

NEODERMYL®

THE “NEEDLE-FREE” COLLAGEN AND ELASTIN FILLER



NEODERMYL[®]

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1. Introduction

Neodermyl[®] is an anti-aging ingredient developed by Induchem Companies which acts like a wrinkles' filler by enhancing skin firmness and elasticity without needle injection. Neodermyl[®] is an energy source for aged cells (see section 4 for Neodermyl[®]'s mode of action).

The present technical report contains the assessment results of Neodermyl[®] *in vitro*, *ex vivo* and *in vivo* (clinical investigation on human volunteers).

Neodermyl[®] treatment permits to achieve in only 15 days the same results than a collagen injection procedure performed by an aesthetic surgeon.

2. Skin biology

2.1. Skin description

The skin contains many specialized cells and structures. The skin is a protective barrier that interfaces with a sometimes-hostile environment. It is also involved in maintaining the proper temperature for the body to function well. It gathers sensory information from the environment, and plays an active role in the immune system protecting people from disease. The skin contains three layers: the epidermis, dermis, and subcutaneous tissue.

2.1.1. Epidermis

The epidermis is the outer layer of skin. The epidermis contains several layers. From bottom to top the layers are: stratum germinatum, stratum spinosum, stratum granulosum, and stratum corneum.

2.1.2. Dermis

The dermis varies in thickness depending on the location of the skin. The two layers of the dermis are the papillary and reticular layers. The papillary dermis is the upper layer containing a thin arrangement of collagen fibers. The reticular dermis is the lower layer. This layer is thicker and made of thick collagen fibers that are arranged parallel to the surface of the skin. The dermis contains many specialized cells and structures. The hair follicles, the sebaceous glands and apocrine glands are associated with the follicle. This layer also contains eccrine (sweat) glands, but they are not associated with hair follicles. Blood vessels and nerves course through this layer. The nerves transmit sensations of pain, itch, and temperature. There are also specialized nerve cells called Meissner's and Vater-Pacini corpuscles that transmit the sensations of touch and pressure. The dermis is composed of different types of tissue that are present throughout, principally collagenous and elastic tissues.

2.1.2.1. Collagen description (Ramshaw and al.)

Collagen is present in almost all organs in the body, especially skin, ligaments, cartilage and bone. Collagen provides strength and structural integrity to these tissues.

Fibroblasts are the cells producing collagen. Usually collagen consists of three chains that are synthesized within the cell and assembled into the characteristic triple helix in the endoplasmic reticulum before secretion. Collagen is a triple helical molecule (1.5 x 300 nm) with a molecular mass of about 330kDa. Collagen consists of typical amino acid sequence repeats Gly-X-Y (where X is frequently Proline and Y is 4-Hydroxyproline). The collagen is a

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superfamily of at least 29 genetically distinct types of collagen known dividing in 6 groups. In dermis the collagens (Collagen type I, type III and type V) are from fibrillar family. Collagen molecules are grouped into fibrils in a head to tail alignment, and are covalently cross-linked to each other (fig 1). Main components of collagen are the amino acids glycine (about 300), proline (about 100) and lysine (about 20) which both are post-translationally modified to hydroxyproline and hydroxylysine, the latter can even be modified by glycosylation (galactose or galactose and glucose). The final collagen fibril is produced by crosslinking tropocollagen via lysyl oxidase which produces aldehyde groups onto lysine and hydroxylysine (desmosine and isodesmosine) which are later on cross-linked to tighten the interaction between the helices. Lysyl-oxidase is a copper-dependent enzyme. The polymer of tropocollagen is called collagen microfibril.

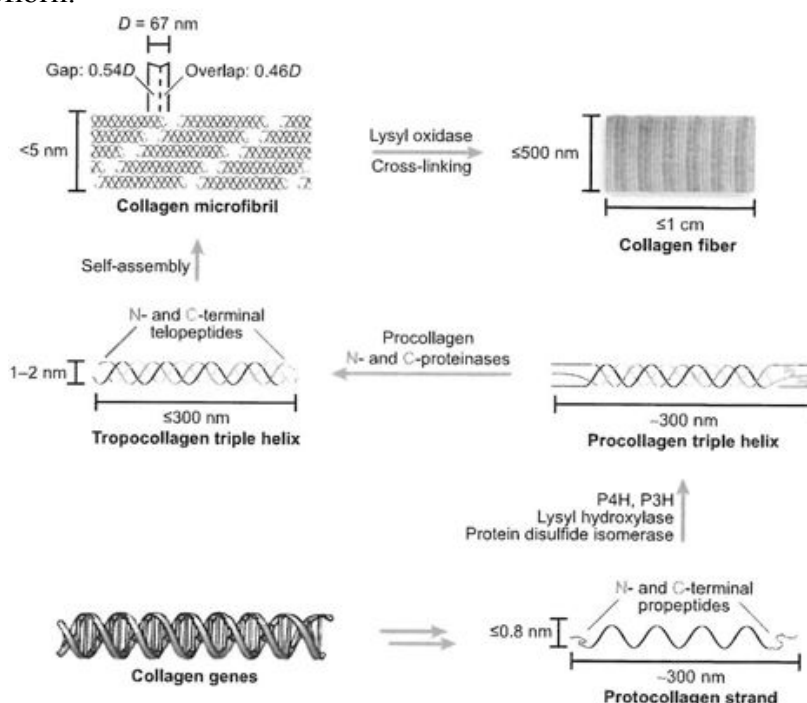


Fig 1: Biosynthetic route to collagen fibers (according Shoulders *and al*)

2.1.2.2. Elastin description

Elastin provides elasticity to organs, especially skin, lung, arteries and ligaments. Elastin can stretch to several times its normal length. In skin, most of the elastin is located in the dermis. Elastin is present in all vertebrates. The high content of hydrophobic amino acids and intermolecular crosslinks (desmosine and isodesmosine) makes elastin one of the most chemically resistant proteins in the body. Its precursor, tropoelastin, is composed of a 72 kDa single polypeptide chain synthesized by fibroblasts. Individual molecules are secreted into the extracellular space, in association with microfibrillar components and cross-linked to each other to form elastic fibers by lysyl-oxidase (a copper dependent enzyme). The expression of the tropoelastin gene in human mainly occurs before birth and in the first years of life. After finishing development the elastin expression is turned down substantially and only a small amount of elastin is produced.

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2.1.3. Subcutaneous tissue

The subcutaneous tissue is a layer of fat, cells and connective tissue that houses larger blood vessels and nerves. This layer is important in the regulation of temperature of the skin itself and the body.

2.2. Skin aging description

Skin aging occurs by two processes. The first one is an inherent process caused by the genes called *intrinsic aging* and the second one is known as *extrinsic aging* and is caused by environmental factors, such as exposure to the sun's rays, smoking...

The macroscopic signs of skin aging are: wrinkles, thin and transparent skin, noticeable loss of firmness and elasticity on the face, hands and neck, sagging skin and dry skin.

Some of macroscopic signs are directly related to microscopic signs of aging as the reduction of cells metabolism/viability (fibroblasts become senescent), the decrease of synthesis and/or degradation of dermis proteins as collagen type I, type III, tropoelastin and elastin. It's the case of wrinkles apparition, thin and transparent skin and loss of skin firmness/ elasticity. Neodermyl® was developed to counteract the effect of skin aging process.

3. Neodermyl®'s Description

Identification:	INCI name	CAS No.
	Glycerin	56-81-5
	Methylglucoside phosphate	15416-98-5/60745-51-9
	Copper Lysinate/Proline	1613132-70-9
Appearance:	Blue liquid	
Solubility:	Soluble in water	
Safety assessment:	Ocular irritation: Human cornea model test; Non-irritant at 100%	
	Skin irritation: Occlusive patch test; Non-irritant at 100%	
	Mutagenicity: Ames assay; Non mutagenic	
	Phototoxicity: 3T3 NRU Phototoxicity; Non-Phototoxic at 5%.	
	Sensitization: HRIPT assay; Non-sensitizing at 100%	
Dosage:	0.5-2 %	
Storage:	Recommended storage temperature: 4-7°C	
	Do not store at temperatures over: 10°C	
	Keep contents under Protective gas.	
Shelf life	2 years	

4. Neodermyl®'s mode of Action

Neodermyl® is an anti-aging active product that induces in the dermis the synthesis of specific key proteins involved in the maintenance of the skin structure by fibroblasts (collagen type I, collagen type III, tropoelastin and elastin). In aged skin, the dermis fibroblasts become senescent, and show therefore a reduced capacity to synthesize these proteins (collagen type I, collagen type III, tropoelastin and elastin). The senescent fibroblasts present a low metabolism and a lack of energy (reduced mitochondrial activity due to e.g. DNA damage, or reduced transport of nutrients). Neodermyl® stimulates these proteins synthesis by providing to cells an energy molecule the Methylglucoside Phosphate (MGP). Neodermyl® provides also specific

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essential amino acids (lysine and proline) and copper needed for collagen and elastin synthesis. These proteins are composed by proline and lysine amino acids. The copper is a cofactor essential for collagen and elastin synthesis as the crosslinking of each protein implies a copper dependent enzyme: the lysyl-oxidase.

