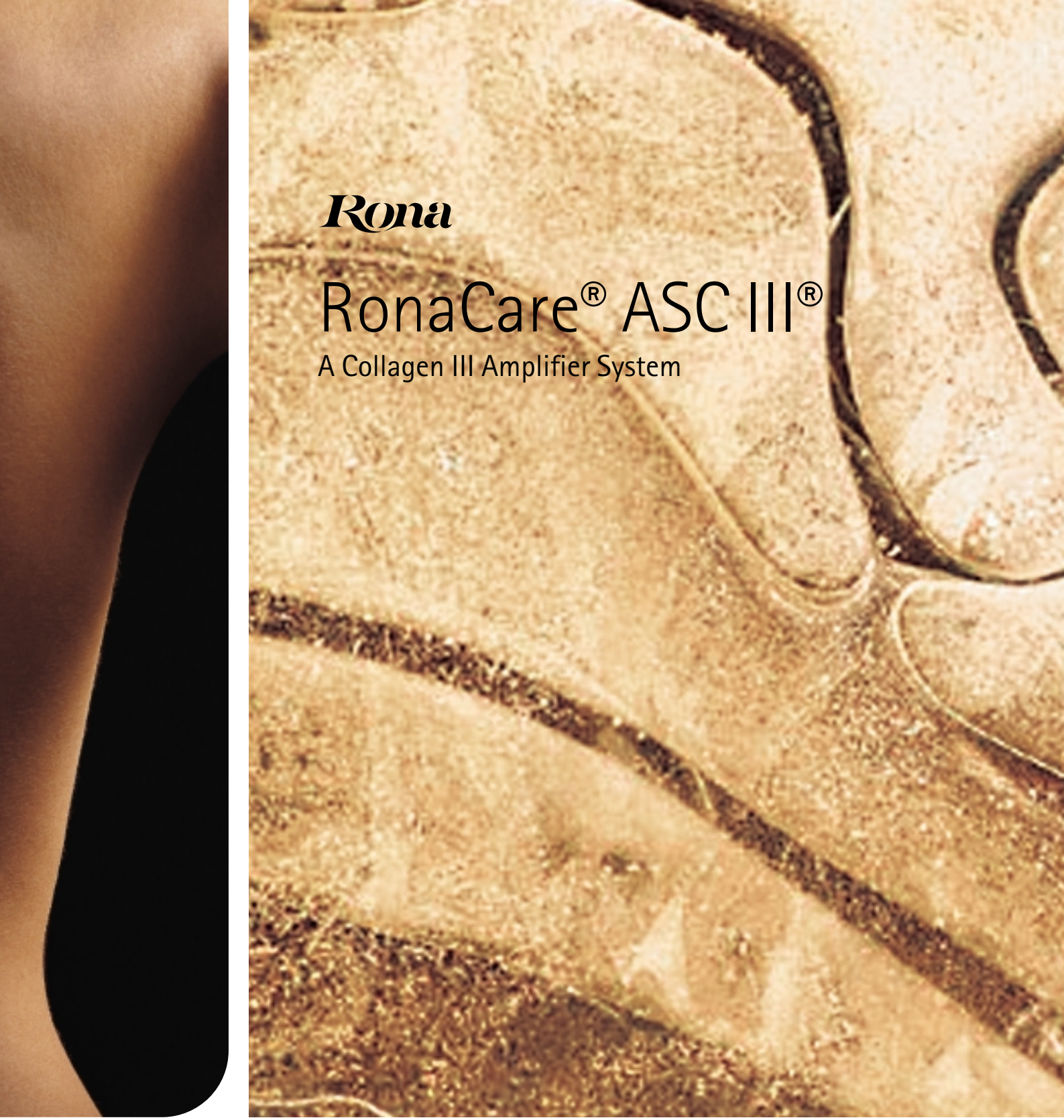
