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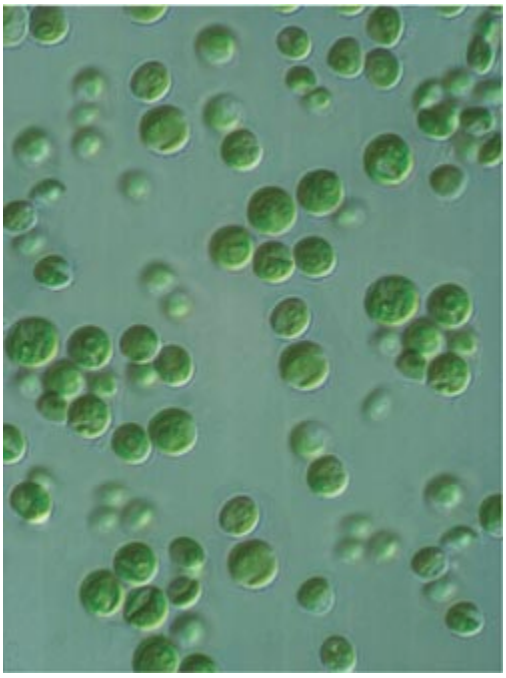
EFFECTS OF CHLORELLA EXTRACT ON SKIN

November 2007



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The ageing process starts from the age of 20 when our cells produce fewer proteins. Skin ageing is characterised in three ways: * By a decrease in the thickness, firmness and elasticity of skin promoting wrinkles. * By the unsightly effect of skin microcirculation in the form of spider veins on the legs or dark circles on the face around the eyes. * By a reduction in the antioxidant capacities with, in particular, lower protection against UV.

The main reasons for these three skin modifications are outlined below. Wrinkles and stretch marks are the result of a lack of skin firmness and elasticity linked to a dramatic alteration of the cells and of the main constituents of the dermis located in the space surrounding the cells. The latter contains macromolecules, polysaccharides, glycosaminoglycans (GAGs), fibrous protein (collagens, elastin), salts and water which together are known as the extra cellular matrix, responsible for tissue cohesion. The main structure proteins are collagens and elastin. The extra cellular matrix components are synthesised and secreted by cells such as the fibroblasts and degraded by enzymes called Matrix MetalloProteinases (MMPs). MMP activity is regulated by various factors including activators like plasminogen, plasmin, tissue plasminogen activator (t-PA) and urokinase plasminogen activator (u-PA), but also by inhibitors like Tissue Inhibitors of Metalloproteinase (TIMPs), anti-plasmin and Plasminogen Activator Inhibitors (PAI 1 and PAI 2).

Angiogenesis is defined as the formation of new blood vessels from preexisting vessels. This excess growth of blood vessels is the cause of unattractive skin problem such as acne rosacea, vascular imperfections and dark circles under the eyes. All these defects are due to the dilation of superficial veins that become visible on the surface of the skin. Vascular imperfections often result in the appearance of unsightly spider veins on the legs. They can become varicose veins due to the stagnation of blood in the lower limbs. Hormone changes (contraception, pregnancy, etc.), constantly standing or sitting, and ageing also contribute to the formation of these small imperfections. Dark circles are due to small veins under the eyes which tend to dilate; blood circulates badly and since the skin is very thin in this site, they rapidly appear. Their appearance is also supported by stress, lack of sleep and pollution.

Skin is also constantly targeted by active forms of oxygen generated by UV radiation exposure and it needs to reinforce its antioxidant defence mechanisms in order to protect itself against cell damage. Thioredoxin (TX) is a group of proteins found extensively in animals, plants and marine bacteria. There are two types of TX: TX1 which is cellular and TX2 which is mitochondrial. They protect cells against the cytotoxicity produced by free radicals. An increase in the thioredoxin production could therefore be an interesting course of action for improving skin cell protection.

