaccharide induces the contraction of fibroblasts which consequently stretch the collagen network for a resulting tensing effect. The marine exopolysaccharide generates a tightening effect on the collagen fibre network.

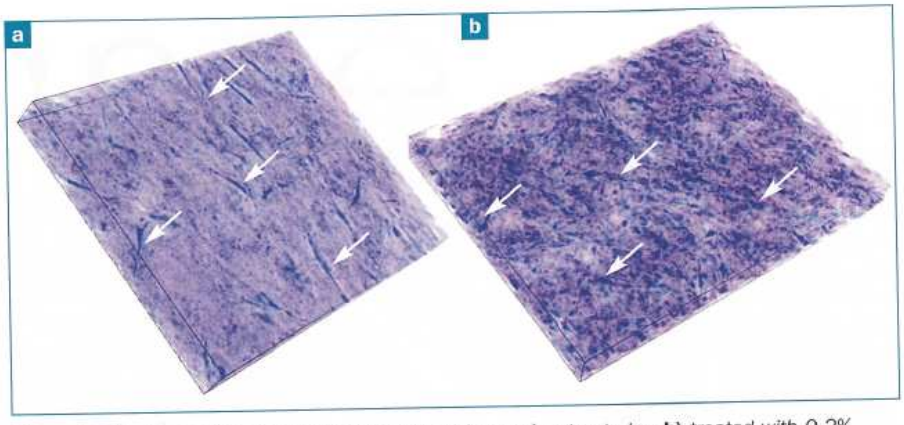


Figure 3: *3D observation of collagen lattices which are* **a)** *untreated or* **b)** *treated with 0.2% of EPS Seafill. The arrows point to fibroblasts in the collagen lattice.*

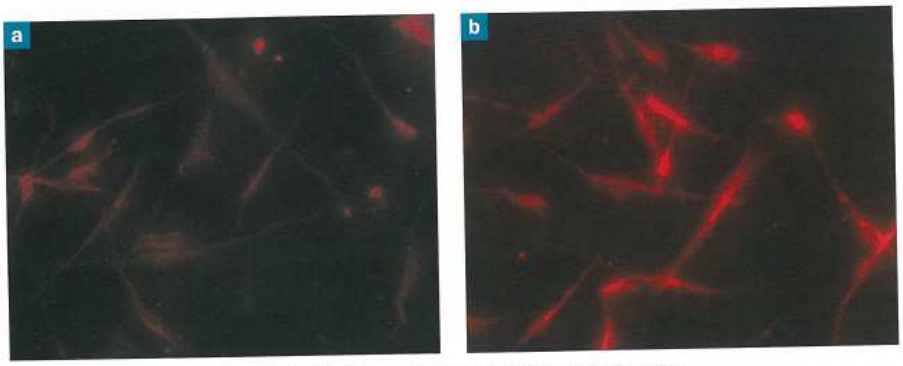


Figure 4: *Immunostaining of elastin fibres produced by human fibroblasts* **a)** *untreated or* **b)** *treated with 0.2% of EPS Seafill.*

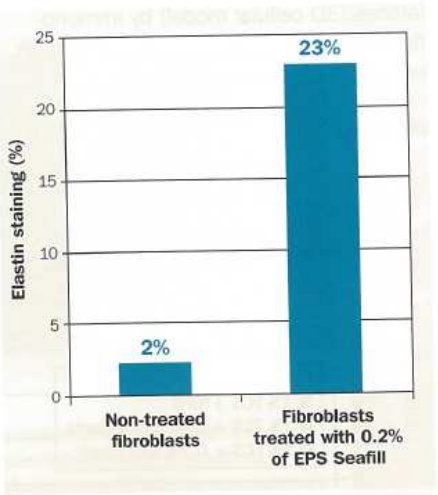


Figure 5: *Quantification of the elastin staining intensity in human fibroblasts untreated or treated with EPS Seafill.*

Effect on elastin synthesis

NHDF were used in passage 4 for elastin synthesis in stretched lattices.

The microscopic observation allows analysing fibroblast morphology in a stretched lattice. As described by Lambert and Bellows[6,10] we observed that fibroblasts are elongated, bipolar, and oriented along the lines of tension.

In the absence of product, the staining of elastin fibres is very low. In the presence of 0.2% the marine exopolysaccharide, an important increase in elastin staining is also observed (see Fig. 4). This effect was confirmed by an image analysis.

