(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
4 February 2010 (04.02.2010)

PCT

(10) International Publication Number
WO 2010/013204 A1

(51) International Patent Classification:
A61P 17/00 (2006.01) *A61K 8/42* (2006.01)
A61P 17/02 (2006.01) *A61K 8/60* (2006.01)
A61P 17/12 (2006.01) *A61K 8/66* (2006.01)
A61P 17/16 (2006.01) *A61K 8/98* (2006.01)
A61K 31/00 (2006.01) *A61K 35/64* (2006.01)
A61K 31/167 (2006.01) *A61Q 19/08* (2006.01)
A61K 31/7036 (2006.01) *A61K 38/48* (2006.01)
A61K 45/06 (2006.01)

(21) International Application Number:
PCT/IB2009/053287

(22) International Filing Date:
29 July 2009 (29.07.2009)

(25) Filing Language: Italian

(26) Publication Language: English

(30) Priority Data:
TO2008A000584 29 July 2008 (29.07.2008) IT

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(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CL, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PE, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MK, MT, NL, NO, PL, PT, RO, SE, SI, SK, SM, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *with international search report (Art. 21(3))*

— *before the expiration of the time limit for amending the claims and to be republished in the event of receipt of amendments (Rule 48.2(h))*

WO 2010/013204 A1

(54) **Title**: COMPOSITION FOR REGENERATING SENESCENT CUTANEOUS AND SUB-CUTANEOUS TISSUE, PRODUCTS AND USES THEREOF

(57) **Abstract**: A cosmetic composition, medical device and pharmaceutical composition for the remodelling and regeneration of lax, atrophic or senescent cutaneous and subcutaneous tissue that characterise the onset of expression or age wrinkles in mammals, animals or humans, comprising at least two among (i) antibiotics, (ii) amino-amidic local anaesthetics, (iii) proteolytic agents, and (iv) antiseptic and salt reconstituents.

"Composition for regenerating senescent cutaneous and subcutaneous tissue, products and use thereof"

* * *

TEXT OF THE DESCRIPTION

FIELD OF THE INVENTION

The present invention relates to a composition for regenerating senescent cutaneous and/or subcutaneous tissue. In particular, the present invention has the object of providing a composition suitable for regenerating cutaneous and/or subcutaneous tissue with wrinkles and striae in the compromised areas.

BACKGROUND OF THE INVENTION

Wrinkles are essentially linked to facial expressions- due to reflective action of the facial muscles - or to senescence - due to irreversible relaxation of the skin.

The processes occurring in the skin, from dermis to epidermis, from the microvascular to the sebaceous - follicular and pigmentary systems, gradually leading first to cutaneous maturity and then to senescence are unavoidable and irreversible.

The cutaneous picture, including wrinkles, crow's feet, sagging and relaxation of the skin, results from the normal organic decline occurring with the passing of the years. Climacteric and the endocrine collapse that characterises it play a major role in the development of these aesthetic imperfections. Furthermore, internal disturbances associated with aging can certainly enhance and impact the condition of the skin.

Aging of the epidermis results in a certain reduction in cellular reproduction, typical of the germinal layer of the skin and reduction in the cells

of the malphighian layer that are extremely important to the epidermis.

The known skin products, when well formulated, serve as preventives and coadjuvants in fighting the appearance of wrinkles, though they can not rejuvenate the skin, but only rehydrate it intensively.

SUMMARY OF THE INVENTION

The present invention has the objects of:

- blocking the unavoidable modification of the mucopolysaccharide component of the ground substance and the loosening of the elastic component and collagen of derma;
- slowing the flattening of the papillary ridges, preventing the slowing of blood circulation, avoiding the accumulation of toxins;
- establishing correct nutritional support in the tissues overlying the papillary zone of the derma.

According to the present invention, such objects are achieved by means of the composition specifically recalled in the claims that follow. The claims form an integral part of the technical teaching provided herein relative to the invention.

The invention is based on the observation of a particular repairing, remodelling and regenerating stimulus exerted on cutaneous and subcutaneous tissues by at least two compounds selected from among:

(i) antibiotics, preferably aminoglycosides,
(ii) local amino amide anaesthetics, preferably lidocaine,
(iii) proteolytic agents, preferably papain, and
(iv) antiseptic and salt reconstituting agents, preferably propolis.

Such activity is induced through the local-

regional interaction of these compounds, inducing recovery of the trophism of senescent or pathological cutaneous and/or subcutaneous tissue.

The above-identified compounds may be advantageous employed both in the cosmetic field, due to their efficacious anti-wrinkle action, and in the medical field for their efficacious action in re-equilibrating the cutaneous and subcutaneous microenvironments.

In the field of cosmetics such compounds may be used advantageously for the cosmetic treatment of wrinkles in a mammal, preferably in a human being.

In the medical field, such compounds may be used advantageously for the regeneration of the cutaneous and subcutaneous tissue of a mammalian, animal or human being, subjected to the damaging of these tissues.

DETAILED DESCRIPTION OF THE INVENTION

The present invention will now be described in detail by way of non-limiting example only, with reference to some preferred embodiments.

The composition object of the present invention comprises at least two compounds selected among:

(i) antibiotics, preferably aminoglycosides, more preferably gentamicin,

(ii) amino amide local anaesthetics, preferably lidocaine,

(iii) proteolytic agents, preferably papain, and

(iv) antiseptic and salt reconstituting agents, preferably propolis.

The aminoglycoside antibiotics contained in the composition of the present invention may advantageously be selected from: gentamicin, micronomicin, spectinomycin, amikacin, kanamycin, tobramycin, neomycin, metilmycin, paromycin, streptomycin, or precursors or natural or synthetic derivative thereof. Among the aminoglycoside antibiotic gentamicin is

particularly preferred.

The amino amidic local anaesthetics contained in the composition object of the present invention may advantageously be selected from: lidocaine, lidocaine hydrochloride, mepivacaine, prilocaine, bupivacaine, etidocaine, ropivacaine, or precursors or natural or synthetic derivatives thereof. Among the amino amide local anaesthetics, lidocaine is particularly preferred.

The proteolytic agents contained in the composition object of the present invention can be advantageously selected from among: papain, papain FU, collagenase (preferably of type Ia, type II, type IV), serratiopeptidases, bromelain, bradykinase, Clostridium peptidases, proteolytic enzymes expressed by Lactobacillus acidophilus, proteolytic enzymes expressed by the genus Aspergillus, proteases, alliinases and fibrinolysin, precursors or natural or synthetic derivatives thereof. Among the proteolytic agents papain FU is particularly preferred.

The antiseptic and salt reconstituents contained in the composition object of the present invention can be advantageously selected from among: propolis, propolis concentrate, de-waxed propolis, concentrated de-waxed propolis, royal jelly and whole beeswax, precursors or natural or synthetic derivatives thereof. Among such compounds propolis is particularly preferred.

The composition may be prepared in the form of a cosmetic composition (for an anti-wrinkle treatment), a pharmaceutical composition, a *medical device*, or in the form of a culture medium for the remodelling and regeneration of cutaneous and/or subcutaneous tissue or for the stimulation of normal trophism and/or pigmentation.

The composition object of the present invention, in addition to containing at least two among compounds (i) to (iv) identified above, may optionally comprise one or more additional active ingredients that operate synergistically with those compounds, increasing the efficacy of the composition in restoring recovering the trophism of pathological or senescent cutaneous and/or subcutaneous tissue.

The additional active ingredients can be selected from amino acids, vitamins, glucosaminoglycans, sugars, peptides, supplements. In addition, such preparations can optionally comprise a physiologically acceptable solvent and/or diluent, as well as common excipients and/or additives for pharmaceutical or cosmetic compositions.