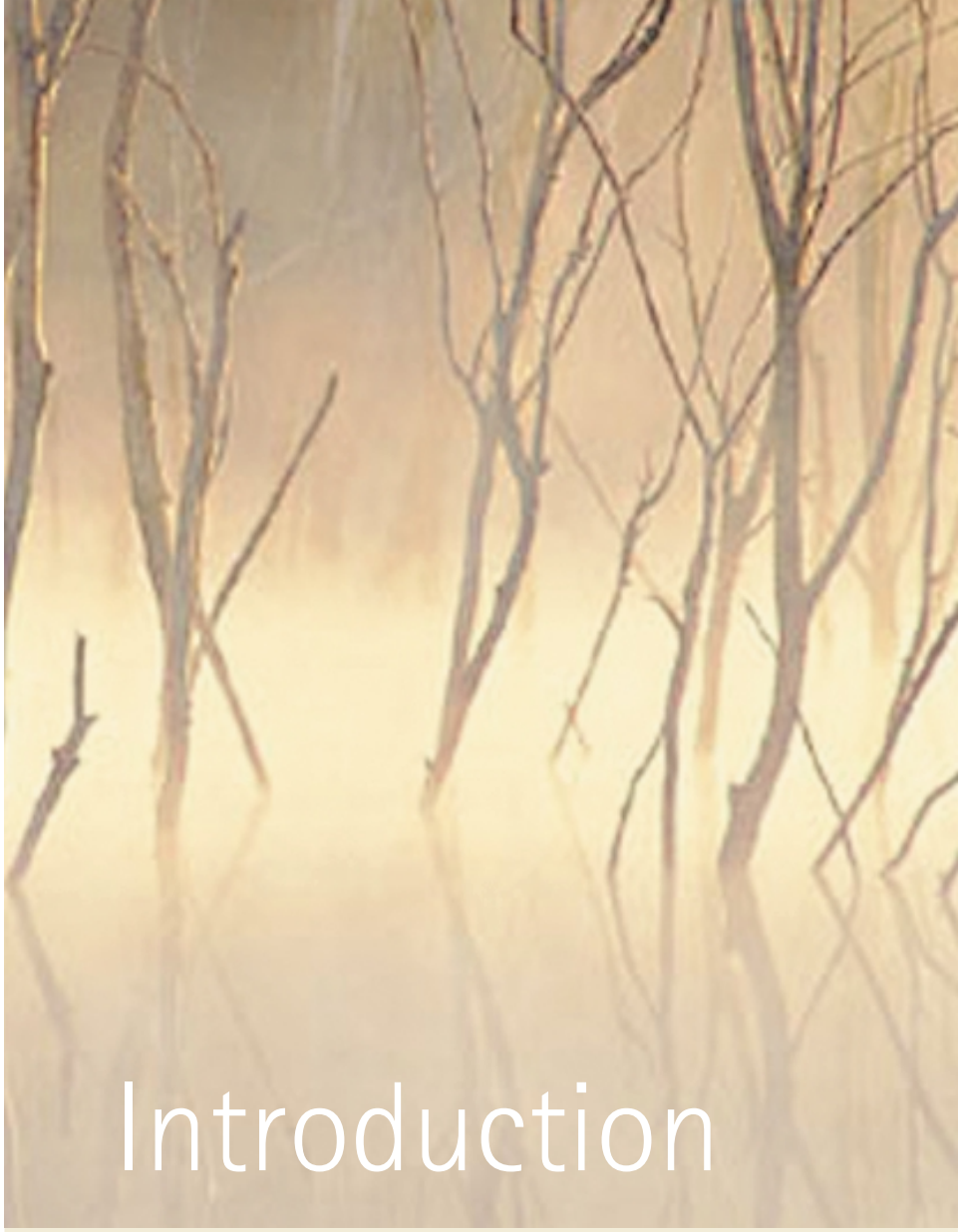