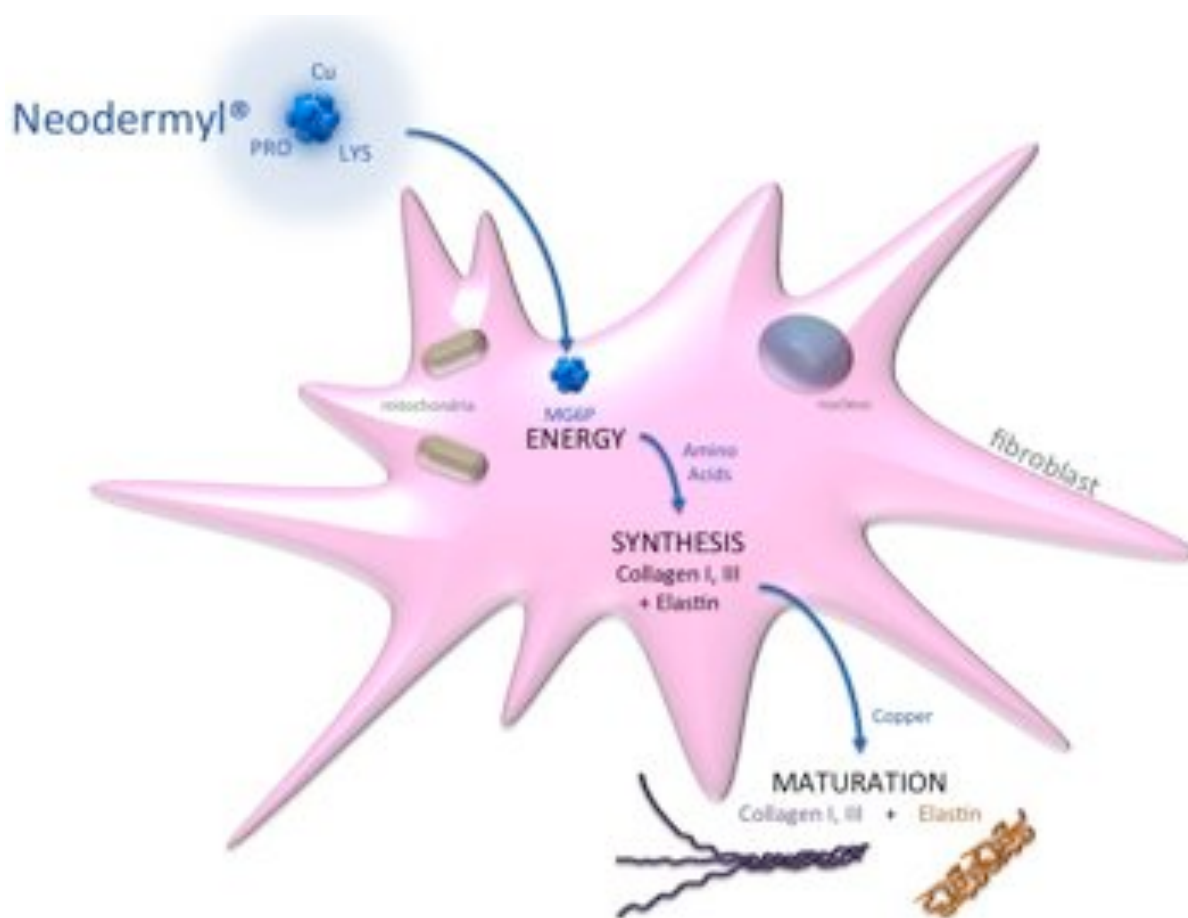


Fig 2: Neodermyl[®]'s mode of action

5. *In vitro* assessment of Neodermyl[®]

5.1. Introduction

The aims of preliminary *in vitro* assessments of Neodermyl[®] were to evaluate its effect on cell viability and its potential biological effect on procollagen synthesis.

To demonstrate these two parameters, *in vitro* assessments were performed on young and aged dermal fibroblasts.

5.2. Materials and methods

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5.2.1. Viability assessment

The MTT test has been used to evaluate the cell viability. The test has been performed on human dermal cells: young fibroblasts (P7-NHDF) and on aged fibroblasts (P17-F). Morphological observations by light microscope were also performed. The aged fibroblasts were obtained by passaging young fibroblasts P7-NHDF several times (17th) thus increasing DNA damages and causing their reduced synthesis capability. To perform the MTT test the cells were incubated for 72 hours in a culture medium “L” consisting of a low Glucose concentration of 1g/l with increasing amounts of Neodermyl[®] at 0.08, 0.4 and 2%. After treatment, cells were incubated with MTT reduced in blue formazan crystals by succinate dehydrogenase (a mitochondrial enzyme). After cell dissociation and formazan crystal solubilization using DMSO, the optical density (OD) of the extracts at 540 nm, proportional to the number of living cells and their metabolic activity, was recorded with a microplate reader (VERSAmax, Molecular Devices). Morphological observations of cells were performed under a microscope. The results of cell viability (or cell proliferation) have been expressed as a percentage of mean improvement viability in comparison to the untreated group results.

5.2.2. Biological effect assessment on procollagen-I synthesis

The biological effect of Neodermyl[®] on procollagen-I synthesis was performed by quantitative Elisa test. Young fibroblasts (P7-NHDF) and aged fibroblasts (P17-NHDF) were seeded in 96-well plates incubated for 24 hours in culture medium. The medium was then removed and replaced by assay medium “N” (N stands for normal amount of Glucose, 4.5g/l) containing or not (control) Neodermyl[®] at concentrations 0.1, 0.5 and 2%, respectively, or the mix of references 10ng/ml of TGF-beta and 20µg/ml of Vitamin C (for positive test validation). The cells were then incubated for 72 hours. All experimental conditions were performed in n=3. After incubation, the supernatants were collected for the quantification of pro-collagen I synthesis, using the procollagen I ELISA kit from Takara (ref. MK101). The results of procollagen I synthesis have been expressed as a percentage of mean improvement synthesis in comparison to the untreated group results.

5.3. Results and discussion on *in vitro* experiments

5.3.1. Viability assessment

The MTT test has demonstrated the non-cytotoxicity of Neodermyl[®] on both cells types (young and aged fibroblasts) and no cell death were observed by morphological observations until 2% Neodermyl[®] treatment. The MTT test has shown that Neodermyl[®] stimulates viability (proliferation) of both cell types. The cells viability increased with Neodermyl[®] concentration up to 2% in comparison to untreated group (fig 1). The improvement of cell viability was respectively 25, 41 and 61% at 0.08, 0.4 and 2% of Neodermyl[®] on young fibroblasts. The improvement of cell viability was respectively 17, 20 and 36% at 0.08, 0.4 and 2% of Neodermyl[®] on aged fibroblasts. The cell viability was more pronounced in young cells as these cells have a more efficient metabolism than aged cells.

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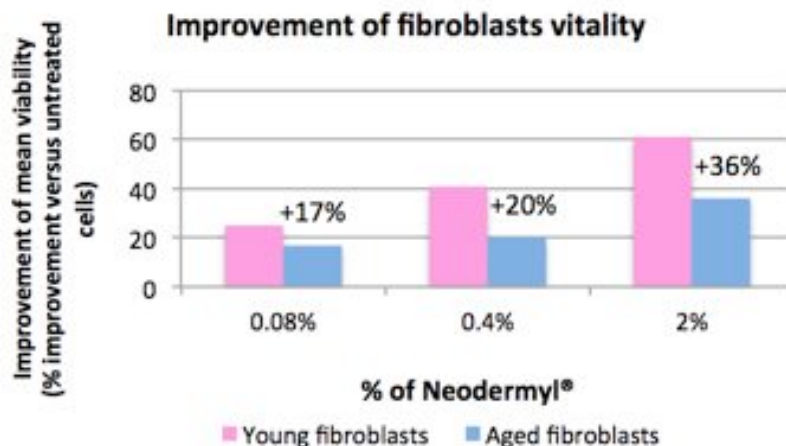


Fig 3: Mean improvement of cell viability on young and aged fibroblasts under Neodermyl® treatment (0.08, 0.4 and 2%) in comparison to control group (MTT test).

The MTT test has demonstrated:

- The non-cytotoxicity effect on both cell types (young and aged fibroblasts) until 2% of Neodermyl®.
- The Neodermyl® efficient effect on the viability of aged fibroblast known to be very difficult to stimulate.