The treatment with 0.2% of the marine exopolysaccharide increases by 23% the synthesis of elastin by human fibroblasts. This result suggests that the marine

exopolysaccharide, thanks to its stimulating effect on elastin synthesis by fibroblasts, aims to improve skin elasticity.

Effect on collagen I synthesis

For the collagen immunostaining in 2D, NHDF were isolated from healthy skin obtained from plastic surgery and used in passage 7, cultured in monolayer on Labtek at a density of 7000 cells by well. NHDF were treated with 0.02% of the marine exopolysaccharide dissolved in DMEM Glutamax medium supplemented with 2% fetal bovine serum and 1% penicillin/streptomycin, or untreated. The cells were cultivated for 120 hours and each experimental condition was performed in triplicate.

In the absence of product, the staining of collagen I fibres is low. In the presence of the marine exopolysaccharide, there was a clear increase of collagen I (see Fig. 6).

Treatment with image analysis was performed on the photographs. The colour intensity is measured by image analysis software and related to 100 cells. The results are shown in Figure 7.

Treatment with 0.02% of the marine exopolysaccharide increases by 20% the synthesis of collagen I by human fibroblasts in monolayer. These results suggest that the marine exopolysaccharide thanks to its stimulating effect on collagen I synthesis by fibroblasts, aims to improve skin firmness.

Affinity for the skin

Human skin explants of 8 mm of diameter were treated with the marine exopolysaccharide for 1 hour. At the end of the incubation period, the samples were freeze-dried to fix and dehydrate the skin. The samples were observed with a JEOL JSM 6301F SEM (field effect scanning electron microscope).

The photographs show scanning electron microscope observations of the surface of an epidermis. Furrows and variations in cutaneous relief can clearly be seen (Fig. 8). After treatment with the marine exopolysaccharide, the furrows are filled in and the differences in relief are smoothed out and made uniform.

Immediate lifting effect on cutaneous relief (*in vivo* Test 2)

Smoothing effect was evaluated on four Caucasian female volunteers, aged between 45 and 65 years, applying once a cream containing 2% of the marine exopolysaccharide (i.e. 0.02% final concentration of the pure exopolysaccharide) to crow's feet selected at random. Evaluation was made by measuring the skin's roughness and the cutaneous relief dispersion using fringe projection

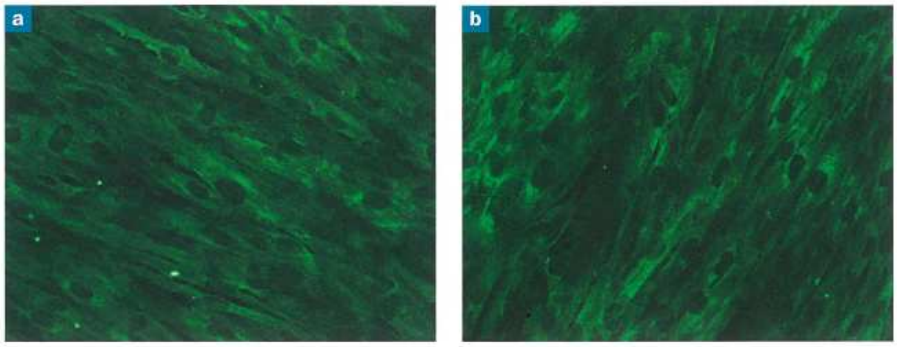


Figure 6: *Immunostaining of collagen I fibres produced by human fibroblasts* **a)** *untreated or* **b)** *treated with 0.02% of EPS Seafill.*

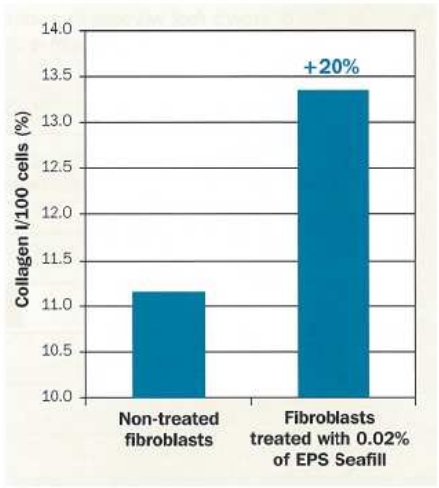


Figure 7: *Quantification of the collagen I staining intensity in human fibroblasts non treated or treated with EPS Seafill.*

before and 15 minutes after the product application.