Rona

RonaCare® ASC III®

A Collagen III Amplifier System

EMD



Introduction

The aging process of human skin is influenced by both intrinsic factors and external influences, such as ultraviolet radiation and environmental pollution[1]. In each case, the connective tissue in the dermis, which is composed of fibroblasts embedded into the extracellular matrix, is altered. Thinning of the dermis occurs due to a decrease in both collagen[2] and glycosaminoglycan[3], which along with elastin, proteoglycans, glycoproteins and fibronectin, form a significant part of the extracellular matrix. Skin integrity also deteriorates due to weakening of the junction between the epidermis and the dermis making it more susceptible to mechanical damage[4].

In human tissues there are at least 10 genetically distinct collagen types that have been well-characterized[5]. Several others are currently under study. Collagen I represents about 80 % of the dermal collagen in an adult's skin, while collagen III accounts for 15 %[6]. The remaining 5 % is mainly made up of collagens IV and V. Collagen III is predominant in young skin and appears first during the wound healing process. Collagen III is, therefore, also known as a "restructuring" collagen. Later it is subsequently replaced by collagen I.

One of the numerous modifications in the extracellular matrix during aging is the significant decrease in collagen synthesis. The ratio of collagen types also changes throughout life. The age-related decrease in the ratio of collagen III/ collagen I is an especially dramatic one (Figure 1)[7].

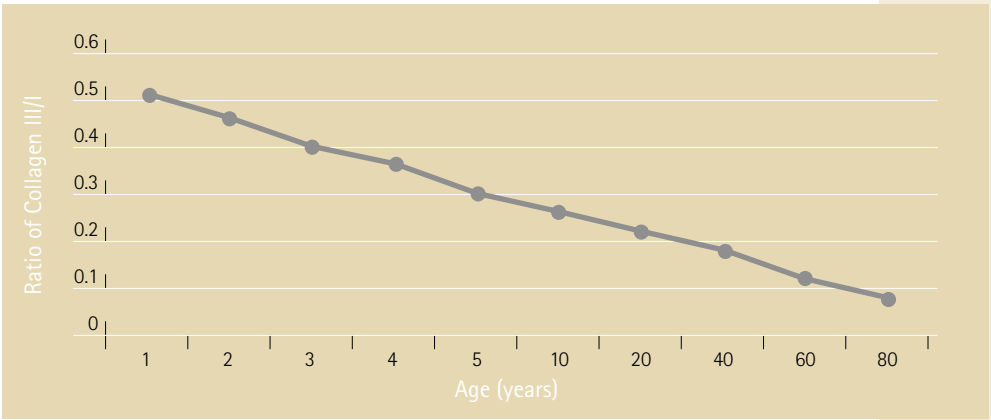


Fig. 1: Ratio of collagen III/ collagen I in human skin in relation to age

The reduction and alteration of the natural collagen support skin layers that lies just beneath the epidermis causes facial lines and wrinkles. Creams and lotions can moisturize or exfoliate the surface of the skin, but they can not diminish the deeper lines or wrinkles that are caused by the reduction and alteration of the dermis.

In summary, three major changes in skin structure involving collagen occur during the aging process:

· Decrease in collagen biosynthesis by fibroblast cells;
· Relative thinning of extracellular matrix, which becomes more pronounced with reduction in collagen III than with collagen I;
· Insolubilization of fibrous collagen leading to loss of skin's biomechanical properties.

Design of RonaCare® ASC III®

Product concept: Keratinocytes are the skin's "antenna" for the reception of external signals. Fibroblasts are the "key cells" that synthesize most of the extracellular matrix. When the skin is damaged by external stress (e.g. UV irradiation), the fibroblasts start to resynthesize the damaged structures. This requires the transduction of a biological signal from the keratinocytes to the fibroblast. These biological signals are called "cytokines". Our product involves influencing these biological signals in such a way that keratinocytes send signals to fibroblasts which cause them to produce collagen III.

We have developed a unique liposomal system that can mimic the biological system to modulate the fibroblast phenotype via keratinocytes. We call this development RonaCare® ASC III® – the Collagen III Amplifier System. When RonaCare® ASC III® is added to human keratinocytes, it appears to express mediators that in human fibroblasts specially induce the synthesis of collagen III.

Interactions keratinocytes-fibroblasts
Demonstration of epidermal mediators

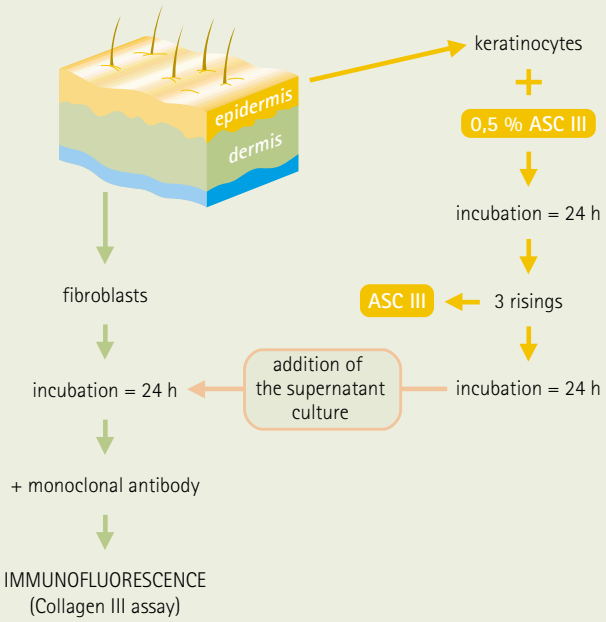


Fig. 2: Mechanism of action of RonaCare® ASC III®

Mode of action: Figure 2 schematically shows the method we have used to demonstrate the activity of RonaCare® ASC III®. This involves transduction of biological signals from keratinocytes to fibroblasts resulting in selective stimulation of collagen III expression in human skin fibroblasts.