5.3.2. Biological effect assessment on procollagen-I synthesis

Procollagen-I synthesis was observed after 3 days. Neodermyl®, at the highest tested concentration (2%), showed a statistically significant stimulating effect on procollagen-I synthesis on both cell types (young and aged). However, its stimulating effect was stronger in aged fibroblasts than in young fibroblasts (47% and 16% of stimulation, respectively). This may be explained by the fact that aged fibroblast use Neodermyl® as a source of energy and nutriment to reactivate their collagen synthesis whereas young fibroblasts use more Neodermyl® for their cellular division.

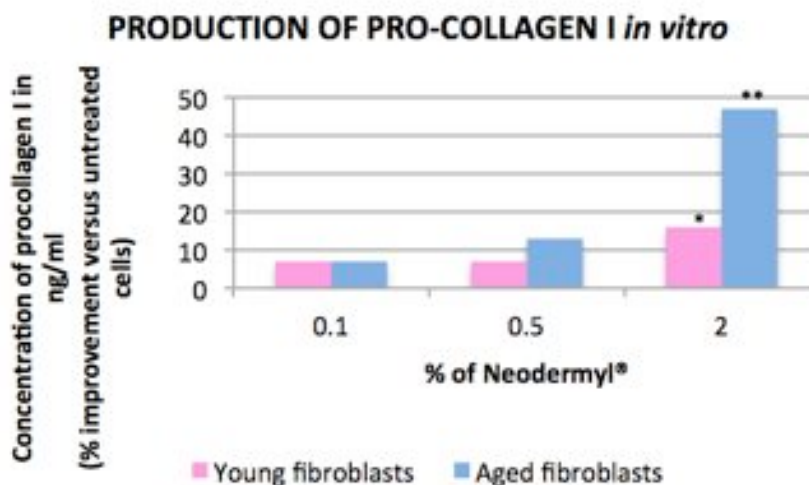


Fig 4: Procollagen I synthesis by young and aged fibroblasts after Neodermyl® treatment at 0.1, 0.5 and 2% (Elisa test)

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-Neodermyl[®], clearly showed a stimulating effect (statistically significant, $p < 0.001$, Student t-test) on Procollagen-I synthesis after 3 days up to +47% by aged fibroblasts (for Neodermyl[®] 2%).

5.4. Conclusions on *in vitro* experiments

The MTT test has demonstrated:

- The non-cytotoxicity effect on both cell types (young and aged fibroblasts) until 2% of Neodermyl[®]
- The Neodermyl[®] efficient effect on the viability of aged fibroblast known to be very difficult to stimulate.

The Elisa test has demonstrated:

A stimulating effect on Procollagen-I synthesis on aged fibroblasts up to +47%.

($p < 0.001$, extremely significant, Student t-test).

6. *Ex vivo* assessment of Neodermyl[®]

6.1. Introduction

Based on promising preliminary *in vitro* results obtained with Neodermyl[®] (increasing viability and Procollagen I synthesis in aged fibroblasts treated by Neodermyl[®]), an *ex vivo* study on aged human skin living explants was performed.

The aim of this *ex vivo* study was to explore the biochemical anti-aging activities of Neodermyl[®] at 0.5 % on the human skin explants maintained in survival. The biochemical parameters studied were related to the synthesis of major dermis proteins impacted by the aging process the collagen type I, collagen type III, tropoelastin and elastin. The general morphology of the skin treated with Neodermyl[®] at each concentration was also observed.

6.2. Materials and methods

6.2.1. Aged skin explants

Aged skin explants (74-year-old Caucasian man) were obtained from an abdomino-plasty. Each explant had an average diameter of 11mm. The explants were kept in a survival BEM culture medium (Bio-EC's Explant Medium) at 37°C in a humid, 5%-CO₂ atmosphere.

6.2.2. Neodermyl[®] treatments of skin explants

Explants were either left untreated for 0, 6 or 11 days ($n = 9$), respectively, or treated for 6 or 11 days with 0.5% Neodermyl[®] ($n=6$). Neodermyl[®] (without any formulation ingredients except water as a solvent) was applied on days 0, 1, 4, 6, and 8 in the mentioned concentrations topically on the basis of 2 mg per cm², using a small spatula. Half of the culture medium (1 ml) was renewed at day 1, 4, 6, and 8, respectively. The control explants did not receive any treatment.

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6.2.3. Histological analysis

6.2.3.1. Preparation of explants for histological analysis

After treatment, explants were cut in three parts, one was fixed in buffered formalin, one was fixed in ordinary Bouin and one was frozen at -80°C. After the fixation for 24 hours in formalin, or 48 hours in Bouin, the samples were dehydrated and impregnated in paraffin. The samples were then embedded. 5 µm thick sections were made using a Leica microtome, and the sections were mounted on Superfrost[®] glass slides. The frozen samples were cut into 7µm thick sections using a Leica CM cryostat. Sections were mounted on Superfrost[®] plus silanized glass slides. The microscopical observations were realized using a Leica DMLB or Orthoplan microscope. Pictures were digitized with a numeric DP72 Olympus camera with CellD storing.

6.2.3.2. General morphology

The observation of the general morphology was performed after staining of Bouin fixed paraffinized sections according to Masson's Trichrome Goldner variant.

6.2.3.3. Total collagen content (Picro-Sirius staining)

The staining of the total collagen was performed by using Picro-Sirius staining. The staining was assessed by microscopic observation. Images were analyzed using the Olympus Cell software were performed to quantify the total collagen content.

6.2.3.4. Collagen type I immunostaining

The immunostaining of collagen type I was performed on frozen sections with a rabbit polyclonal anti-collagen I antibody (Monosan, ref. PS047); diluted at 1/200, for 1 hour at room temperature, and revealed using AlexaFluor AF488 (Lifetechnologies, A11008). The nuclei were post-stained with propidium iodide. The immunostaining was performed using an automated horizontal slide-processing system (Dako, AutostainerPlus). The immunostaining was assessed by microscopic observation and image analysis using the Olympus CellD storing software.

6.2.3.5. Collagen type III immunostaining

The immunostaining of collagen type III was performed on frozen sections with a goat polyclonal anti-collagen III antibody (SBA, ref. 1330-01); diluted at 1/100, for 2 hours at room temperature; the staining was enhanced with a streptavidin/biotin system and revealed using VIP (Vector, SK-4600). The nuclei were post-stained with Masson's hemalun. The immunostaining was performed using an automated horizontal slide-processing system (Dako, AutostainerPlus). The immunostaining was assessed by microscopical observation and image analysis using the Olympus CellD storing software.

6.2.3.6. Tropoelastin Immunostaining

The immunostaining of tropoelastin was performed on frozen sections with a mouse anti-tropoelastin antibody (Chemicon, ref. MAB2503), diluted at 1/25, overnight at 4°C; the staining was enhanced with a streptavidin/biotin system and revealed using FITC (Invitrogen, SA 1001). The nuclei were post-stained with propidium iodide. The immunostaining was assessed by microscopical observation and image analysis using the Olympus CellD storing software.

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6.2.3.7. Elastin Immunostaining

The immunostaining of elastin was performed on frozen sections with a rabbit polyclonal anti-elastin antibody (Novotec, ref. 25011); diluted 1/800, for 2 hours at room temperature, the staining was enhanced with a streptavidin/biotin system and revealed using FITC (Invitrogen, SA 1001). The nuclei were post-stained with propidium iodide. The immunostaining was performed using an automated horizontal slide-processing system (Dako, AutostainerPlus). The immunostaining was assessed by microscopical observation and image analysis using the Olympus CellD storing software.

6.3. Results and discussion on *ex vivo* assessments

6.3.1. General morphology and total collagen content

6.3.1.1. General morphology

The general morphology study demonstrate that Neodermyl[®] at 0.5% induced from D6 an epidermal stimulation (5 to 6 cellular layers/3 to 4), without modification in the papillary dermis (fig 5).

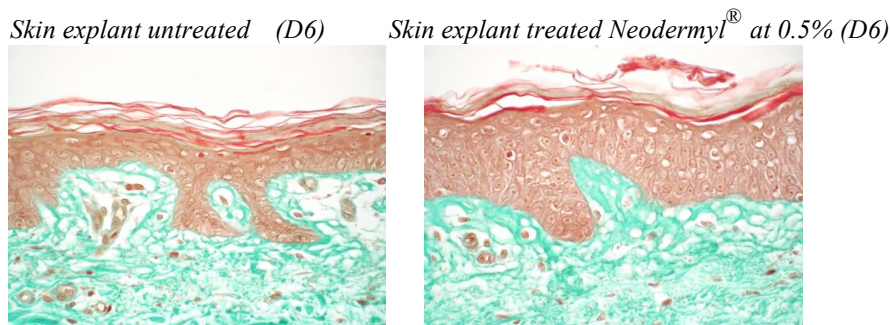


Fig 5 : General morphology at D6 D11 post Neodermyl[®] at 0.5% treatment on human skin explants (Tissue coloration, Masson's Trichrome Goldner variant)

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6.3.1.2. Total collagen content (image analyses from Picro-Sirius staining)

By histological observations it has been shown that compared to untreated explant at D6 and D11, Neodermyl[®] at 0.5% induced an increase of total collagen expression (slight to moderate, from D0 to D11, see fig 6). By image analysis, it has been shown that compared to untreated explant at D11 an increase of 6.8% is seen with Neodermyl[®] at 0.5% (fig 7).