Dermochlorella

Green micro-algae are rich in proteins, mainly in the amino acids that constitute the dermis fibres and the expressed specific proteins that produce antioxidant activity. We therefore studied the effect of an extract obtained from the micro-algae Chlorella vulgaris, named Dermochlorella, on skin restructuring, skin microcirculation and skin protection against UV irradiations.

Different studies were carried out to assess the following:

- Effect on skin restructuring: an approach was tested by molecular biology (RNAm) according to the DNA mini-chips and RT-PCR methods. It allowed studying the effect of Chlorella vulgaris on human dermal fibroblast genes expression and thus measuring its effect on the genes expression of the different extra-cellular matrix components. Moreover, clinical tests assessed the effect of a cream containing Chlorella vulgaris at 1% on stretch marks, skin firmness and tone.
- Effect on skin microcirculation: an in vitro test assessed its efficacy on angiogenesis. Clinical studies assessed the efficacy of a cream containing Chlorella vulgaris at 1% on vascular imperfections, dark circles and lymphatic drainage.
- Effect on skin protection: an in vitro test was carried out by molecular biology (RNAm) according to the DNA mini-chips and RT-PCR methods.

It allowed studying the effect of Chlorella vulgaris on human epidermal keratinocytes Thioredoxin 1 expression. A complementary study was done on the protein TX2 by Western blot. A complementary in vitro test was carried out in order to measure, by immunofluorescence, the protection of Langerhans cells into a skin treated by UV irradiations.

Materials and methods

Test product

The Chlorella extract was obtained by alkaline hydrolysis of Chlorella vulgaris. It was filtered on hollow fibres and concentrated by nanofiltration, and thus an extract purified out of salt and enriched out of proteins and peptides was obtained.

Skin restructuring

- Expression of collagens, TIMPs, proteins of the epidermal differentiation complex (EDC), elastin and elafin:

Human fibroblasts or keratinocytes, isolated from normal human skin and cultivated in monolayer, were treated over 24 hours with Chlorella vulgaris (without preservative) tested at 1%. At the end of the experiments, cells were recovered and RNA was prepared for genomic analysis. The gene expression of these fibroblasts or keratinocytes was analysed by a transcriptomic screening on mini-chips. The analysis is based on the use of total RNA as a template for reverse transcription and 33P label (optimal sensitivity). Labelled cDNA targets were hybridised to the specific cDNA probes conveniently fixed to the mini-chips. After extensive washing, the relative amount of each specific target hybridised to its probe was revealed by phosphor imaging. The results for TIMPs and proteins of the epidermal differentiation complex were confirmed using real-time PCR (quantitative PCR, Q-PCR). This confirmation was performed with RNA extracted and stored at -80°C. G3PDH was used as the housekeeping gene. The result for elastin was confirmed by a test on cells. The production of elastin by human dermal fibroblasts, treated by Chlorella vulgaris at 0.5% over 24 hours, was quantified by the Fastin Elastin Assay kit.

- Expression of genes which regulate MMPs: PAI-1, PAI-2, t-PA and u-PA:

Two experiments were conducted: the first with normal human dermal fibroblasts (NHDF), cultured in co-culture on the lower face of the membrane of 27 human reconstituted epidermis (Skinethic), 13 days old. The product Chlorella vulgaris (without preservative) tested at 1% was introduced in the culture medium and deposited on the surface of epidermis over 24 hours. The second experiment used normal human epidermal keratinocytes (NHEK), cultured in monolayer. Cells were cultivated over 24 hours at 37°C and 5% CO2. At the end of the experiments, cells were recovered and RNA was prepared for genomic analysis. The gene expression of these cells was analysed by a transcriptomic screening on mini-chips.

- Effect on skin firmness and anti-stretch mark effect:

Eleven volunteers applied a cream containing Chlorella vulgaris at 1% twice daily for 84 days. The parameters studied were the variations of the skin's firmness and tone with ballistometry, and the variations of the morphology and of the colour of the stretch marks with cross-polarised photographs.