In general, the aminoglycoside antibiotic, preferably gentamicin, is used in a quantity comprised between 4 mg/Kg and 650 mg/Kg with respect to the total weight of the composition, preferably between 40 mg/Kg and 320 mg/Kg, more preferably between 40 mg/Kg and 160 mg/Kg for the substantially solid (creams), gel or liquid compositions.

In general, the amino amide local anaesthetic, preferably lidocaine hydrochloride, is used in a quantity comprised between 1 mg/Kg and 400 mg/Kg with respect to the total weight of the composition, preferably between 1 mg/Kg and 100 mg/Kg, more preferably between 10 mg/Kg and 80 mg/Kg for the substantially solid , gel or liquid compositions.

In general, the proteolytic agent, preferably papain FU, is used in a quantity comprised between 1 mg/Kg and 100 mg/Kg with respect to the total weight of the composition, preferably between 4 mg/Kg and 80 mg/Kg, more preferably between 40 mg/Kg and 80 mg/Kg for the substantially solid, gel or liquid

compositions.

In general, the antiseptic and salt reconstituent, preferably propolis, is used in a quantity comprised between 0.1% (w/v) and 50% (w/v) with respect to the total volume of the composition, preferably between 1% (w/v) and 30% (w/v), more preferably between 10% (w/v) and 20% (w/v) for the substantially solid, gel or liquid compositions.

The composition may be formulated as a solid or liquid, anhydrous or aqueous, for example in the form of a cream, unguent, ointment, powder, patch, impregnated membrane, solution, emulsion, suspension, vesicular dispersions, lotion, gel, spray.

Generally, a cream base is used as diluent or vehicle in the substantially solid preparations (such as creams, unguents, ointments); a physiological solution is used as diluent in the substantially liquid preparations.

As additional ingredients, which may be used in combination with the above identified compounds (i) to (iv) by way of example are cited:

- amino acids such as methionine, cystine, N-acetylcysteine, cysteine, glycine, leucine, isoleucine, proline, glutamine, arginine, glutamic acid, histidine, histidine-HCl, lysine, lysine-HCl, phenylalanine, serine, threonine, tryptophan, tyrosine, tyrosine-disodium salt, valine, hydroxyproline. Such amino acids are often used in mixtures comprising a large number of different amino acids.

- vitamins and cofactors, such as retinoic acid, retinaldehyde, retinol, alpha-tocopherol, beta-carotene, ascorbic acid, pantothenic acid, dexpanthenol, D-calcium pantothenate, cocarboxylase tetrahydrate, pyridoxine, pyridoxine-HCl, folic acid, niacinamide, riboflavin, cobalamin, para-aminobenzoic acid, biotin and as vitamin-

related nutrients inositol and myoinositol;

- glucosaminoglycans, such as hyaluronic acid, chondroitin sulphates;

- sugars, such as rice starch, glucose, sucrose, glucans, mannan, glucomannan, fucose, fructose, heparan sulfates, pectin, starches, their alcohol derivatives;

- peptides, such as glutathione, collagen, elastin, wheat extract;

- supplements, such as selenium, coral powder or *Corallium pulvis*, mother-of-pearl powder or *permula pulvis*, plant extracts (for example, *Centella asiatica*, *Aesculus hippocastanum*, *Calendula officinalis*, *Menyanthes trifoliata*, *Malva silvestris*, *Fucus vesiculosus*, *Ginkgo biloba*, *Hieracium pilosella*, *Melilotus officinalis*, *Papaver nudicaule*), triterpine acids or ethers (for example madecossoside, asiaticoside and their derivatives and precursors), glycyrrhetic acid, glycyrrhizin, glycocolic acid, soy lecithin.

The preferred embodiments of the composition object of the present invention are represented by the composition denominated ASX described hereinafter in the form of a cream, gel and infusion.

Examples of tissue-specific compositions

Compositions in the form of cream, gel and infusion are indicated hereinafter as compositions ASX-1-GEL, ASX-1-CREMA and ASX-1-INFUS and ASX-2-GEL, ASX-2-CREMA and ASX-2-INFUS and are illustrated by way of example only in tables 1 to 6. Such composition may comprise, in addition to at least two of the above identified compounds (i) to (iv) and to the possible additional ingredients, a physiologically acceptable vehicle such as a cream base, a gel or a physiological solution.

Table 1. Composition ASX-1-GEL (medical device) for the treatment of senescent cutaneous and subcutaneous tissue.

Substance	Concentration
Active Principles	mg/kg
Gentamicin	40 mg/kg
Lidocaine	10 mg/kg
Papain FU	56.00 mg/kg
Additional active ingredients	mg/kg
Mixture of L-amino acids	900 mg/kg
Vitamin A	4 mg/kg
B Vitamins (B1, B2, B5, B6, B9, B12)	48 mg/kg
Vitamin C	4.5 g/kg
Vitamin D	0.0055 mg/kg
Vitamin E	10 mg/kg
Vitamin H	0.069 mg/kg
Vitamin PP	46 mg/kg
BASE in gel (carbopol or cellulose derivatives)	q.s. per kg of product

Table 2. Composition ASX-1-CREMA for the treatment of senescent cutaneous and subcutaneous tissue.

Substance	Concentration
Active Principles	mg/kg
Gentamicin	40 mg/kg
Lidocaine	10 mg/kg
Papain FU	56.00 mg/kg
Additional active ingredients	mg/kg
Mixture of L-amino acids	900 mg/kg
Vitamin A	4 mg/kg
B Vitamins (B1, B2, B5, B6, B9, B12)	48 mg/kg
Vitamin C	4.5 g/kg
Vitamin D	0.0055 mg/kg
Vitamin E	10 mg/kg
Vitamin H	0.069 mg/kg
Vitamin PP	46 mg/kg
Whole beeswax	5 mg/kg
BASE in the form of a cream or emulsion (O/A or A/O) (such as: water, white vaseline, cetostearyl alcohol, mineral oil, ceteth-20, sodium phosphate, p-chloro-m-cresol, phosphoric acid)	q.s. per kg of product

Table 3. Composition ASX-1-INFUS for the treatment of senescent cutaneous and subcutaneous tissue.

Substance	Concentration
Active Principles	mg/kg
Gentamicin	40 mg/kg
Lidocaine	10 mg/kg
Papain FU	56.00 mg/kg
Additional active ingredients	mg/kg
Mixture of L-amino acids	900 mg/kg
Vitamin A	4 mg/kg
B Vitamins (B1, B2, B5, B6, B9, B12)	48 mg/kg
Vitamin C	4.5 g/kg
Vitamin D	0.0055 mg/kg
Vitamin E	10 mg/kg
Vitamin H	0.069 mg/kg
Vitamin PP	46 mg/kg
Physiological solution	q.s. per L of solution

Table 4. Composition ASX-2-GEL (medical device) for the treatment of senescent cutaneous and subcutaneous tissue.

Substance	Concentration
Active Principles	mg/kg
Propolis	10% (w/v)
Papain FU	56.00 mg/kg
Additional active ingredients	mg/kg
Mixture of L-amino acids	900 mg/kg
Vitamin A	4 mg/kg
B Vitamins (B1, B2, B5, B6, B9, B12)	48 mg/kg
Vitamin C	4.5 g/kg
Vitamin D	0.0055 mg/kg
Vitamin E	10 mg/kg
Vitamin H	0.069 mg/kg
Vitamin PP	46 mg/kg
Coral powder	0.1 mg/kg
Mother-of-pearl powder	0.01 mg/kg
Tincture of Aesculus hippocastanum	1% (w/v)
BASE in gel (carbopol or cellulose derivatives)	q.s. per kg of product

Table 5. Composition ASX-2-CREMA for the treatment of senescent cutaneous and subcutaneous tissue.