The mean collagen content usually observed after short culture times is on average from 2 to 10%, thus confirming the strong efficacy of Neodermyl[®].

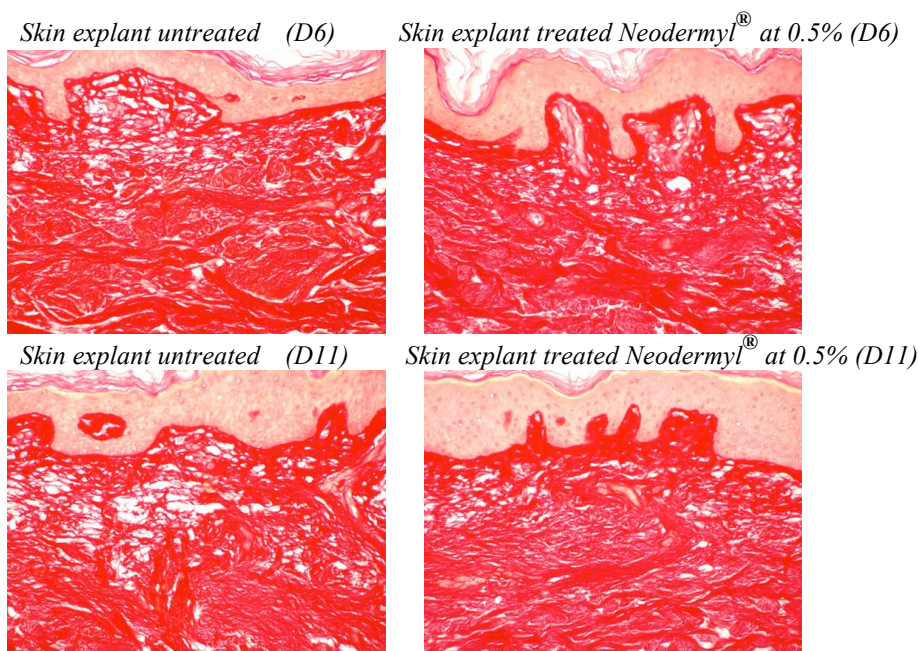


Fig 6 : Total collagen content at D6 and D11 post Neodermyl[®] at 0.5% treatment on human skin explants (Tissue coloration, Picro-Sirius staining)

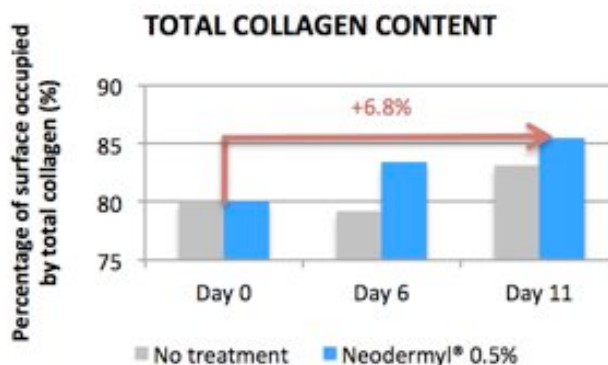


Fig 7: Total collagen content in human skin explants with or without Neodermyl[®] treatment at 0.5% at D0, D6 and D11 (Image analysis)

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6.3.2. Collagen type I synthesis

Histological observations and image analysis on collagen type I synthesis

By histological observations it has been shown that compared to untreated explant at D6 and D11, Neodermyl[®] 0.5% induced a clear increase of collagen I synthesis (see fig 8).

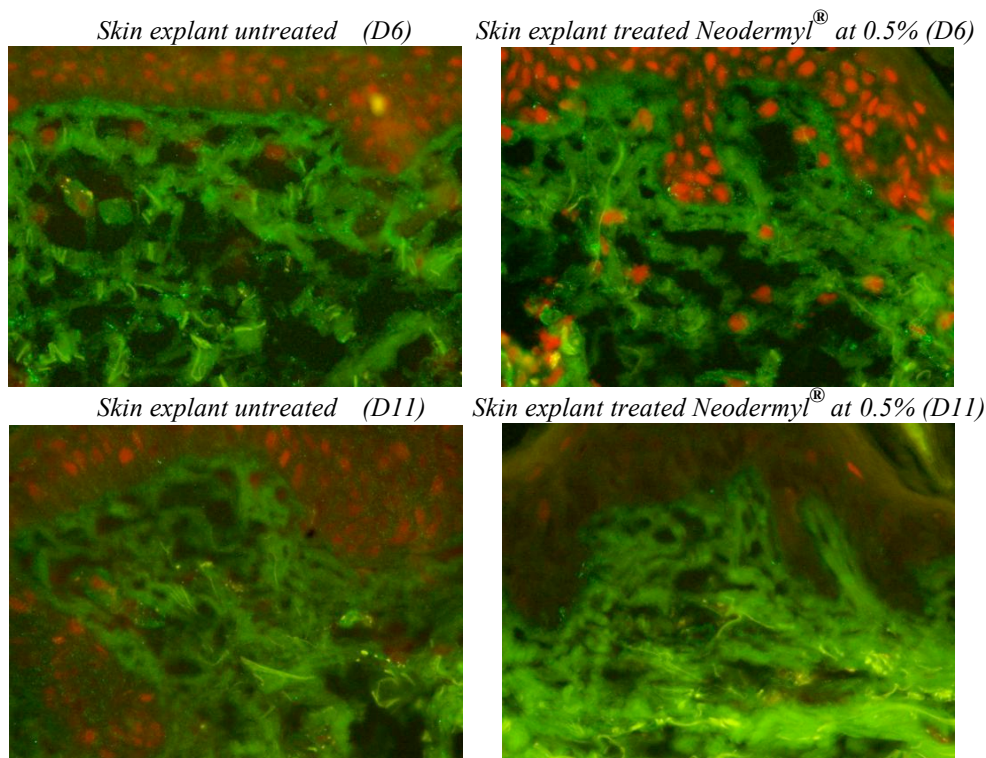


Fig 8: Collagen type I visualization in human skin explants with or without Neodermyl[®] treatment 0.5%) at D6 and D11 (immunostaining - green fluorescence)

By image analysis, it has been shown that compared to untreated explant at D11, Neodermyl[®] at 0.5% induced an increase statistically significant (Student t-test; *p < 0.01) of collagen type I by 179% (see fig 9).

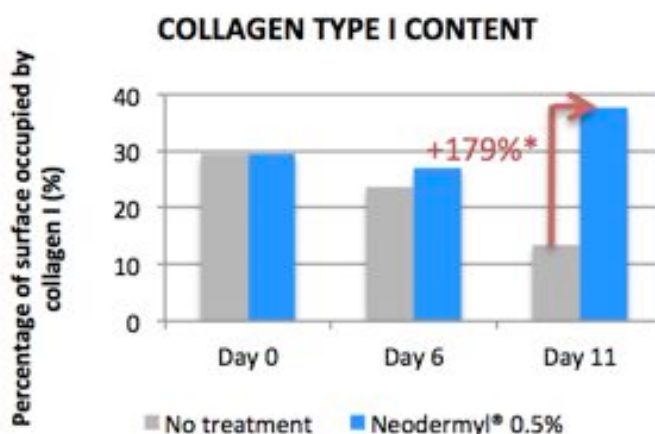


Fig 9: Collagen Type I content in human skin explants with or without Neodermyl[®] treatment 0.5% at D0, D6 and D11 (Image analysis)

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6.3.3. Collagen type III synthesis

Histological observations and image analysis on collagen type III synthesis

By histological observations, it has been shown that compared to untreated explant Neodermyl® at 0.5% induced at D6 a clear increase and at D11 a very clear increase of collagen type III synthesis (see fig 10).