Skin microcirculation

- Effect on angiogenesis:

The system of test for this study was an in vitro model of angiogenesis, constituted of endothelial human cells in co-culture with human fibroblasts. The effect of Chlorella vulgaris was studied by the measurement of the length of tubules. The vascular endothelial growth factor (VEGF) was used as reference activator, and the suramin as reference inhibitor of the formation of tubules.

- Effect on spider veins:

This study consisted of analysing vascular imperfection variations using Chromameter Minolta of the type CR321. Seventeen women aged between 35 and 65 years old applied a cream containing Chlorella vulgaris at 1% twice a day for 84 days. The volunteers had apparent lesions related to a bad venous function with heavy leg sensation; e.g. small varicose veins, small stellar angiomas, varicosities, petechia. The effect of the product on spider veins was evaluated after 28 and 84 days of application.

- Effect on dark circles:

Fifteen women aged between 50 and 65 years old applied a cream containing Chlorella vulgaris at 1% twice a day for 28 days. The effect of the product on the dark circles was measured by colorimetric analysis. The skin colour was defined according to its luminosity (L) and its individual typological angle or ITA, which defines the degree of pigmentation of the skin of an individual; the higher the ITA, the lighter the skin.

- Effect on lymphatic drainage:

Fourteen women aged between 25 and 54 years old, having problems of heavy legs and water retention, applied a cream containing Chlorella vulgaris at 1% twice a day for 28 days. The evaluation was performed using B mode ultrasound imaging 7.5MHz. The dermo-hypodermis thickness was measured in order to assess the reduction of the oedema in the cases of subjects with heavy legs coupled with a swelling of ankles.

Protection against UV irradiation

- Effect on Thioredoxin expression:

Normal human epidermal keratinocytes (NHEK) were treated for mini-chips and RT-PCR analysis using a thioredoxin-1 (TX1) specific probe. The protein expression was quantified in human fibroblasts by Western blot. Proteins were dissociated and dosed according to standard protocols and samples were separated on a gel with 18% acrylamide. Proteins were transferred on nitrocellulose. Membranes were saturated and after several washings, proteins were labelled with an anti-thioredoxin-2 (TX2) antibody or an anti-actin antibody and then an anti-immunoglobulin-peroxidase. After washing, the peroxidase activity was detected.

- Effect on Langerhans cells:

The protection of Langerhans cells (LC) was evaluated in human skin by immunofluorescence, using an anti-CD1a antibody.

Results

Effect of Chlorella vulgaris on skin restructuring

- Effect on collagens 1 and 3:

Collagen is the main fibre protein of the body that gives tissues their elasticity. Its role may be compared to that of a frame. It is composed of different types depending on their location and it is essential forthe healing process. Chlorella vulgaris, tested at 1%, increased collagen-1 (COL1) expression by +333% and collagen-3 (COL3) expression by +150% in human fibroblasts (Fig. 1).

- Effect on the proteins of the dermoepidermal junction (DEJ):

In fibroblasts, Chlorella vulgaris increased collagen-4 (COL4) and laminin-6 (LAM6) expression by +77% by +41%, respectively. In keratinocytes, Chlorella vulgaris increased laminin-5 (LAM5) expression by +37% and collagen-7 (COL7) expression by +65% (Fig. 2).

- Effect on the proteins of the epidermal differentiation complex (EDC):

Chlorella vulgaris increased the expression of EDC proteins in keratinocytes and particularly the small prolin rich proteins (SPRRs) and the cysteine-rich c-terminal (CRCT) protein also called NICE. These results show that this extract supports the epidermal differentiation (Fig. 3).