Substance	Concentration
Active Principles	mg/kg
Propolis	10% (w/v)
Papain FU	56.00 mg/kg
Additional active ingredients	mg/kg
Mixture of L-amino acids	900 mg/kg
Vitamin A	4 mg/kg
B Vitamins (B1, B2, B5, B6, B9, B12)	48 mg/kg
Vitamin C	4.5 g/kg
Vitamin D	0.0055 mg/kg
Vitamin E	10 mg/kg
Vitamin H	0.069 mg/kg
Vitamin PP	46 mg/kg
Coral powder	0.1 mg/kg
Mother-of-pearl powder	0.01 mg/kg
Tincture of Aesculus hippocastanum	1% (w/v)
Whole beeswax	5 mg/kg
BASE in the form of a cream or emulsion (O/A or A/O) (such as: water, white vaseline, cetostearyl alcohol, mineral oil, ceteth-20, sodium phosphate, p-chloro-m-cresol, phosphoric acid)	q.s. per kg of product

Table 6. Composition ASX-2-INFUS for the treatment of senescent cutaneous and subcutaneous tissue.

Substance	Concentration
Active Principles	mg/kg
Propolis	10% (w/v)
Papain FU	56.00 mg/kg
Additional active ingredients	mg/kg
Mixture of L-amino acids	900 mg/kg
Vitamin A	4 mg/kg
B Vitamins (B1, B2, B5, B6, B9, B12)	48 mg/kg
Vitamin C	4.5 g/kg
Vitamin D	0.0055 mg/kg
Vitamin E	10 mg/kg
Vitamin H	0.069 mg/kg

Vitamin PP	46 mg/kg
Physiological solution	q.s. per L of solution

The cosmetic, pharmaceutical and *medical device* type compositions object of the present invention may also comprise further accessory elements such as excipients and vehicles whose choice and employment fall within the capacity of one skilled in the art with no need to exert inventive activity.

The results obtained with the ASX compositions demonstrate that the state of senescence or cutaneous and subcutaneous atrophy occurring in degenerative processes can be recuperated. The ASX compositions, in fact, have been shown capable of inducing excellent remodelling, repair and regeneration of cutaneous and subcutaneous tissues with normal histo-functional characteristic.

The experiments performed are described in greater detail in the following section.

EXAMPLE 1. Biopsies and prototype-solutions

The animal biopsy samples under study are constituted by atrophic-senescent surgical specimens removed for the purpose of correcting major functional cutaneous or subcutaneous alterations.

All samples were washed three times with physiological solution containing antibiotics (100 Units/ml penicillin + 100 µg/ml streptomycin + 160 mg/L gentamicin + fluconazole 0.2 mg/ml) for ten minutes at room temperature.

The biopsies were then sectioned into three parts (two controls, 1 and 2, and one sample 3 to be treated for each subject) and suspended in a final volume equal to 25 ml of the corresponding culture solution in 10 cm plates (Lab-Tek chamber slides, Nunc, Kamstrup, Denmark).

Two types of controls were prepared, an untreated negative control (1), that is, treated with physiological

solution and antibiotics only (as described above), and a positive control (2) treated with cell culture medium commonly used for cutaneous biopsies.

1. Negative control: the control 1 biopsy specimens were suspended in physiological solution in 10 cm plates (Lab-Tek chamber slides, Nunc, Kamstrup, Denmark).

2. Positive controls: the control 2 biopsy specimens were placed in 10 cm plates (Lab-Tek chamber slides, Nunc, Kamstrup, Denmark) in D-MEM medium supplemented with:

10% FBS (Celbio, Milan, Italy)
160 mg/L gentamicin (Schering-Plough, Milan, Italy)
2 mM L-glutamine (Life Technologies; growth medium)
50 ng/mL EGF (Sigma Aldrich, Milan, Italy).

3. Samples: the sample biopsy specimens 3 were placed in 10 cm plates (Lab-Tek chamber slides, Nunc, Kamstrup, Denmark) in *Culture media ASX-1-INFUS*.

All samples were incubated for 15 days in a Heraeus incubator thermostatically controlled at the temperature of 37°C with a constantly supplied atmosphere containing 5% CO_2 (v/v in air). Two-thirds of the culture medium were replaced every 7 days. All biopsy tissues used in culture constitute a possible co-conditioning optional support for the three dimensional growth of the cell samples under study.

Staining protocol

After three 10 minute washes at room temperature in PBS (pH 7,4), the samples were resuspended in a 4% para formaldehyde fixing solution in D-MEM (Gibco) pH 7.4 for one hour at room temperature. All biopsies object of study were treated with Alcian blue. This stain is made of a group of water soluble polyvalent basic dyes. The colour blue is due to the present of copper in the molecule. Alcian blue at a final concentration of 1% w/v in PBS (pH 7.4) is added to a 3% acetic acid solution (pH

2.5). After a two hour incubation at room temperature this composition permanently stains acidic mucopolysaccharides and glycoproteins both sulfonated and carboxylated. Specific controls were set up for each sample. All samples were washed three times with PBS (pH 7,4) at room temperature for five minutes and then observed with light microscopy. A net increase in type 1 collagen and type 4 collagen that stain in blue is noted in the samples treated with the solution *Culture medium ASX-1-INFUS* with respect to control 1 and control 2.

RESULTS

Staining with the Alcian blue colorimetric method

- Negative controls 1 treated with physiological solution: diffuse blue staining (score = ++++) alternating with cytolytic and necrotic areas.
- Positive controls 2 treated with common biopsy culturing medium, as described above. A very strong diffuse Alcian blue staining (score = +++++).
- Sample treated with the solution *Culture medium CHEL1-INFUS*. It is noted that the cells, in which a re-deposition of mucopolysaccharides and glycoproteins, stain well with Alcian blue growing in superimposed and ordered layers with physiological distribution of mucopolysaccharides and GAG (Score = +++).

Western blot

Samples were subjected to phenotypic analysis with Western Blot for the markers (Santa Cruz Biotechnology, America, California) anti-collagen type I, anti collagen type IV, anti cytokeratins 1, 5, 10, 14. After five washes, the membranes were incubated with the corresponding secondary antibodies (1:1000) conjugated with horseradish peroxidase (HRP, Santa Cruz, Calif. USA) for 1h at room temperature as reported in table 7 below.

Characterisation of samples treated with Culture medium ASX-1-INFUS versus controls

The results relative to the expression of collagen type I, collagen type IV and of the cytokeratins 1, 5, 10 and 14 are expressed on a quantitive scale as presented in Table 7 below:

Table 7

Markers	Control 1	Control 2	Sample
collagen typo I	-/+	+++	+++++
collagen typo IV	-/+	+++	+++++
Cytokeratin 1	++++	++++	+++++
cytokeratin 5	++++	+++	+++++
cytokeratin 10	++	++++	+++++
cytokeratin 14	+++	++	+++

Legend
--- = band absent
-/+ = light presence of band
+ = band present thin
++ = band present medium
+++ = band present extensive
++++ = band present high
+++++ = band present effuse

EXAMPLE 2. IN VIVO CLINICAL STUDIES

Formulation ASX-1 CREMA.

IN VIVO CLINICAL STUDY 1.

A clinical study was performed in order to evaluate the tolerability and therapeutic efficacy in cutaneous and subcutaneous regeneration and repair of a product denominated ASX-1-CREMA (composition reported in table 2).