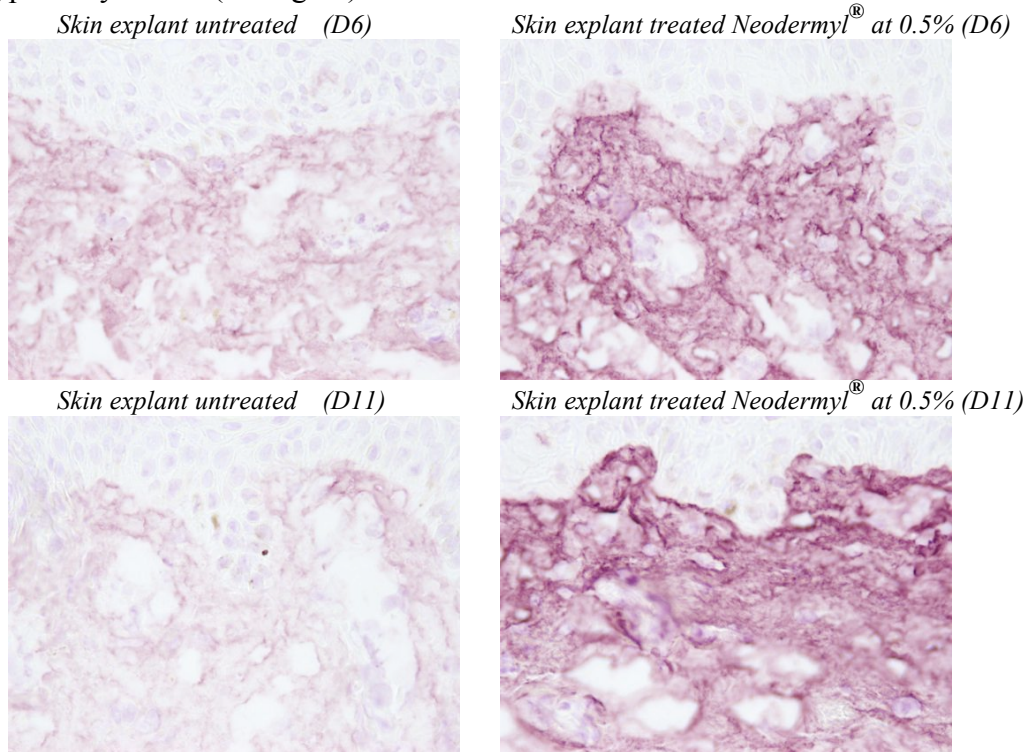


Fig 10: Collagen type III visualization in human skin explants with or without Neodermyl® treatment (0.5%) at D6 and D11 (Immunostaining- pink staining)

By image analysis, it has been shown that compared to untreated explant at D11, Neodermyl® at 0.5% induced an increase statistically significant (Student t-test; *p <0.01) of collagen type III synthesis about 194% (see fig 11).

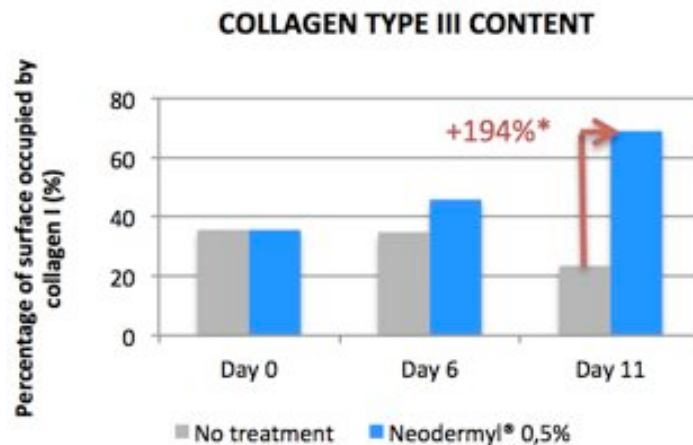


Fig 11: Collagen type III content in human skin explants with or without Neodermyl® treatment (0.5%) at D0, D6 and D11 (Image analysis).

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6.3.4. Synthesis ratio of collagen type I/ collagen type III

The synthesis ratio of collagen type I/collagen type III is a major parameter to evaluate in the skin aging process. This ratio gives information on the extracellular matrix renewal or repair. In skin repair mechanism, the collagen type III is firstly and predominantly synthesized in the dermis. In a young skin the ratio collagen type I/collagen type III is inferior to that observed in an aged skin.

The synthesis ratio collagen type I/collagen type III is calculated from image analysis data (see section 5.3.2 and 5.3.3) (fig 12).

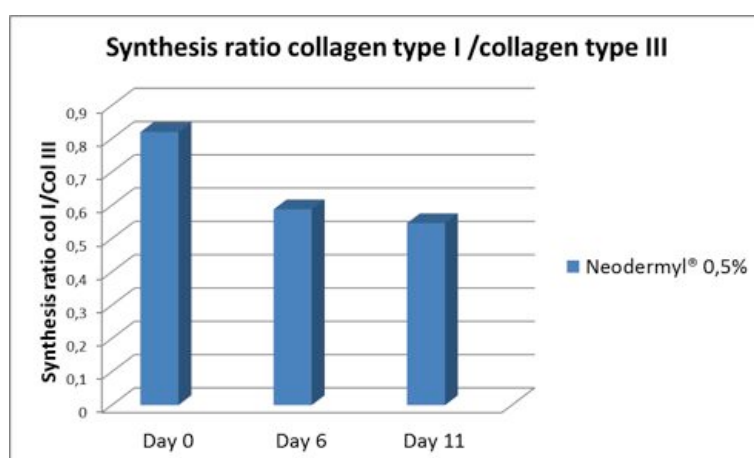


Fig 12: Ratio of collagen type I/collagen type III synthesis in human skin explants non treated with Neodermyl® at D0, and after 6 or 11 days of treatment with Neodermyl® at 0,5% (calculated from Image analysis data).

The aged human explants treated by Neodermyl® at 0.5 %, showed younger skin properties (low synthesis ratio collagen type I/collagen type III).

6.3.5. Conclusions on collagen synthesis after Neodermyl® treatment

Histological observation (Picro-sirius staining, collagen type I and collagen type III immunostaining) have shown:

Compared to untreated explant Neodermyl® 0.5% induced:

- at D6 and D11 a slight to moderate increase of total collagen expression
- at D6 and D11 a clear increase of collagen I synthesis
- at D6 a clear increase and at D11 a very clear increase of collagen type III synthesis

Image analyses have shown:

Compared to untreated explant Neodermyl® 0.5% induced:

- at D11 an increase of +6.8% of total collagen
- at D11 a statistically significant increase (Student t-test; $p < 0.01$) of collagen type I synthesis (+179%).
- at D11 a statistically significant increase (Student t-test; $p < 0.01$) of collagen type III synthesis (+194%)

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The aged human explants treated by Neodermyl® at 0.5 %, showed a reactivated collagen type I and type III synthesis and a younger skin properties (low synthesis ratio collagen type I/collagen type III)

6.3.6. Synthesis of proteins involved in elastic tissue (tropoelastin and elastin)

6.3.6.1. Tropoelastin synthesis

By histological observations, it has been shown that compared to untreated explant Neodermyl® at 0.5% induced at D11 a slight increase of the expression of tropoelastin on all the elastic fibers (see fig 13). The newly synthesized tropoelastin presents the typically candelabra structure of elastic fibers, showing an adequate ongoing maturation process.

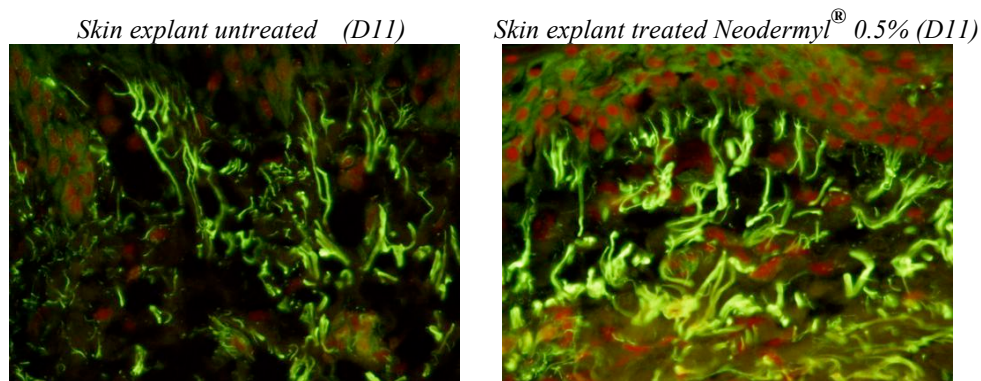


Fig 13: Tropoelastin visualization in human skin explants with or without Neodermyl® treatment (0.5%) at D11 (Immunostaining-green fluorescence)

By image analysis, it has been shown that compared to untreated explant at D11, Neodermyl® at 0.5% induced an increase statistically significant (Student t-test; *p <0.1) of +57% of tropoelastin synthesis (see fig 14).