In this method, the epidermis is separated from the dermis of a normal human skin sample. The epidermal cells (consisting mainly of keratinocytes) are then incubated in the culture media with RonaCare® ASC III®, stimulating the production of cytokines. The supernatant of the keratinocyte containing the cytokines is collected, filtered, and then added to the fibroblast cultures. If the keratinocytes were stimulated successfully, they would synthesize cytokines, concomitantly enabling the fibroblasts to produce collagen.

Our data (immunofluorescence staining using a solution of murine antiimmunoglobulin antibodies from rabbits, coupled to fluorescein) shows that RonaCare® ASC III® appears capable of increasing collagen III synthesis selectively in elderly fibroblasts to similar levels as in newborn cells, even though basal levels of collagen synthesis are age-dependent.

In Vitro Studies

Immunostaining of collagen III: The selective increase of collagen III in elderly fibroblasts was observed via selective immunostaining of collagen III[8]. Qualitative identification of the antibody/collagen III precipitant was achieved using a solution of murine anti-immunoglobulin antibodies from rabbits coupled to fluorescein. The intensity of the fluorescence is proportional to the content of collagen III in human fibroblasts.

Figure 3a shows young fibroblasts (4 years old) with a high collagen III content. Figure 3b shows lower content in older fibroblast culture (66 years old). Interestingly, when the same 66-year-old fibroblast culture was treated with RonaCare® ASC III®, an intense staining (indicating the increased collagen III concentration) was observed, similar to the intensity of 4-year-old fibroblasts, as shown in Figure 3c.

Selective immunostaining of collagen III in human fibroblasts

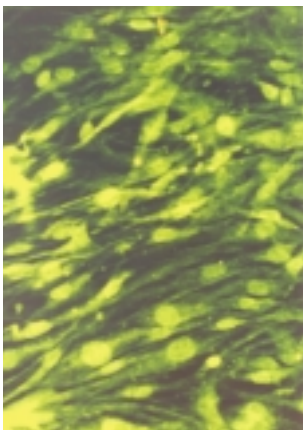


Fig. 3a: Human fibroblasts, 4 years old

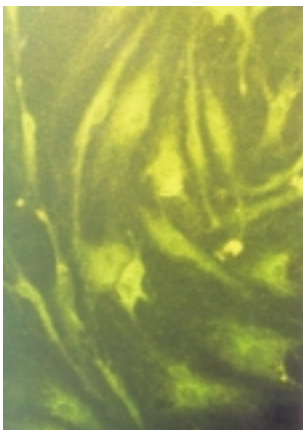


Fig. 3b: Human fibroblasts, 66 years old

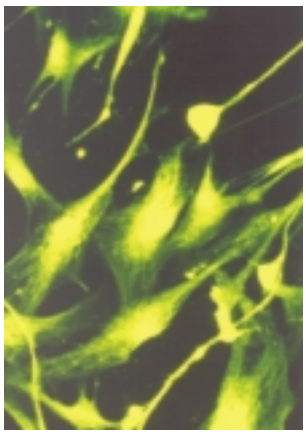


Fig. 3b: Human fibroblasts, 66 years old treated with 0.5 % RonaCare® ASC III®

Cell proliferation of fibroblasts: We studied cell proliferation in human fibroblasts[9]. Optical density correlates to the number of cells present in the human fibroblast culture. This was recorded over time (Figure 4), using RonaCare® ASC III® having different levels of dipalmitoyl hydroxyproline (DPHP) and a control (containing all the ingredients of RonaCare® ASC III® without the three-dimensional structure). The results of both the control and RonaCare® ASC III® do not show cell proliferation. We therefore conclude that the increase in collagen III biosynthesis in the dermis (after application of RonaCare® ASC III®) is due to the induction of fibroblast activity and not due to fibroblast proliferation.