The present study was performed on a sample constituted by four dogs of different races and sizes presenting cutaneous lesions attributable to cutaneous laxity (genetically determined or acquired wrinkling) with consequent infective or fibrotic processes, ulceration or atrophy.

At the screening visit subjects were selected with the lesions indicated above.

The animals were brought to the clinical follow-up visits weekly until healing and subjected to cytological examination and evaluation of the extension and depth of the cutaneous lesions.

Case 1

An 8-year-old female Shar Pei was brought to the clinical visit for ocular lesions from excessive skin laxity in the peri-orbital teguments on the skin. After surgical correction with removal of the excessive cutaneous mass a cutaneous laxity from atrophic-senescent fibrosis persisted around the scar, complicated by ulceration.

Clinical Examination Cutaneous thickening due to fibrosis.

Diagnosis. Clinical picture referable to chronic atrophy complicated by superficial secondary infections. The cytological examination revealed a bacterial infection of the ulcerated lesion.

Therapy. Systemic antibiotic therapy with enrofloxacin 5mg/kg *per os* for three weeks. The wound was cleaned with povidone-iodine the first day, proceeding then with wound cleaning with physiological solution and application of the preparation ASX-1 CREMA twice daily for 30 days.

Follow-up visits. After one week an improvement in the infection and 50% regeneration of tissue was noted. After two weeks the infection had disappeared and further reduction of the lesion were observed.

Clinical result. At the next follow-up visit after four weeks from the initiation of treatment the complete disappearance of the dermal fibrosis was observed.

Case 2

A 10-year-old German Shepherd dog brought to the clinic for multiple caudal burns.

Clinical Examination. Dorsal-caudal geographic map lesions with exudate and the formation of atrophic-fibrous scars including fur.

Diagnosis. The cytological examination indicated bacterial superinfection of the burn wound with hypertrophic scars.

Therapy. Marbofloxacin was administrated *per os* at 2.5 mg/Kg for 21 days. The fur was shaved and wound cleaned with povidone-iodine followed by application of the preparation ASX-1-CREMA twice daily for 60 days.

Follow-up visits. After one week it tissue granulation was observed that the

After one week granulation tissue was observed with a circa 30% reduction in the lesion dimensions and at the end of the second week complete healing of the wound was observed, with the formation of a stable regenerated tissue and a 50% reduction of the atrophic-senescent scar.

Clinical result. At the successive visit after 30 days from the initiation of treatment all the scars appeared whitish in colour, of normal resistance and consistency without atrophic-fibrotic components.

Case 3

A 10-month-old female Shar Pei dog.

Anamneses From birth problems with spontaneous cutaneous laceration with secondary atrophic scars in the dorsal peri-caudal area. Following numerous attempts at surgical revision, the patent was subjected to numerous therapies: tetracycline associated with cortisone deposit, local disinfection of the lesion area with paromomycin sulfate + prednisolone and clostebol acetate: no appreciable result and induction of cutaneous hyperextensibility. Multiple cutaneous

biopsies were performed revealing an alteration of the collagen fibres compatible with Ehlers-Danlos syndrome.

Day 0. The cutaneous wounds and lacerations were treated with polyvinylpyrrolidone twice daily and preparation ASX-1-CREMA with occlusive bandage.

In order to control secondary infections, systemic antibiotic therapy with amoxicillin and clavulanic acid 12.5mg/kg Bid was associated.

Day 7. After 7 days re-epithelisation of all wounds treated with preparation ASX-1-CREMA was observed, such scars did not present tissue atrophy or laxity.

The use of preparation ASX-1-CREMA allowed the attainment of eutrophic remodelling of broad cutaneous wounds that had been complicated by super infections for months within only 30 days.

Case 4

Description a 12-year-old male Shar Pei dog.

Anamneses The dog had cutaneous laceration areas (mainly of the adnexal structure type) of the posterior limbs for about one year that had progressively increased in diameter in the last 2 months. Surgical removal of the ulcer tissue and of the excess cutaneous mass was performed; antibiotic therapy with cefalexin for 15 days at 20 mg/kg bid was set up.

Day 0. Disinfection of the affected areas was performed with povidone iodine and preparation ASX-1-CREMA was applied once a day with occlusive bandage until follow-up.

Systemic antibiotic therapy with cefalexin was continued at the same dosages.

Day 10 At the follow-up visit remission of the lesions was noted.

Day 20. Application of preparation ASX-1-CREMA allowed attainment of eutrophic re-epithelisation of the surgically treated cutaneous areas.

Day 180. Application of preparation ASX-1-CREMA allowed attainment of remission-free healing of the surgical wound.

Formulation ASX-2-CREMA.

IN VIVO CLINICAL STUDY 2.

We used the ASX-2-CREMA composition with anti-wrinkle action described in table 5 in the context of compassionate use for 40 subjects affected by:

A) marked expression wrinkles;

B) wrinkles caused by relaxation of senescent skin.

Table 8 below summarises the most significant data.

Lesions almost exclusively concerned the face.

Table 8.

Treated Subjects	N.	male	female	Mean age
Group A	20	10	10	29 +/- 8
Group B	20	8	12	53 +/- 10
Total	40	20	20	41 +/- 9

The composition with anti-wrinkle action ASX-2-CREMA was administered with two daily applications for a period of no less than 30 days up to a maximum of 90 days. In no case was the onset of allergy or intolerance phenomena observed, instead in most cases a few days after starting therapy patients reported a feeling of local tension. Initially, in almost all subjects, elasticity was observed as an affect of the superficial vasodilation induced by the composition ASX-2-CREMA; definitely a positive effect considering the state of

occlusion of many vessels observed both in expression grooves and in the atrophic senescent wrinkles. In the greater part of the subjects the significant regression of the wrinkles (90%) with respect to time zero was obtained. Highly significant results with respect to time zero were observed in the majority of subjects enrolled in group A (98%). A significant reduction (80%) in the dimensions was observed in the subjects enrolled in group B with macroscopic modifications (variations in pigmentation, increase in elasticity, reduction of atrophy and laxity, etc.) with a strong response to the anti-wrinkle treatment under study.

EXAMPLE 3. COMPARATIVE STUDIES. ANTI-WRINKLE COMPOSITION ASX-1-CREMA AND ASX-2-CREMA.

Instrumental, subjective and dermatological evaluations or hydration, elasticity and tone, thickness and cutaneous roughness in the two groups of subjects subjected to differential treatment with the anti-wrinkle compositions ASX-1 CREMA andASX-2 CREMA.

The first group under study is formed by subjects with age comprised between 20-35 years presenting facial expression wrinkles and which were treated for thirty days exclusively with ASX-2 CREMA.

The second group under study is formed by subjects of age comprised between 60-75 years presenting deep facial wrinkles linked to senescence and which were treated for thirty days exclusively with ASX-1 CREMA.

Each subject applies the product to the face twice per day (morning and evening).

The evaluation of benefits (subjective and objective evaluation) was performed by instrumental measures, objective clinical evaluation and a self-evaluation questionnaire, for example VAS (Subjective Analogy Evaluation).

Selection of volunteers

The test is performed according to the principles of the Helsinki declaration, on 40 consenting subjects (all female), a group of 20 subjects with age comprised between 20 and 35 years (treatment ASX-2 CREMA) and a group of 20 subjects with age comprised between 60 and 75 years (treatment with ASX-1 CREMA). At the beginning of the test every subject provided informed consent as written by the investigators.