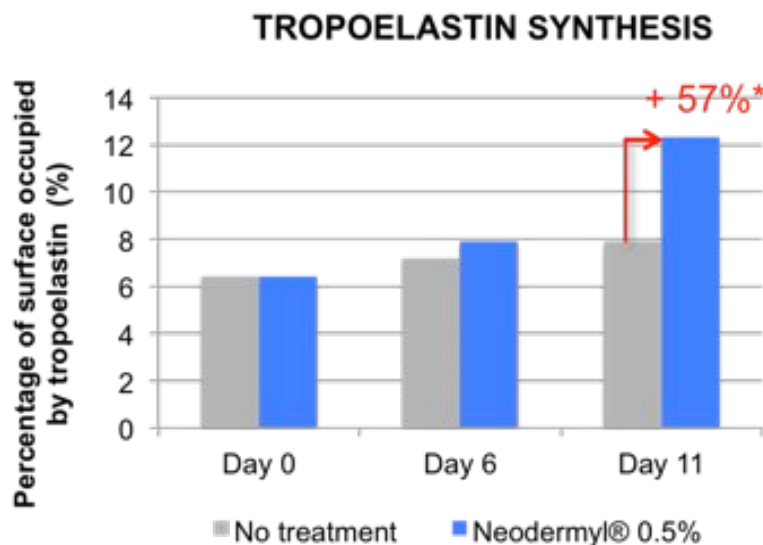


Fig 14: Tropoelastin synthesis in human skin explants with or without Neodermyl® treatment (0.5%) at D0 and D11 (Image analysis).

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6.3.6.2. Elastin synthesis

By histological observations, it has been shown that compared to untreated explant Neodermyl[®] at 0.5% induced at D11 a very clear increase of elastin expression on all the elastic fibers (see fig 15). The newly synthesized elastin presents the typically candelabra structure of elastic fibers, showing its proper maturation.

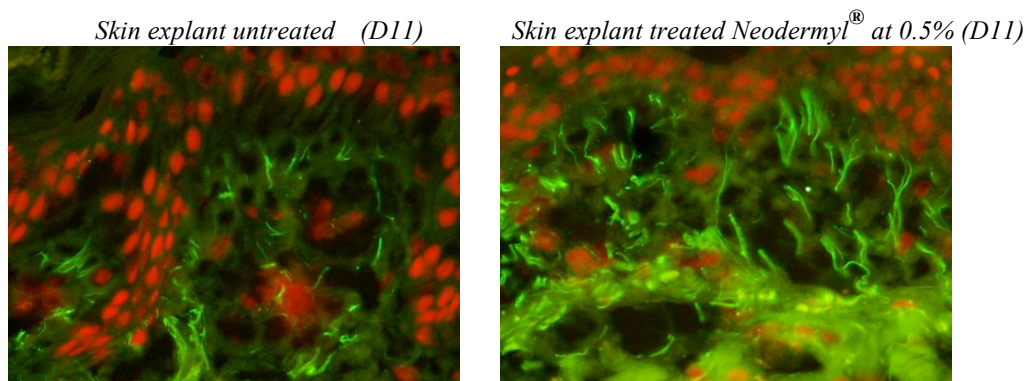


Fig 15: Elastin visualization in human skin explants with or without Neodermyl[®] treatment (0.5%) at D11 (Immunostaining-green fluorescence)

By image analysis, it has been shown that compared to untreated explant at D11, Neodermyl[®] at 0.5% induced an increase statistically significant (Student t-test; **p < 0.05) of +190% of elastin synthesis (see fig 16).

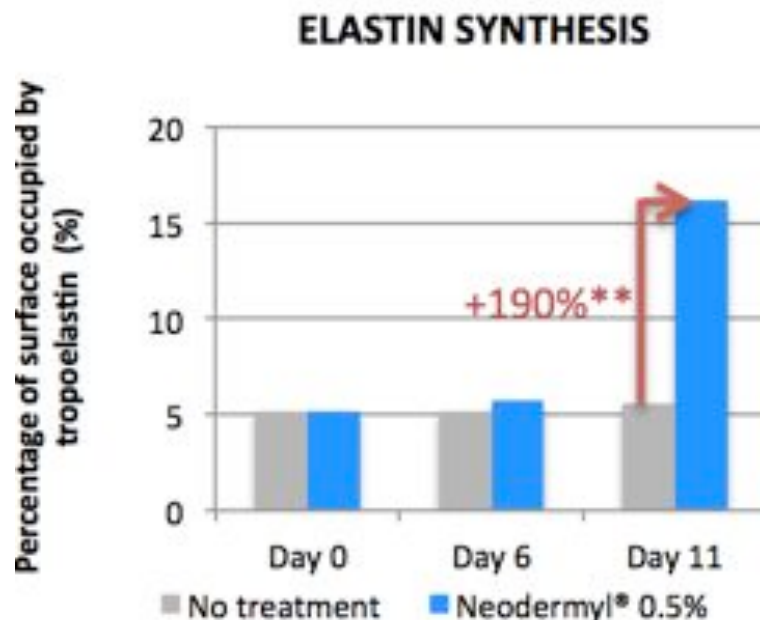


Fig 16: Elastin synthesis in human skin explants with or without Neodermyl[®] treatment (0.5%) at D0 and D11 (Image analysis).

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6.3.6.3. Conclusions on tropoelastin and elastin synthesis

Histological observations (tropoelastin and elastin immunostaining) have shown at D11:

Compared to untreated explant Neodermyl® 0.5% induced:

- a slight increase of tropoelastin synthesis
- a clear increase of elastin synthesis

Image analyses have shown:

Compared to untreated explant Neodermyl® 0.5% induced at D11:

- a statistically significant increase of tropoelastin (+57%, Student t-test; p <0.1) and elastin synthesis (+190%, Student t-test; p <0.05)

After 11 days of treatment:

Neodermyl® at 0.5% stimulates both the tropoelastin (up to +57%) and elastin (up to +190%) synthesis showing its anti-aging properties (tropoelastin and elastin are two major proteins involved in skin elasticity properties).

6.4. Conclusions on *ex vivo* experiments

Histological observation (Picro-sirius staining, collagen type I and collagen type III, tropoelastin and elastin immunostaining) have shown:

Compared to untreated explant Neodermyl® at 0.5% induced at D11:

- a slight to moderate increase of total collagen expression
- a clear increase of collagen I synthesis
- a very clear increased of collagen type III synthesis
- a slight increase of tropoelastin synthesis
- a clear increase of elastin synthesis

Image analyses have shown:

Compared to untreated explant Neodermyl® at 0.5% induced at D11:

- an increase of +6.8% of total collagen
- a statistically significant increase of collagen type I synthesis by +179% (Student t-test; p <0.01).
- a statistically significant increase of collagen type III synthesis about +194% (Student t-test; p <0.01)
- a statistically significant increase of tropoelastin (+57%; Student t-test; p <0.1) and elastin synthesis (+190%; Student t-test; p <0.05)

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Neodermyl® at 0.5% has shown anti-aging properties on 74 years old skin explants:

-Neodermyl® stimulates collagen synthesis very quickly and only 11 days post-treatment: collagen type I (up to +179%) and collagen type III (up to +194%). Both proteins are involved in skin structure properties.

-Neodermyl® stimulates two major proteins involved in skin elasticity properties: the tropoelastin (up to +57%) and elastin synthesis (up to +190%).

-The aged human explants treated by Neodermyl® at 0.5 % showed young skin properties: a lower ratio collagen type I/collagen type III and a higher amount of new elastin and tropoelastin synthesized.

7. Clinical investigation of Neodermyl®

7.1. Introduction

First encouraging *in vitro* results (increasing viability and procollagen I synthesis in aged fibroblasts) and *ex vivo* results (increasing collagen type I, III, tropoelastin and elastin synthesis in an aged human living skin explant) have shown the potential application of Neodermyl® 0.5% as an anti-aging molecule.

To confirm its effectiveness as anti-aging active molecule a clinical investigation was performed with a cream containing Neodermyl® (1%). The following clinical parameters were assessed: collagen density, anisotropy index, skin firmness and anti-wrinkle activity.

These parameters were selected as they are impacted by the skin aging process. With the skin aging, the collagen density and the skin firmness decrease, the skin anisotropy increases and wrinkles appear.

7.2. Materials and methods of clinical tests

7.2.1. Description of the cream used

Cream formula: AQUA, OCTYLDODECYL NEOPENTANOATE, OCTYLDODECANOL, MYRISTYL MYRISTATE, ACRYLATES/C10-30 ALKYL ACRYLATE CROSSPOLYMER, SODIUM HYDROXIDE, PHENOXYETHANOL, METHYLPARABEN, ETHYLPARABEN, BUTYLPARABEN, PROPYLPARABEN, ISOBUTYLPARABEN (+/-) NEODERMYL® 1%

7.2.2. Description of the panel and study condition

20 women volunteers (ages: 61 ± 7 years) were involved in the clinical investigation. The volunteers have a face with fine lines or expression lines visible at the bridges. They agree not to use other product during the study and they have no dermatological problems. The volunteers applied the placebo on one side of their face and a cream containing Neodermyl® at 1% on the other side of the face twice per day for 60 days. The various measurements were recorded at D0, D15 and D60. The methods used are described in detail in the following sections. The study was performed in comparison to a placebo (same formula without Neodermyl®).