Fifteen minutes after applying the cream containing 2% of the marine exopolysaccharide, a reduction in average roughness of –8.7% on average and up to –17.5% is observed as well as a reduction in roughness with regard to the average quadratic variation of –9.9% on average and up to –18%.

After only one application of a cream containing 2% of the marine exopolysaccharide on crow's feet wrinkles we showed a roughness smoothing effect as well as a cutaneous relief lifting effect 15 minutes after application as shown in Figure 9.

Immediate anti-wrinkle effect (*in vivo* Test 2)

Anti-wrinkle efficacy was evaluated on 17 Caucasian female volunteers, aged between 45 and 65 years, applying once a cream containing 2% of the marine exopolysaccharide to facial crow's feet selected at random versus a base cream alone (placebo). Evaluation was made by measuring the depth of the crow's feet wrinkles using fringe projection and with illustrative photographs before application and 15 minutes, 3 hours and 6 hours after the product application.

A unique application of a cream containing a concentration of 2% the marine exopolysaccharide reduces the depth of the main wrinkle by –5.1% on average and up to –16% after 15 minutes (significant variation), –1.9% on average and up to –15% after 3 hours, –0.1% on average and up to –20% after 6 hours (see Fig. 10). This effect is visible as shown in Figure 11.

In comparison, application of the placebo cream reduces the depth of the main wrinkle by only –0.6% on average and up to –15%, after 15 minutes (significant variation), and –0.2% on average and up to –24% after three hours. After six hours, the control cream has a tendency to increase the depth of the wrinkle.

Thus the marine exopolysaccharide

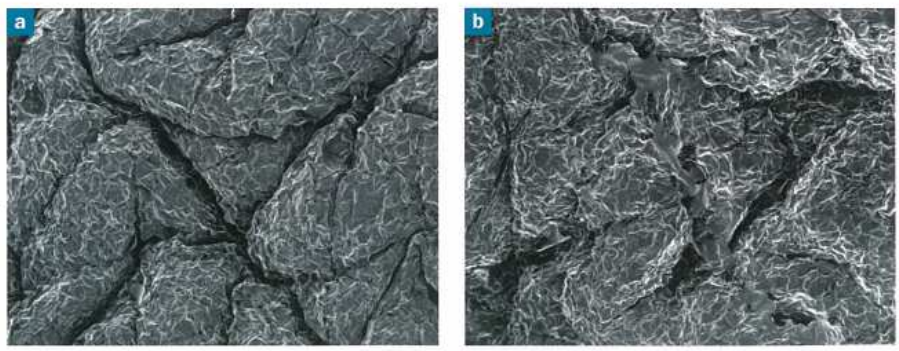


Figure 8: *SEM observation of human skin explants* **a)** *treated or* **b)** *not with EPS Seafill (enlargement x100).*

reduces the depth of the wrinkles after 15 minutes and for a period of six hours. The self evaluation questionnaire revealed that more than 70% of the volunteers found their skin smoothed out and their expression lines softened.

Conclusion

The marine exopolysaccharide EPS Seafill increases the synthesis of collagen I and the synthesis of elastin by human fibroblasts. Thanks to its stimulating effect, it aims to improve skin firmness and elasticity. Moreover, it improves the contraction of a collagen lattice, showing that it generates a tightening effect on the collagen fibre network for a resulting tensing effect.

These *in vitro* effects were confirmed by two *in vivo* studies that demonstrated that EPS Seafill lifts cutaneous relief and has a smoothing effect. Due to its novel structure and its skin affinity, it can reduce wrinkles 15 minutes after application. Its tightening effect lifts crow's feet roughness to smooth out cutaneous relief.

This marine exopolysaccharide has no land based equivalent and represents a new and original source of molecules since it helps to fight against one of the main intrinsic factors of skin ageing. PC