Subject selection - Inclusion criteria

- Group 1 = women between 20 and 35 years of age with mild cutaneous expression wrinkles (grade I on Larnier photographic scale);
- Group 2 = women between 60 and 75 years of age with cutaneous wrinkles of different degrees of cutaneous senescence (grade V-VI on the Larnier photographic scale);
- Subjects that had not applied topical retinoids during the 6 months prior to the study;
- Subjects that had not taken systemic retinoids during the year prior to the study;
- Subjects that had not applied topical products based on alpha or beta hydroxyl acids in the 45 days prior to the study;
- Subjects that had not taken systemic antioxidant and/or photosensitising substances during the 2 weeks prior to the study;
- Subjects agreeing not to apply any product on the face during the 7 days prior to the study;
- Subjects agreeing to not have exposure to sun or UV lamps during the study;
- Subjects agreeing to have expose to the sun only after applying sunscreens of a degree not lower than SPF 15 during the study.
- Subjects agreeing not to use other anti-aging

cutaneous treatments during the study.

Exclusion criteria

- Pregnant or nursing women;
- Subjects affected by systemic pathologies with cutaneous manifestations;
- Subjects with a history of intolerance to cosmetic products;
- Subjects with actinic keratosis of the face;
- Subjects have undergone filler treatments on the face (ex. collagen, hyaluronic acid, etc);
- Subjects that have applied topical retinoids during the 6 months prior to the study;
- Subjects that had taken systemic retinoids during the year prior to the study;
- Subjects that had applied topical products based on alpha or beta hydroxyacids in the 45 days prior to the study;
- Subjects that had taken antioxidants and/or systemic photosensitising substances during the two weeks prior to the study;
- Subjects that did not agree to not apply any product to the face during the 7 days previous to the study;
- Subjects on topical or systemic treatments (for example, corticosteroids or photosensitising substances) with any drug that could influence the result of the test;
- Subjects with dermatologic pathology (eczema, psoriasis, cutaneous lesions, acne, etc.);
- Subjects that had participated in clinical studies in the previous 30 days.

Objective dermatological evaluation

At the beginning, during and at the end of the clinical study (T_0 and T_{4weeks} with evaluation of the merit of the product) the visual examination of the

selected areas.

Global cutaneous evaluation: hydration, elasticity and wrinkles.

Evaluation of tolerability: appearance of possible undesirable effects such as erythema, desquamation, itching and swelling.

Instrumental evaluation

Instrumental measurements were performed inside a climate controlled room (24° + 2 C°; 50 +10% rh) after an acclimatisation period of 30 minutes.

At the above-detailed times (T_0 and T_{4weeks} with evaluation of the merit of the product), the investigators perform the following instrumental evaluations:

i) cutaneous hydration with the Corneometer CM 825 Combi 3 Courage & Khazaka, which measures the variation in the quantity of water in the corneal layer before and after the treatment;

ii) cutaneous elasticity with Cutometer SEM 575 Courage & Khazaka, which measures the variation in cutaneous elasticity before and after treatment;

iii) cutaneous thickness with the DERMASCAN C (Cortex Technology) Sonography device in A-scan mode;

iv) cutaneous wrinkles using analysis of silicone replicates of the cutaneous zone subjected to treatment. The image analysis software (Quantilines, Monaderm) is based on the measurement principle described by Corcuff.

Subjective evaluation

The volunteers expressed their subjective judgement at the end of the period on a dedicated questionnaire relative to:

- cosmetic appeal (perfume, ease of absorption and application);

- product efficacy (hydration, elasticity and wrinkles);

- tolerability: possible reaction of intolerance or discomfort to the product perceived during the treatment;

- VAS (Subjective Analogy Evaluation).

All subjects tested expressed a judgement of good tolerability of the cream.

Concerning the other parameters, the results are reported in table 9.

Table 9.

SENSATION UPON APPLICATION				
appearance	*negative*	*average*	*good*	*excellent*
COLOUR	0	2	21	17
AROMA	0	10	15	15
TACTILE SENSATION UPON APPLICATION				
CONSISTENCY	0	0	25	15
SPREADABILITY	0	1	23	16
FRESHNESS	0	1	24	15
ABSORBENCY	0	2	25	13
SENSATION AFTER APPLICATION				
AROMA	2	18	13	7
EFFECT ON SKIN	1	9	15	15
PRODUCT RESIDUE	1	8	12	19
GENERAL JUDGEMENT				
TOLERABILITY	0	1	13	26
EFFICACY	0	0	17	23

Table 10. Sonography with measurement of dermal hypodermal thickness.

CHARACTERISTIC of the woman enrolled		1° CONTROL TIME ZERO		2° CONTROL TIME 30 days		VARIATION D = DERMA H = HYPODERM mm = millimetre	
Subject	Age	D (mm)	D-H (mm)	D (mm)	D-H (mm)	D (mm)	D-H (mm)
1	30	1.40	7.50	1.60	7.80	0.20	0.30
2	35	1.70	10.80	2.60	10.80	0.90	0.00

CHARACTERISTIC of the woman enrolled		1° CONTROL TIME ZERO		2° CONTROL TIME 30 days		VARIATION D = DERMA H = HYPODERM mm = millimetre	
3	21	2.20	10.00	2.70	10.80	0.50	0.80
4	28	1.90	9.10	2.80	9.80	1.10	0.70
5	30	2.30	7.50	2.80	7.50	0.50	0.00
6	22	1.40	10.50	1.90	10.90	0.50	0.40
7	34	2.60	10.30	2.80	10.80	0.20	0.50
8	30	1.80	9.40	2.90	9.90	1.10	0.50
9	33	2.50	10.20	2.70	10.30	0.30	0.10
10	29	2.50	10.30	2.90	10.20	0.40	0.90
11	20	2.20	9.20	2.90	10.00	0.70	0.80
12	23	1.60	7.30	2.70	7.80	1.10	0.50
13	25	1.70	9.00	2.70	10.30	1.00	1.30
14	32	2.00	9.30	3.00	10.30	1.00	1.00
15	32	2.30	10.70	2.50	11.00	0.20	0.30
16	27	2.70	9.00	2.90	9.50	0.20	0.50
17	22	1.90	8.60	2.20	8.80	0.30	0.20
18	22	2.20	9.20	2.90	9.70	0.70	0.50
19	30	1.80	8.80	2.40	8.80	1.60	0.00
20	30	1.80	9.70	2.90	10.70	1.10	1.00
21	75	2.30	7.50	2.90	8.00	0.60	0.50
22	74	2.30	9.50	2.70	10.00	0.40	0.50
23	74	1.90	7.50	2.50	8.00	0.60	0.50
24	71	1.80	7.10	1.90	8.40	0.10	1.30
25	73	1.70	10.70	1.80	11.00	0.10	0.30
26	65	1.80	10.00	2.80	12.00	1.00	2.00
27	60	2.30	9.00	2.70	10.00	0.30	1.00
28	75	1.30	8.50	1.60	8.90	0.30	0.30
29	61	2.70	10.10	3.00	10.60	0.30	0.50
30	64	1.60	10.00	2.90	10.80	1.30	0.80
31	75	1.80	8.10	2.80	8.80	1.00	0.70
32	63	1.80	7.80	2.80	7.80	1.00	0.00
33	68	2.60	10.60	2.80	10.80	0.20	0.20
34	72	2.50	10.90	2.60	10.90	0.10	0.00
35	61	2.40	9.30	2.90	10.00	0.50	0.70
36	70	2.50	10.30	2.80	10.70	0.30	0.40
37	65	2.20	10.70	2.80	11.00	0.60	0.30
38	69	1.80	10.00	2.40	10.30	1.20	0.30

CHARACTERISTIC of the woman enrolled		1° CONTROL TIME ZERO		2° CONTROL TIME 30 days		VARIATION D = DERMA H = HYPODERM mm = millimetre	
39	75	1.80	7.30	1.90	8.80	0.10	1.50
40	70	1.90	9.90	2.90	10.00	1.00	0.10

CONCLUSIONS

ASX-2 treatment of expression wrinkles (grade I-II, on the Larnier scale) for 30 days *bis in die* in 20 women with mean age equal to 27.75 years:

Mean value of DERMAL INCREASE = 0.68 mm

Mean value of HYPODERMAL INCREASE = 0.51 mm

ASX-1 treatment of marked age wrinkles (grade V-VI, on the Larnier scale) for 30 days *bis in die* in 20 women of mean age equal to 69 years:

Mean value of DERMAL INCREASE = 0.55 mm

Mean value of HYPODERMAL INCREASE = 0.59 mm

All 40 subjects treated registered a net INCREASE in the dermis and hypodermis.