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7.2.3. Collagen density assessment by SIAscope

The SIAscope V is a digital epiluminescence microscopy system designed for taking shadow-free magnified images of skin. It is generally used to measure and image the amount of collagen present in the epidermal layer of skin. The instrument consists of a handheld probe containing light sources and a digital imaging sensor. The SIAscope V measurement consists of several images taken in quick succession. Instrument specific software performs calculations of the different wavelength images using knowledge about the layered nature of skin tissue and spectral characteristics. It produces images for melanin, blood, and collagen which are then used for analysis purposes. Images of each of the sites were captured using the SIAscope V instrument. The SIAscope was used to track changes in the organization of collagen fibers during the study at D0, D15 and D60.

7.2.4. Anisotropy index

The aging process of the skin results in a change in the organization of skin lines. They pass by a homogeneous isotropic (lines oriented in all directions), for a young person, a state where the furrows gradually disappear, giving rise to a persistent state of deep furrows leading to the formation of highly oriented wrinkles. The state of micro-relief is reflected in the anisotropy index, it allows the assessment of the effect of a product on the skin surface topography. This index is calculated from the evaluation of grayscale image at each study time D0, D15 and D60. The measurements were performed using the Skinevidence.

7.2.5. Skin firmness assessment

The study is performed using the Cutometer® MPA 580 by Courage & Khazaka. The measuring principle is based on the suction method. Negative pressure is created in the device and the skin is drawn into the cylindrical aperture (2 mm in diameter) of the probe.

Inside the probe, the penetration depth is determined by an optical measuring system. Each suction phase is followed by a relaxing phase. The evaluation was performed on the cheekbone with the following program: (Length of the cycle: 4 seconds; Suction: 2 seconds; Relaxation: 2 seconds; Negative pressure: 500 millibars; Diameter of the chamber: 2 mm).

The resistance of the skin to be sucked up by the negative pressure and its ability to return to its original position are displayed as curves at the end of each measurement. From these curves the parameters (R0 and R5) can be calculated. R0 parameter represents the skin firmness and R5 parameter represents the skin elasticity. The closer to 1 the R5 value is, the better the elasticity is. During the suction phase, the deformation of the skin by the negative pressure measures first the elastic resistance, then the viscous component, which taken together represent skin firmness. During the relaxation phase, the immediate recovery of the skin measures sheer cutaneous elasticity, whereas the delayed return of the skin to its initial position measures the visco-elastic component.

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7.2.6. Anti-wrinkle activity

Evaluation of the anti-wrinkle activity was performed by the fringe projection technique on a silicone replica. It is carried out by measuring the depth, width and volume of wrinkles using the fringe projection technique. The Dermatop[®] system by Eotech relies on profilometry by fringe projection interference combined with the Toposurf[®] surface processing software. 3D reconstitutions are then analyzed by the Toposurf[®] software, which enables the calculation of the average depth, width and volume of the wrinkles.

The results of the effect of the product on the evolution of the various parameters were expressed in μm for the average depth, mm for the average width, and mm^3 for the average volume, respectively. The location of the wrinkles on the replica is performed so as to analyze the same area for each time point. A picture of each replica was taken with a fringe projection profilometer, and transferred to the Toposurf[®] software and finally the depth; width and volume of each wrinkle were calculated. Calculation of the mean values of depth, width and volume of the wrinkles was done for each subject before and after application of the product. Calculation of the overall effect of the product studied was performed by determining the variation percentage compared to the initial measurement for each parameter. Statistical analysis by using Student t-test on paired data.

7.3. Results and discussion

7.3.1. Collagen assessment by SIAscope

Neodermyl[®] 1% in comparison to placebo induced after 15 days and 60 days statistically significant improvements of collagen density respectively by $\times 7.5^*$ and $\times 7.6^{**}$ times ($*p < 0.05$ compared to placebo and $**p < 0.1$ compared to placebo, Student t-test) (fig 17):

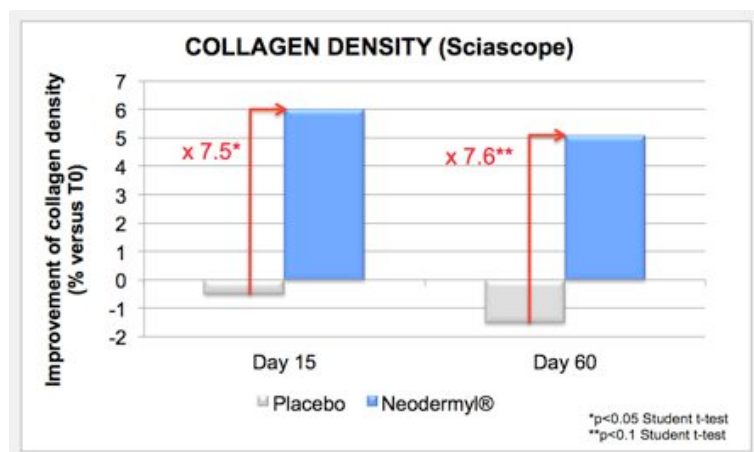


Fig 17: Collagen density assessment at D15 and D60 after Neodermyl[®] 1% and Placebo treatment in comparison to normal skin at D0 (Siascope)

Neodermyl[®] improves by about 7.5 times the skin collagen density after 15 days and gives equivalent results after 60 days

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7.3.2. Skin micro-relief anisotropy index

Neodermyl® at 1% in comparison to placebo induced after 15 days and 60 days statistically significant improvements of skin index anisotropy respectively by 25* and 2.5 times compared to placebo at D15 and D60 (*p<0.05, Student t-test) (fig 18).

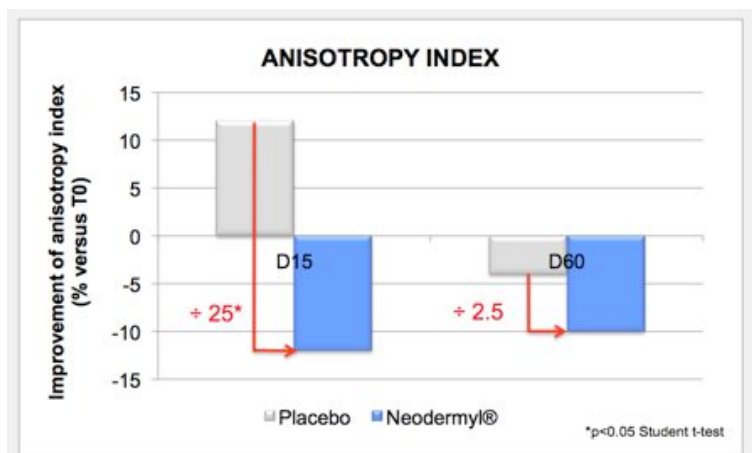


Fig 18: Skin anisotropy index assessment at D15 and D60 after Neodermyl 1% and Placebo treatment in comparison to normal skin at D0 (gray scale image)

Neodermyl® at 1% improves the skin microrelief by reducing the anisotropy of the skin respectively by 25 and 2.5 times after 15 days and 60 days.

7.3.3. Skin Firmness

Neodermyl® 1% in comparison to placebo induced after 15 days, and 60 days statistically significant improvements of skin firmness respectively by x13* and x12** times (*<0.1 and **p <0.01 compared to placebo, Student t-test) (fig 19).

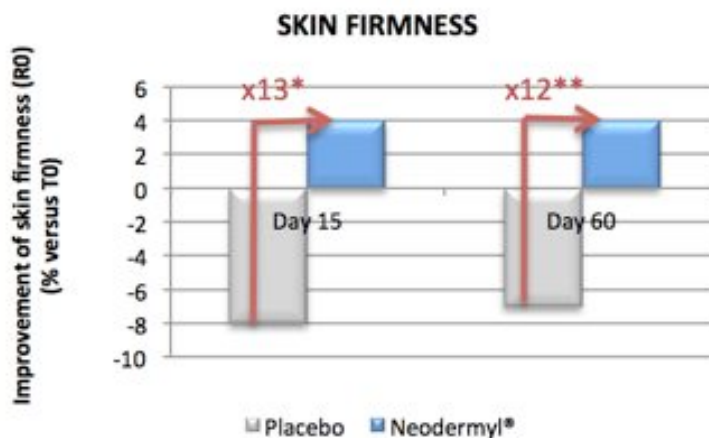


Fig 19: Skin firmness assessment at D15 and D60 after Neodermyl 1% and Placebo treatment in comparison to normal skin at D0 (cutometer measurements, parameter R0)

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Neodermyl® 1% in comparison to placebo induced after 15 days and 60 days statistically significant improvements of skin elasticity respectively of +60%* and +44%* (*p<0.1 compared to placebo, Student t-test) (fig 20).