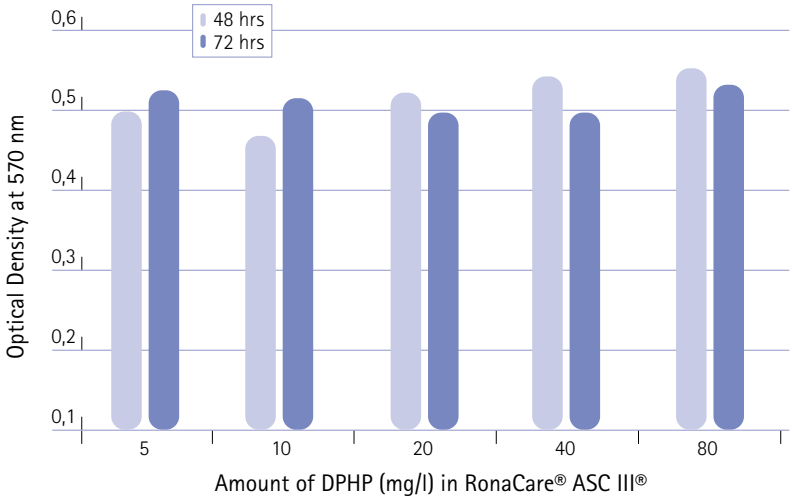


Fig. 4: Cell proliferation study in human fibroblasts

Selective amplification of collagen III: We studied selective amplification of collagen III after induction of fibroblasts[10]. Collagen I and III can be simultaneously quantified in human fibroblasts using radioimmunoassay. Figure 5 summarizes the selectivity of results obtained after 48 hours incubation using RonaCare® ASC III® (with two different levels of dipalmitoyl hydroxyproline) and a mixture of all the ingredients in RonaCare® ASC III® (reference solution). The results show a ratio of about 60/40 (collagen III/I) with RonaCare® ASC III® irrespective of dipalmitoyl hydroxyproline concentration. The total increase of collagen I and III was found to be well over 500 µg/l in a non-dose dependent manner. Meanwhile, the reference solution showed a ratio of about 27/73 (collagen III/I) with a total increase of collagen III and I well below 100 µg/l.

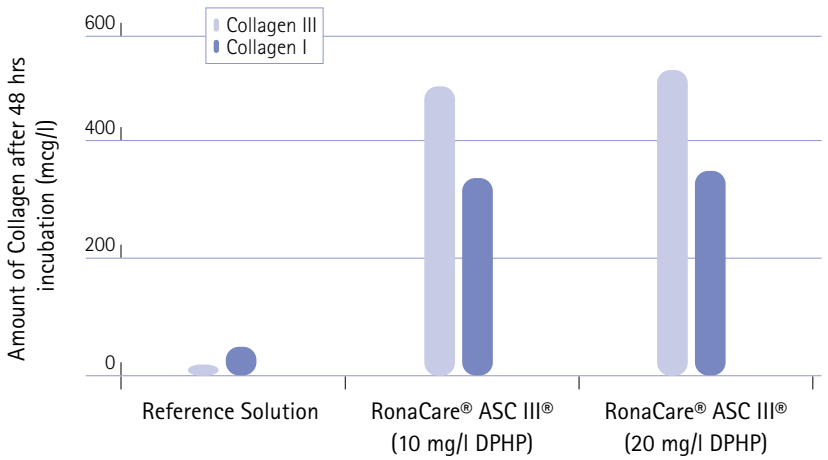


Fig. 5: Quantitative determination of collagen III and I in fibroblast culture

Comparative studies: In an in vitro comparative study, we incubated cultured human dermal fibroblasts (obtained from a 54-year-old woman, cells at 4th passage) in Dulbecco's modified eagle medium (purchased from Life Technologies Inc., Rockville, Maryland, USA) in the presence of RonaCare® ASC III® (0.2 %) or vitamin C (50 µg/ml) or retinoic acid (1 µg/ml)[10-13]. As a control, we used human fibroblast culture in Dulbecco's modified eagle medium supplemented with 10 % fetal calf serum, penicillin and streptomycin. At the start of the experiments, the number of cells was about 50,000/well.

In the cell proliferation assay (Coulter Counter method), no effect was observed compared to the control when cells were treated with RonaCare® ASC III®. On the other hand, retinoic acid caused a reduction in cell count, whereas vitamin C caused an increase in cell count. The results (based on fluorescence intensity) showed that both collagen I expression and collagen III expression are strongly increased in the presence of each of the three products (Table 1). In the case of retinoic acid and ascorbic acid, collagen I was altered to a larger extent than collagen III. However, in the case of RonaCare® ASC III®, collagen III increased more than collagen I.