EXAMPLE 4. COMPARATIVE STUDIES

Biopsies treated with formulation ASX-1 complete versus partial formulations.

Twenty animal biopsies from surgical removal of excess cutaneous folds were analysed (Shar Pei dogs, excessive peri-ocular wrinkles and of the face in general) for the purpose of correcting major cutaneous and subcutaneous functional deficits (conjunctivitis, blepharitis, entropion, tear disorders, chronic dermatitis).

All samples were washed three times with physiological solution containing antibiotics (100 units/ml penicillin + 100 ug/ml streptomycin + 160 mg/L gentamicin + fluconazole 0.2 mg/ml) for 10 min at room temperature.

Every single biopsy of the twenty specimens collected for the experiment was sectioned into ten parts (eight controls, 1 - 2 - 3 - 4 - 5 - 6 - 7 and 8, and two samples, 9 and 10, to be treated for each subject) and suspended in a final volume of 25 ml of the respective culture solutions in 10-cm plates (Lab-Tek chamber slides, Nunc, Kamstrup, Denmark).

Eight types of controls were prepared: (1) an untreated negative control-1 resuspended in physiological solution containing antibiotics as described below; (2) a positive control-2 treated with cell culture media commonly used for cutaneous biopsies; (3) a positive control-3 treated with incomplete ASX-1 medium containing only gentamicin as active principle; (4) a positive control-4 treated with incomplete ASX-1 medium containing only lidocaine as active principle; (5) a positive control-5 treated with incomplete ASX-1 medium containing only papain as active principle; (6) a positive control-6 treated with incomplete ASX-1 medium containing only gentamicin and lidocaine as active principles; (7) positive control-7 treated with incomplete ASX-1 medium containing only papain and lidocaine as active principles; (8) a positive control-8 treated with incomplete ASX-1 medium containing only papain and gentamicin as active principles.

A type (9) sample-9 was prepared, treated with COMPLETE ASX-1 medium containing gentamicin, lidocaine and papain.

A type (10) sample-10 was prepared, treated with COMPLETE ASX-1 + ASX-2 medium containing gentamicin, lidocaine, papain and propolis.

The ten parts of each of the twenty biopsies analysed were treated as follows.

1. **Negative Control-1**: the control 1 biopsy specimens were suspended in physiological solution in 10-

cm plates (Lab-Tek chamber slides, Nunc, Kamstrup, Denmark).

2. Positive Control-2: the control 2 biopsy specimens were placed in 10-cm plates (Lab-Tek chamber slides, Nunc, Kamstrup, Denmark) in D-MEM medium supplemented with:

10% FBS (Celbio, Milan, Italy)

160 mg/L gentamicin (Schering-Plough, Milan, Italy)

2 mM L-glutamine (Life Technologies; growth medium)

50 ng/mL EGF (Sigma Aldrich, Milan, Italy).

3. Positive Control-3: the control 3 biopsy specimens were placed in 10-cm plates (Lab-Tek chamber slides, Nunc, Kamstrup, Denmark) in the solution whose composition is reported in table 11. In addition, the above-said solution contains additional active ingredients as reported in table 12.

Table 11.

Substance	Concentration
Active Principle	mg/kg
Gentamicin	40 mg/kg

Table 12.

Substance	Concentration
Additional active ingredients	mg/kg
Mixture of L-amino acids	900 mg/kg
Vitamin A	4 mg/kg
B Vitamins (B1, B2, B5, B6, B9, B12)	48 mg/kg
Vitamin C	4.5 g/kg
Vitamin D	0.0055 mg/kg
Vitamin E	10 mg/kg
Vitamin H	0.069 mg/kg
Vitamin PP	46 mg/kg
Whole beeswax	5 mg/kg
BASE in the form of a cream or emulsion (O/A or A/O) (such as: water, white vaseline, cetostearyl alcohol, mineral oil, ceteth-20, sodium phosphate, p-chloro-m-cresol, phosphoric acid)	q.s. per kg of product

4. Positive Control-4: the control 4 biopsy specimens were placed in 10-cm plates (Lab-Tek chamber slides, Nunc, Kamstrup, Denmark) in the solution whose composition is reported in table 13. In addition, the above-said solution contains additional active ingredients as reported in table 12.

Table 13.

Substance	Concentration
Active Principle	mg/kg
Lidocaine	10 mg/kg

5. Positive Control-5: the control 5 biopsy specimens were placed in 10-cm plates (Lab-Tek chamber slides, Nunc, Kamstrup, Denmark) in the solution whose composition is reported in table 14. In addition, the above-said solution contains additional active ingredients as reported in table 12.

Table 14.

Substance	Concentration
Active Principle	mg/kg
Papain FU	56.00 mg/kg

6. Positive Control-6: the control 6 biopsy specimens were placed in 10-cm plates (Lab-Tek chamber slides, Nunc, Kamstrup, Denmark) in the solution whose composition is reported in table 15. In addition, the above-said solution contains additional active ingredients as reported in table 12.

Table 15.

Substance	Concentration
Active Principles	mg/kg
Gentamicin	40 mg/kg
Lidocaine	10 mg/kg

7. Positive Control-7: the control 7 biopsy specimens were placed in 10-cm plates (Lab-Tek chamber

slides, Nunc, Kamstrup, Denmark) in the solution whose composition is reported in table 16. In addition, the above-said solution contains additional active ingredients as reported in table 12.

Table 16.

Substance	Concentration
Active Principles	mg/kg
Lidocaine	10 mg/kg
Papain FU	56.00 mg/kg

8. Positive Control-8: the control 8 biopsy specimens were placed in 10-cm plates (Lab-Tek chamber slides, Nunc, Kamstrup, Denmark) in the solution whose composition is reported in table 17. In addition, the above-said solution contains additional active ingredients as reported in table 12.

Table 17.

Substance	Concentration
Active Principles	mg/kg
Gentamicin	40 mg/kg
Papain FU	56.00 mg/kg

9. Samples: the sample 9 biopsy specimens were placed in 10-cm plates (Lab-Tek chamber slides, Nunc, Kamstrup, Denmark) in Culture medium *ASX-1-INFUS COMPLETE* whose composition is reported in table 18. In addition, the above-said solution contains additional active ingredients as reported in table 12.

Table 18.

Substance	Concentration
Active Principles	mg/kg
Gentamicin	40 mg/kg
Lidocaine	10 mg/kg
Papain FU	56.00 mg/kg

10. Samples: the sample 10 biopsy specimens were placed in 10-cm plates (Lab-Tek chamber slides, Nunc, Kamstrup, Denmark) in Culture medium *ASX-1-ASX-2-INFUS COMPLETE* whose composition is reported in table 19. In addition, the above-said solution contains additional active ingredients as reported in table 12.