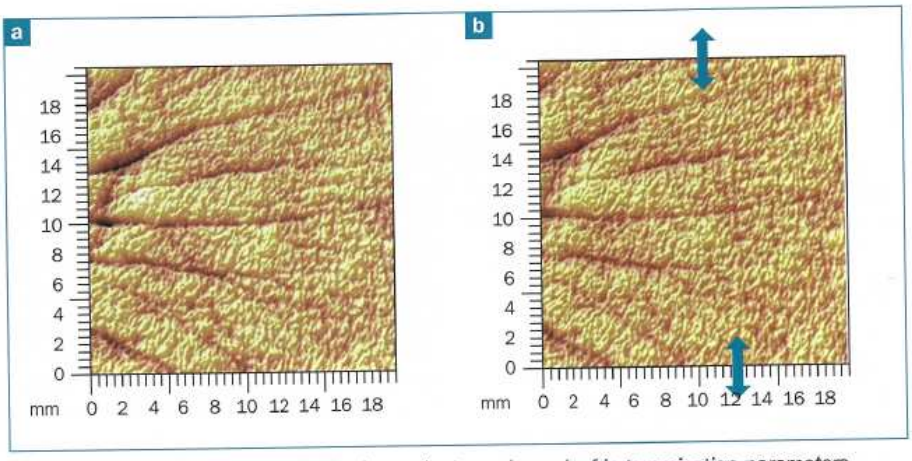


Figure 9: *View of crow's feet wrinkles in a volunteer, shown by fringe projection parameters* **a)** *before (T0), and* **b)** *after treatment with a cream containing 2% EPS Seafill (T+15 minutes).*

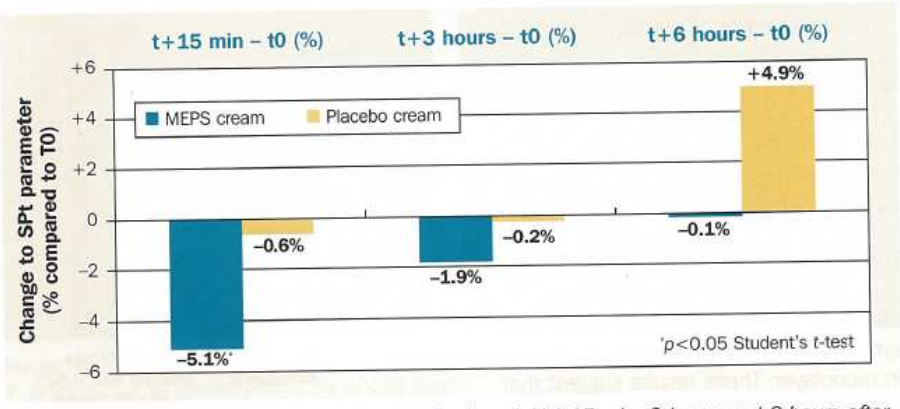


Figure 10: *Change in SPt parameter (depth of main wrinkle) 15 min, 3 hours and 6 hours after application of a cream containing 2% EPS Seafill versus a placebo cream.*

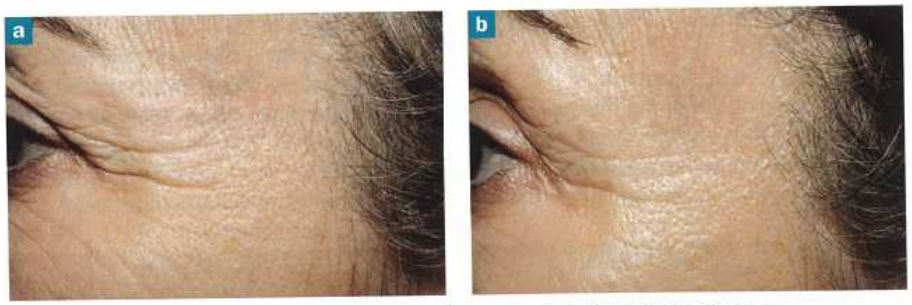
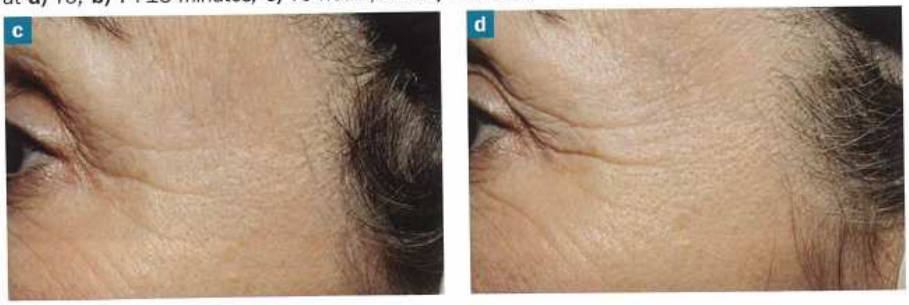


Figure 11: *Photographs of crow's foot on a volunteer treated with EPS Seafill cream at* **a)** *T0,* **b)** *T+15 minutes,* **c)** *T3 hours, and* **d)** *T6 hours.*



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