- Effect on elastin and elafin:

Elastin is a glycoprotein secreted by dermis cells that have elastic properties. Its synthesis decreases with age, resulting in the appearance of stretch marks under the action of mechanical constraints. Elafin is a specific inhibitor of elastase, an enzyme responsible for elastin fibre degradation. Chlorella vulgaris, tested at 1%, increased elastin expression in fibroblasts by +35% and elafin expression in keratinocytes by +183% (Fig. 4). Chlorella vulgaris, tested at 0.5%, increased the elastin production in human dermal fibroblasts by 55% (Fig. 5).

- Effects on TIMPs expression:

TIMPs are able to inhibit all MMPs. Therefore, they play a key role in maintaining a balance between extracellular matrix formation and degradation in various physiological processes. Chlorella vulgaris at 1% increased TIMP-1 (+50%), TIMP-2 (+25%) and TIMP-3 (+31%) in dermal cells (Fig. 6).

- Effect on MMPs activators and inhibitors:

Chlorella vulgaris at 1% decreased the expression of t-PA and increased the expression of PAI-2 in human fibroblasts; it decreased the expression of u-PA, and increased the expression of PAI-2 in keratinocytes (Fig. 7).

- Effect on skin firmness and tone:

The analysis of a bead propelled onto the skin (ballistometry) allowed the measurement of cutaneous firmness and tone. The results obtained with Chlorella vulgaris were compared to an untreated control.

Three parameters were studied, as follows:

Cutaneous penetration: -7.4% on average and up to -26% after 84 days of use. A decrease in this parameter indicates that the bead creates only a slight depression on the skin. The skin is firmer.

Absorption of bounces by skin: +6.5% on average and up to +66% after 84 days of use. An increase in this parameter indicates that the bounces stop sooner. The skin tone increased. Amplitude of bounce: -10.5% on average and up to –46% after 84 days of use. A decrease in this parameter indicates that the bead bounces less intensely. The firmness and tone of the skin increased (Fig. 8).

- Anti-stretch mark effect:

Chlorella vulgaris at 1% significantly decreased the colour of stretch marks (chromametry analysis): –10.4% on average and up to –32% after 84 days of use.

It decreased the morphology of stretch marks (analysis from photographs): –2.9% on average and up to –7.9% after 84 days of use (Fig. 9).

Effect of Chlorella vulgaris on skin microcirculation

- Effect on angiogenesis:

VEGF is known to activate vessel formation (+128%) while suramin is an inhibitor of this mechanism (–72%). Chlorella vulgaris, tested at 0.1% significantly decreased the length of vessels: –46% (Fig. 10 and Fig. 11).

- Effect on spider veins:

Chlorella vulgaris at 1% visibly decreased the redness of the vascular lesions: –15% on average and up to –64% after 28 days of use; –25% on average and up to –77% after 84 days of use (Fig. 12). This effect was observed in 75% of volunteers at the end of the test. The treatment improved the size and the colour of the spider veins (Fig. 13).

- Effect on dark circles:

Chlorella vulgaris increased the transparency of the skin: +1.84% on average and up to +6.8% after 28 days of use. It decreased the pigmentation of the skin: +11.5% on average and up to +113% after 28 days of use (Fig. 14). This effect was observed in 73% of volunteers at the end of the test.

- Effect on lymphatic drainage:

Chlorella vulgaris decreases the ankle adipose tissue thickness: 0.2 mm on average (i.e.1.8%) and up to 1.2 mm (i.e. 11.0%) after 28 days of use (Fig. 15 and Fig. 16). This effect was observed in 64% of volunteers at the end of the test.

Effect of Chlorella vulgaris on skin protection

- Effect on the expression of thioredoxin (TX):

The treatment by Chlorella vulgaris (tested at 1%) increased respectively by 286% and 40% the TX1 mRNA expression in human keratinocytes and fibroblasts (Fig. 17). The increase obtained with keratinocytes has been confirmed by quantitative RT-PCR: Chlorella vulgaris increased by 382% the Thioredoxin-1 mRNA expression (data not shown). On the level of protein, Chlorella vulgaris clearly increased the TX2 protein production by human fibroblasts (Fig. 18). By stimulating the endogenous level of thioredoxin, Chlorella vulgaris should protect skin cells against several types of free radical generating systems and could be an interesting and original way to reduce skin accelerating ageing.