Table 19.

Substance	Concentration
Active Principles	mg/kg
Gentamicin	40 mg/kg
Lidocaine	10 mg/kg
Papain FU	56.00 mg/kg
Propolis	10% (w/v)

All samples were incubated for 15 days in a Heraeus incubator thermostatically controlled at the temperature of 37°C with an constantly supplied atmosphere containing 5% CO_2 (v/v in air). Two-thirds of the culture medium were replaced every 7 days. All biopsy tissues, used in culture constitute a possible co-conditioning optional support for the three dimensional support of the cell samples under study.

Characterisation of samples treated with complete ASX-1-INFUS culture medium versus controls treated with incomplete ASX-1-INFUS culture medium

The results relative to the expression of type I collagen, type IV collagen and of cytokeratins 1, 5, 10 and 14 are presented with a quantitative scale in table 20.

Table 20.

Markers	Contr. 1	Contr. 2	Contr. 3	Contr. 4	Contr. 5	Contr. 6	Contr. 7	Contr. 8	Samp. 9
collagen type I	-/+	+	+	+	+	+	++	+	++++
collagen type IV	-/+	+++	++	+	+	++	+++	++	+++

Markers	Contr. 1	Contr. 2	Contr. 3	Contr. 4	Contr. 5	Contr. 6	Contr. 7	Contr. 8	Samp. 9
cytokeratin 1	+	+++	+	+	+	+	++	+	++++
cytokeratin 5	+	+++	++	+	++	+	++	++	+++
cytokeratin 10	+	+++	+	+	+++	++	++	+	++++
cytokeratin 14	++	+++++	+++	+++	+	+++	++++	++	++

Legend
--- = band absent
-/+ = light presence of band
+ = band present thin
++ = band present medium
+++ = band present extensive
++++ = band present high
+++++ = band present effuse

The culturing difference between use of the re-equilibrating solution ASX-1 COMPLETE (Sample-9) and the other culture media tested (Controls 1-8) which do not induce the correct synthesis and secretion of the proteins typical of eutrophic cutaneous and subcutaneous extracellular matrix.

It is specified that the solution ASX-1 COMPLETE (Sample-9) supplies in a single solution:

- said amino glycoside antibiotic, for example gentamicin or a derivative thereof; and
- said amino-amidic anaesthetic, lidocaine or a derivative thereof; and
- a proteolytic enzyme, for example papain.

The colorimetric method used to evaluate the entire cutaneous and subcutaneous matrix produced was described above ("Alcian blue"), the method of choice for evaluating the extracellular matrix in its entirety [2]. The results are reported in table 21.

Table 21.

Culture Media tested	Results at day 90 of incubation
CTRL1 = physiological solution	matrix stained at 0.1% and not corresponding morpho-histologically to intact cutaneous and subcutaneous matrix

Culture Media tested	Results at day 90 of incubation
CTRL2 = Commercial culture medium (DMEM, F12, EGF)	matrix stained at 30% and not corresponding morpho-histologically to intact cutaneous and hypodermal matrix, lack of peri-cellular and inter-zonal matrix
CTRL3 = Solution containing only one active principle, *salicylic acid*	matrix stained at 25% and not corresponding morpho-histologically to intact cutaneous and hypodermal matrix with broad areas free of collagen fibres
CTRL4 = Solution containing only one active principle: *zinc oxide*	matrix stained at 25% and not corresponding morpho-histologically to intact cutaneous and hypodermal matrix with broad areas free of collagen fibres
CTRL5 = Solution containing only one active principle: *papain*	matrix stained at 25% and not corresponding morpho-histologically to intact cutaneous and hypodermal matrix with broad areas free of collagen fibres
CTRL6 = Solution containing only two active principles, *gentamicin + zinc oxide*	matrix stained at 50% and not corresponding morpho-histologically to intact cutaneous and hypodermal matrix with broad areas free of *inter-zonal matrix*
CTRL7 = Solution containing only two active principles, *papain + zinc oxide*	matrix stained at 50% and not corresponding morpho-histologically to intact cutaneous and hypodermal matrix for the presence of broad areas free of *inter-zonal matrix*
CTRL8 = Solution containing only two active principles, *gentamicin + papain*	matrix stained at 50% and not corresponding morpho-histologically to intact cutaneous and hypodermal matrix for the presence of broad areas free of *inter-zonal matrix*
SAMPLE 9 = Solution *ASX-1 INFUS* COMPLETE containing three active principles, *salicylic acid + zinc oxide + papain*	matrix stained at 90% and corresponding morpho-histologically to intact cutaneous and hypodermal matrix

Such data were collected at days 7, 10, 15, 30, 45, 60, 75, and 90 of culture in parallel for controls and samples.

Furthermore, also at days 7, 10, 15, 30, 45, 60, 75, and 90 of culture, using such methodologies as Western Blot (WB), continuous monitoring was performed on the capacity of the fibroblasts present in the tested cultures to actively product collagen fibres, such as collagen type I, which is present at high

concentration in the matrix of healthy covering tissues and of the sample cells treated with solution *ASX-1 INFUS* complete.

It seems appropriate to point out that the culture treated with complete culturing media supplemented only with growth factors (control-2) or with complete culture media supplemented only with gentamicin (control-3) or with complete culture media supplemented with only lidocaine (control-4) or with complete culture media supplemented only with papain (control-5) or with complete culture media with only two active principles such as gentamicin + lidocaine (control-6), or with only two active principles such as papain + lidocaine (control-7), or, finally, with only two active principles such as gentamicin + papain (control-8), do not result in the deposition of normal cutaneous or subcutaneous extracellular matrix equal to or comparable with the results achieved with the use of all three synergistic active principles, such as gentamicin + lidocaine + papain (Sample-9) of the solution Culture medium *ASX-1-INFUS COMPLETE*.

Characterization of samples treated with Culture medium ASX-1-INFUS complete versus controls treated with Culture medium ASX-1-ASX-2-INFUS complete.

The results regarding expression of collagen type I, collagen type IV and the cytokeratins 1, 5, 10 and 14 were expressed with a quantitative scale in table 22.

Table 22.

Markers	Sample 9	Sample 10
collagen type I	++++	+++++
collagen type IV	+++	+++
cytokeratin 1	++++	+++++
cytokeratin 5	+++	+++
cytokeratin 10	++++	++++
cytokeratin 14	++	+++

Legend
--- = band absent
-/+ = light presence of band
+ = band present thin

++ = band present medium
+++ = band present extensive
++++ = band present high
+++++ = band present effuse

The culture difference between use of solution ASX-1 COMPLETE (Sample-9) and solution ASX-1-ASX-2 COMPLETE (Sample-10) results in a perfecting of the correct secretion of the proteins typical of eutrophic cutaneous and subcutaneous extracellular matrix.

It is specified that the solution ASX-1-ASX-2 COMPLETE (Sample-10) comprises in a single solution:

- an aminoglycoside antibiotic, for example gentamicin or a derivative thereof; and
- an amino amide anaesthetic, lidocaine or a derivative thereof; and
- a proteolytic enzyme, for example papain; and
- an apitherapeutic product, for example propolis.

The colorimetric method used to evaluate the entire cutaneous and subcutaneous matrix produced was described above ("Alcian blue"), the method of choice for evaluating the extracellular matrix in its entirety [2]. The results are reported in table 23.

Table 23.