Effects on fibroblast cells [a]	*RonaCare® ASC III®*	*Retinoic acid*	*Vitamin C*
Collagen I amplification [b]	75.0 %	67.0 %	82.0 %
Collagen III amplification [b]	83.0 %	42.0 %	67.0 %
Collagen I/III	1.2 %	0.7 %	0.9 %
Collagenase formation [b]	4.0 %	63.0 %	43.0 %
Net collagen III increase vs collagenase [c]	44.0 %	1.4 %	3.3 %

*Table 1: Summary of comparative studies of RonaCare® ASC III®, retinoic acid and vitamin C**

* Cell type: Human dermal fibroblasts; 54 year-old woman; incubation time = 72 hr

a Coulter Counter method

b Selective Immunostaining method

c These are calculated by normalizing the data for % amplification of collagen III and % formation of collagenase, and dividing accordingly.

Regarding collagenase synthesis, our in vitro studies showed an increase of the enzyme expression in the cultured human dermal fibroblasts with retinoic acid or vitamin C. In contrast, when cells were treated with RonaCare® ASC III®, the collagenase synthesis was not at all affected resulting in positive net production of collagen III.

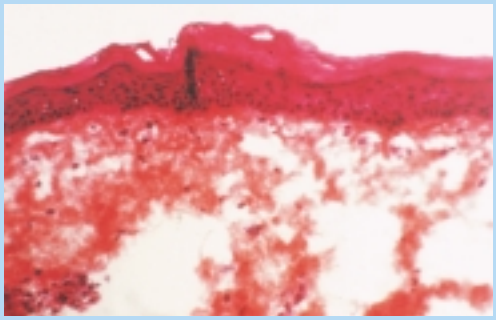
In Vivo Studies

Cutometer study of skin elasticity: This analysis quantifies the degree of elasticity in the upper layers of the human skin. In this procedure, skin is sucked into the orifice of a test-tube using constant vacuum pressure for a set time. Two optical lenses located at the test-tube orifice measure the depth to which the skin penetrates into the probe.

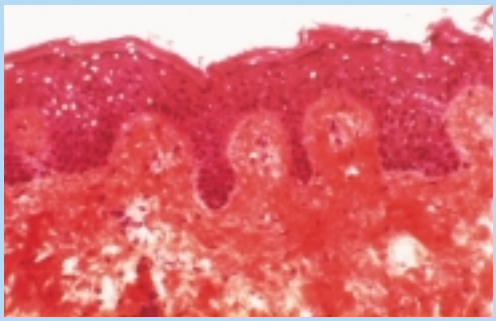
Two gels – one containing 5 % RonaCare® ASC III® and one without – were applied daily for 27 days on each half of the face (panel size 6, average age 50 years, females with fine wrinkle and dry skin). Measurements were carried out before and after the 27 days of treatment. For a gel containing RonaCare® ASC III®, we observed a 35 % increase in skin elasticity over the 27 days. The gel containing no RonaCare® ASC III® showed only a 10 % increase in skin elasticity during the treatment period.

Biopsy studies for RonaCare® ASC III® efficacy: We used human biopsy studies to evaluate the efficacy of RonaCare® ASC III® on the skin[12,13]. A study lasting five weeks was conducted. Under local anesthesia, samples of both dermis and epidermis were taken from the face of a 44-year-old male volunteer at the end of week two, three and five. Treatments occurred on a daily basis. Between two and five weeks, the left side of the volunteer's face was treated daily with a cream containing 5 % RonaCare® ASC III®, while the right side was treated with an identical cream without RonaCare® ASC III® (control). In order to avoid possible cytokine-mediated effects by RonaCare® ASC III® carried over from the treated left face side to the untreated side, the control skin was taken from the right face side before starting ASCIII-treatment.

Fig. 6: Study of human skin after biopsy:



a. before treatment



b. after three weeks of treatment with RonaCare® ASC III®

Target parameters for measurement of RonaCare® ASC III® efficacy on skin were the thickness of epidermis and relative content of pro-collagen III. Pro-collagen III is a precursor of collagen III and allows one to distinguish between newly formed and pre-existing collagen III.