- Protection of Langerhans cells (LC):

LC were detected in human epidermis using an anti-CD1a (Fig. 19A). After UVexposure, the number of LC dramatically decreased and the morphology was different: cells lost their numerous dendrites and became more spherical (Fig. 19B). A topical application of the Chlorella extract at 5% during three days before UV exposure protected LC, the number of LC and their morphology were similar to the control without UVB (Fig. 19C).

Conclusion

It has previously been described that Chlorella vulgaris has an anti-collagenase and an anti-elastase effect, and a stimulation effect on total collagen synthesis (Communication by CODIF at the IFSCC Berlin in 2000). In this paper, we have shown that it acts by increasing the expression of TIMP1, TIMP2 and TIMP3 that inhibit MMP and thus prevent the breakdown of collagens and elastin through an indirect action. Moreover, we detected that it also acts by the plasminogen pathway since it inhibits u-PA by keratinocytes and t-PA by fibroblasts, and also stimulates both PAI-1 and PAI-2. All these effects are summarised in Figure 20.

In addition to the effect on elastin expression, Chlorella vulgaris increases elafin expression and thus prevents the elastin degradation by elastase inhibition. It also increases expression of the epidermal-dermal junction proteins (Collagen-4 and -5 and Laminin-5 and -6) and stimulates the proteins of the epidermal differentiation complex, showing a restructuring effect not only on dermis but also on epidermis. Chlorella vulgaris is thus an excellent firming active ingredient on both epidermal and dermal structure but also on epidermal-dermal junction. Several clinical tests show that a cream containing Dermochlorella at 1% increases the skin's firmness and tone and decreases the morphology and the colour of stretch marks after 84 days of use. Chlorella vulgaris inhibits angiogenesis significantly. This result is of interest for all dermatological applications, mainly for improving skin problems such as psoriasis, which is characterised by an elevated formation of new vessels, and rosacea which is the consequence of face oedema, skin redness and an excessive dilation of face superficial vessels. Another application is the treatment of superficial varicosities that appear in the legs, preventing or updating elimination by surgery or sclerotherapy. This effect could be the consequence of a reduction of VEGF action through the over expression of TIMP3 since TIMP3 is an antagonist of VEGF receptor (Qi et al., 2003). However, a negative regulation of VEGF effect is interesting to solve skin problems usually treated by retinol because retinoids are known to decrease VEGF production by keratinocytes (Weninger et al., 1998). This down regulation should be partially responsible for the benefits of retinoids in psoriasis and rosacea, and also in acne and photo-ageing. The advantage of Chlorella vulgaris is its very good tolerance.

Chlorella vulgaris clearly increases thioredoxin expression in keratinocytes. It protects human skin cells against

ageing induced by free radicals, thanks to the stimulation of the natural defences of the cutaneous cells; it increases the expression of the thioredoxin-1 and thioredoxin-2. By stimulating the endogenous level of thioredoxins, this micro-algal extract protects skin cells against several types of free radical generating systems, and could be an interesting and original way of action to reduce skin accelerating ageing. As revealed in this article, Chlorella vulgaris has many properties. It can therefore be used as a natural active ingredient for anti-ageing, anti-wrinkle, anti-cellulite, anti-stretch mark and sun care products.

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ABSTRACT

This article provides the data obtained on an extract of the micro-algal Chlorella vulgaris, which was developed and named Dermochlorella. Some data were already known, and others were obtained after new in vitro tests on different skin cells and clinical tests made on skin microcirculation, skin restructuring and skin protection against UV irradiations. All these data conclude that Chlorella vulgaris is a useful cosmetic ingredient for antiageing, anti-wrinkle, anti-cellulite and anti-stretch mark products.