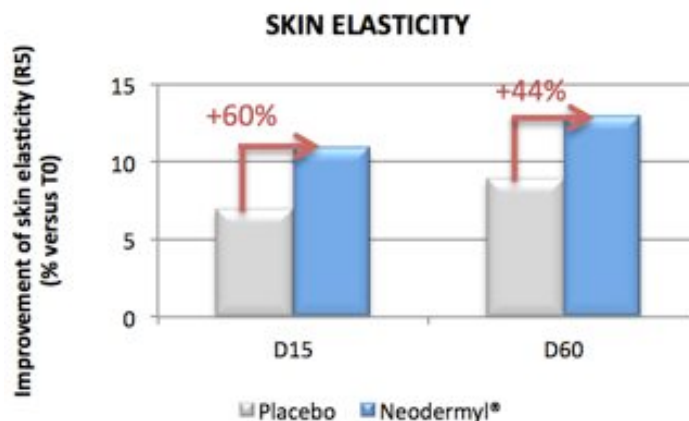


Fig 20: Skin elasticity assessment at D15 and D60 after Neodermyl 1% and Placebo treatment in comparison to normal skin at D0 (cutometer measurements, parameter R5)

Neodermyl® 1% significantly improves skin firmness (by 12 to 13 times) and elasticity (at least 44% corresponding at least to 1.6 times) at D15 and D60 in comparison to placebo.

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7.3.4. Anti-wrinkle activity

Neodermyl® at 1% in comparison to placebo induced after 15 days:

- a statistically significant improvement of the wrinkles' depth compared to placebo of -15% corresponding to 7.5 fold reduction of wrinkles' depth (Student t-test, * $p < 0.05$) (fig 21 and 22)
- a statistically significant improvement of the wrinkles' volume compared to placebo of -13% corresponding to 6.5 fold reduction of wrinkles' volume (Student t-test, * $p < 0.05$) (fig 21 and 22)

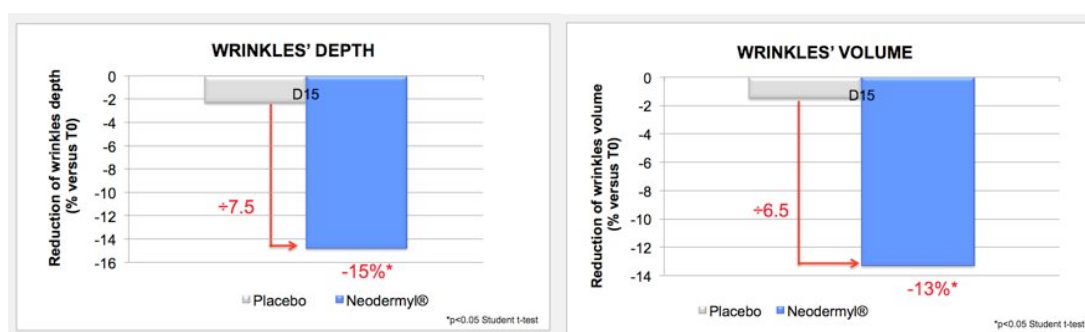


Fig 21: Wrinkles' depth and volume assessment at D15 after Neodermyl 1% and placebo treatments in comparison to normal skin at D0 (fringe projection technique on silicone replica)

Wrinkle profile of skin untreated (D0)

Wrinkle profile of skin treated Neodermyl® 1% (D15)

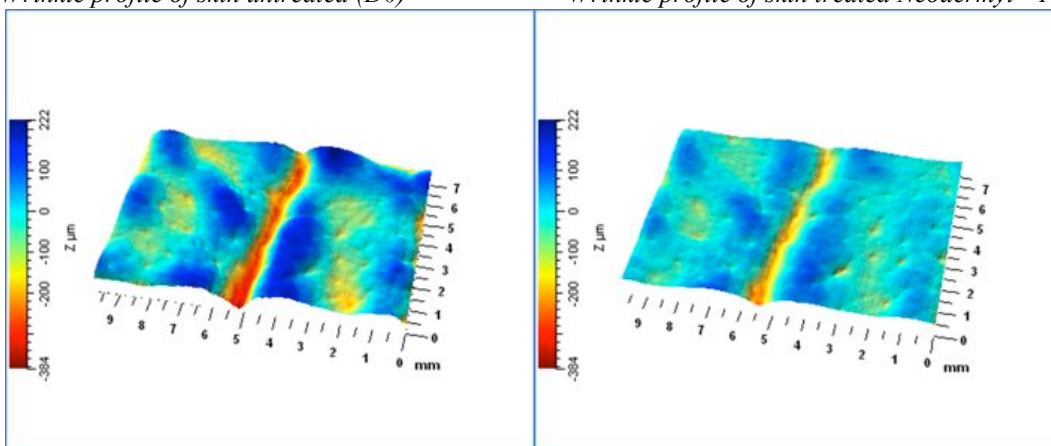


Fig 22: Wrinkles' profile of skin untreated (D0) and skin treated by Neodermyl 1% (D15) (fringe projection technique on silicone replica)

Neodermyl® at 1% significantly decreases the depth and volume of the wrinkles at D15 in comparison to placebo (-15% of wrinkles' depth and -13% of wrinkles' volume).

7.4. Conclusions on clinical investigations

The clinical investigation has shown that after 15 days to 60 days of treatment, the cream with 1% Neodermyl® significantly statistically improves the following clinical parameters:

- increasing collagen density
- improvement of skin micro relief by reducing skin anisotropy
- increasing skin firmness and elasticity
- reduction of the depth and volume of wrinkles

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8. General Conclusions

Neodermyl® is an active ingredient containing Methylglucoside-6-phosphate (MG6P), Proline, Lysine and Copper. Methylglucoside-6-phosphate is a biotechnology activated sugar which provides energy after its metabolization (Krebbs cycle). Proline and lysine are essential amino-acids which are needed for collagen and elastin synthesis and copper is the lysyl-oxidase co-factor. Without copper, the collagen and elastin couldn't be cross-linked by lysyl-oxidase.

In vitro experiments have shown the capabilities of Neodermyl® (0.5 and 2%) to reactivate aged fibroblasts (senescent) by increasing their metabolism (increased viability and procollagen I synthesis). The effect of Neodermyl® (0.5 and 2%) was visible *in vitro* very quickly just after 3 days of treatment.

Ex vivo experiments have shown the capabilities of Neodermyl® (0.5 and 2%) to stimulate fibroblasts in aged living skin explants by stimulating their synthesis of collagen type I, collagen type III, tropoelastin and elastin. The effect of Neodermyl® (0.5 and 2%) was also visible *ex vivo* very quickly just after 6 and 11 days of treatment. The aged explants showed after Neodermyl® treatment younger skin properties (decreased ratio of collagen type I/collagen type III).

The clinical investigation performed on human volunteers confirms the effects of Neodermyl® seen during *in vitro* and *ex vivo* experiments. The clinical parameters (see fig 23) are improved by Neodermyl® treatment. Neodermyl® promotes the synthesis in dermis of key proteins involved in skin structure and elasticity: collagen type I, collagen type III, tropoelastin and elastin. The stimulation of these proteins synthesis by Neodermyl® at microscopic level has an impact clearly visible on the macroscopic level leading to a rejuvenation of human aged skin.

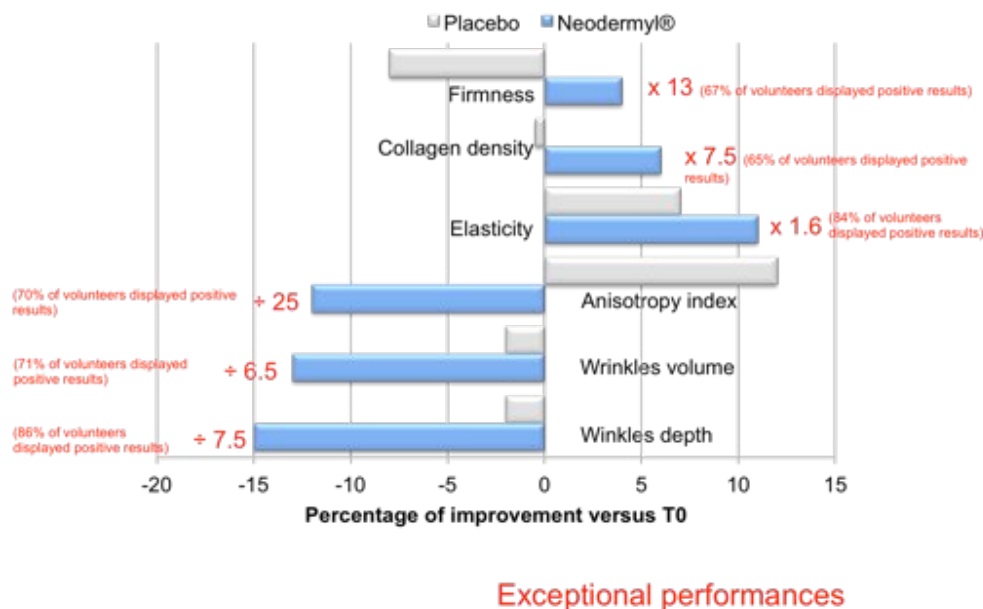


Fig 23: Summary of results from clinical investigation performed on human volunteers