Results of immunofluorescence staining of the skin show that untreated skin contained practically no pro-collagen III at day 1 (control) and showed a very weak response for the two-week control (placebo formulation without RonaCare® ASC III®). That was done with treatment of the right side with an identical cream without RonaCare® ASC III® and evaluation of facial skin after one day and two-week intervals. In contrast, the application of RonaCare® ASC III® resulted in very significant increase in pro-collagen III content. This increase of pro-collagen III content was accompanied by an equally pronounced increase in thickness of epidermis, and improvement in dermal/epidermal junction area with reformation of collagen fibers in the dermis (Figure 6).

Formulation Guidelines

Fig. 7: Scanning electron microscopy of RonaCare® ASC III®

- RonaCare® ASC III® can easily be incorporated into lotions, creams and gels.
- Use level about 2 – 6 %
- Incompatibilities: Ionic emulsifiers, surfactants, short chain ethoxylates, bivalent cations, fruit acids and high alcohol concentrations (> 15 %) disrupt the liposome structure over time.
- pH requirements: slightly acidic to neutral, preferred pH 5.5 – 6.5
- RonaCare® ASC III® should be added to emulsions after cooling down to 35 °C. Homogenization is possible but should be kept to a minimum.
- The preferred storage temperature of RonaCare® ASC III® is at 5 – 15 °C.
- In formulated products, RonaCare® ASC III® can be stable over a broad temperature range from 40 °C to –10 °C.

Technical Data

Product description

The RonaCare® ASC III® liposome is a suspension of phospholipidic vesicular carriers, in which the external lipophilic wall contains the amphiphilic dipalmitoyl hydroxyproline. The unique liposome is shaped like a hexagonal pyramid. Its membrane always shows angled structures (Figure 7).

INCI Name

Water, Lecithin, Dipalmitoyl Hydroxyproline, Phenoxethanol, Tall Oil Sterol, Linoleic Acid, Tocopherol, Sodium Ascorbate, Mannitol, Methylparaben, Ethylparaben, Propylparaben, Butylparaben

Stability

RonaCare® ASC III® is stable at least for 18 months, if unopened and stored at +5 to 30 °C. The three-dimensional structure is lost on prolonged heating at temperatures above 35 °C or storage below freezing.

Conclusion

RonaCare® ASC III® is an effective amplifier of collagen III as it selectively increases collagen III production by enhancing the fibroblast activity via modulating cytokine production. The significant increase in pro-collagen III accompanied by a pronounced increase of the epidermal layer thickness results in an improvement in dermal/epidermal junction area and reformation of collagen fibers in the dermis. Skin care products containing RonaCare® ASC III® can, therefore, repair the natural collagen support layer that lies just beneath the skin, resulting in increased skin elasticity and smoothness and reduction of facial lines and wrinkles.

Ordering Information

Item No.	*Name*	*Pack Sizes*
110154	RonaCare® ASC III®	1 kg

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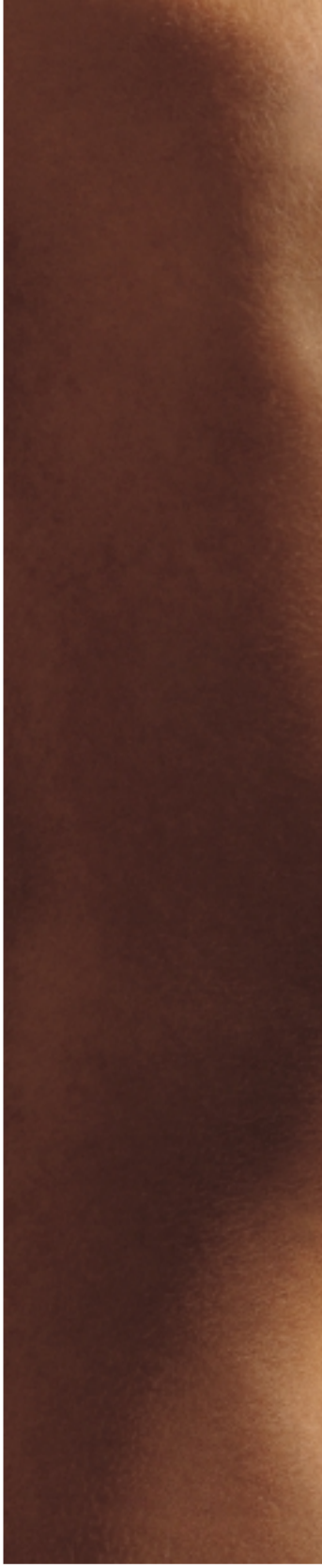

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