Culture Media tested	Results at day 90 of incubation
SAMPLE 9 = Solution *ASX-1 INFUS* COMPLETE containing three active principles, *salicylic acid + lidocaine + papain*	matrix stained at 90% and corresponding morpho-histologically to intact cutaneous and hypodermal matrix
SAMPLE 10 = Solution *ASX-1-ASX-2 INFUS* COMPLETE containing four active principles, *salicylic acid + lidocaine + papain + propolis*	matrix stained at 100% and corresponding morpho-histologically to intact cutaneous and hypodermal matrix

Such data were collected in parallel at days 7, 10, 15, 30, 45, 60, 75, and 90 of culture for the Controls and Samples.

Furthermore, also at days 7, 10, 15, 30, 45, 60, 75, and 90 of culturing, methods such as Western Blot (WB) were used to continuously monitor the ability of

fibroblasts present in the tested culture to actively produce collagen fibres, such as type I collagen, present at eutrophic concentrations in the matrix of healthy covering tissues and also in the sample cells treated with the *ASX-1-ASX-2 INFUS* complete.

It seems appropriate to point out that the cultures treated with all three synergistic active principles, such as gentamicin + lidocaine + papain (Sample-9) of the solution Culture medium *ASX-1-INFUS COMPLETE*, result in the deposition of normal cutaneous or subcutaneous matrix, but the use of all four synergistic active principles, such as gentamicin + lidocaine + papain + propolis (Sample-10) of the Culture medium *ASX-1-ASX-2 INFUS COMPLETE*, results in the deposition of an excellent corresponding to eutrophic cutaneous and subcutaneous tissue.

Naturally, the details of realisation and embodiments may vary even appreciably from what has been described and illustrated without departing from the field of protection of the present invention, as defined by the annexed claims.

BIBLIOGRAFIA

1. Robinson M, Reynolds AJ, Gharzi A, Jahoda CA. *In vivo* induction of hair growth by dermal cells isolated from hair follicles after extended organ culture. J Invest Dermatol. 2001 Sep;117(3):596-604.
2. Griffith, L. G. & Naughton, G. Tissue engineering--current challenges and expanding opportunities. Science. 2002;295:1009-1014 .
3. Wagers, A. J., Christensen, J. L., & Weissman, I. L. Cell fate determination from stem cells. Gene Ther. 2002;9:606-612 .
4. Bianco, P. and Cossu, G. Uno, nessuno e centomila: searching for the identity of mesodermal progenitors. Exp Cell Res. 1999 Sep 15;251(2):257-63.
5. Rabinovitch, M. & De Stefano, M. J. Cell shape changes induced by cationic anesthetics. J. Exp. Med. 1976;143: 290-304.
6. Parker F.: "Cute e ormoni" in Williams R.H. eds: "Trattato di Endocrinologia". III° edizione italiana, Piccin, Padova. 1979; vol II°, cap 23: 1115-19.

CLAIMS

1. A composition comprising at least two among:

(i) at least one antibiotic, the precursors or natural derivatives thereof, preferably and aminoglycoside antibiotic;

(ii) at least one amino amidic-local anaesthetic the precursors or natural or synthetic derivatives thereof,

(iii) at least one proteolytic agent, the precursors or natural or synthetic derivatives thereof, and

(iv) at least one antiseptic agent and salt reconstituent , the precursors or natural or synthetic derivatives thereof, preferably an apitherapeutic antiseptic agent.

2. The composition according to claim 1, wherein said at least one antibiotic is selected from among: gentamicin, micronomicin, spectinomycin, amikacin, kanamicina, tobramycin, neomycin, metilmycin, paromycin, streptomycin, preferably gentamicin.

3. The composition according to claim 1 or claim 2, wherein at least on amino-amidic local anaesthetic is selected from among: lidocaine, lidocaine hydrochloride, mepivacaine, prilocaine, bupivacaine, etidocaine, ropivacaine, preferably lidocaine.

4. The composition according to any of the previous claims, wherein said at least one proteolytic agent is selected from among: papain, papain FU, collagenases, serratiopeptidases, bromelain, bradykinase, *Clostridium* peptidases, proteolytic enzymes expressed by *Lactobacillus acidophilus*, proteolytic enzymes expressed by the genus *Aspergillus*, proteases, alliinases and fibrinolysin, preferably papain FU.

5. The composition according to any of the previous claims, wherein said at least on antiseptic and salt reconstituent is selected from among: propolis, concentrated propolis , de-waxed propolis, concentrated de-waxed propolis, royal jelly and beeswax, preferably propolis.

6. The composition according to any of the previous claims, wherein said at least one antibiotic is present in a quantity comprised between 4 mg/Kg and 650 mg/Kg with respect to the total weight of the composition, preferably between 40 mg/Kg and 320 mg/Kg.

7. The composition according to any of the previous claims, wherein said at least one amino-amidic local anaesthetic is present in a quantity between 1 mg/Kg and 400 mg/Kg with respect to the total weight of the composition, preferably between 1 mg/Kg and 100 mg/Kg.

8. The composition according to any of the previous claims, wherein said at least one proteolytic agent is present in a quantity comprised between 1 mg/Kg and 100 mg/Kg with respect to the total weight of the composition, preferably between 4 mg/Kg and 80 mg/Kg.

9. The composition according to any of the previous claims, wherein said at least one antiseptic and salt reconstituent is present in a quantity comprised between 0.1% (w/v) and 50% (w/v) with respect to the total weight of the composition, preferably between 1% (w/v) and 30% (w/v).

10. The composition according to any of the previous claims, wherein said composition also comprises at least one among a physiologically acceptable vehicle, an amino acid, a sugar, a vitamin, a glucosaminoglycan, a peptide, a supplement.

11. The composition according to any of the

previous claims, wherein said composition is formulated as a cream, unguent, ointment, powder, patch, impregnated membrane, solution, emulsion, suspension, vesicular dispersion, lotion, gel, spray.

12. A pharmaceutical composition for stimulating the repair, remodelling and/or regeneration of lax, atrophic, senescent cutaneous and/or subcutaneous tissue and/or surgical scars with lax, atrophic margins and/or with cutaneous grooves and/or red cutaneous striae comprising a composition according to any of the claims 1 to 11.

13. An *in vitro* cell or tissue culture medium, preferably for cutaneous and/or subcutaneous tissue comprising a composition according to any of the claims 1 to 11.

14. A cosmetic method for the repair, remodelling and/or regeneration of a lax, atrophic and/or senescent cutaneous and/or subcutaneous tissue in a mammal envisioning the use of a composition according to any of the claims 1 to 11.

15. The use of a composition according to any of the claims 1 to 11 for the anti-wrinkle cosmetic treatment of a lax, atrophic and/or senescent cutaneous and/or subcutaneous tissue in a mammal.

16. The use of a composition comprising i) proteolytic agent, the precursors or natural or synthetic derivatives thereof, and ii) at least one antiseptic and salt reconstituting agent, the precursors or natural or synthetic derivatives thereof, preferably an apitherapeutic antiseptic agent, for the cosmetic anti-wrinkle treatment of a lax, atrophic and/or senescent cutaneous and/or subcutaneous tissue in a mammal.

17. The use according to claim 16, wherein said at least one proteolytic agent is selected from among:

papain, papain FU, collagenases, serratiopeptidases, bromelain, bradykinase, *Clostridium* peptidases, proteolytic enzymes expressed by *Lactobacillus acidophilus,* proteolytic enzymes expressed by the genus *Aspergillus,* proteases, alliinases and fibrinolysin, preferably papain FU.

18. The use according claim 16 or claim 17, wherein said at least one antiseptic and salt reconstituent is selected from among: propolis, concentrated propolis , de-waxed propolis, concentrated de-waxed propolis, royal jelly and beeswax, preferably propolis.

19. The use according to any of the claims 16 to 18, wherein said at least one proteolytic agent is present in a quantity comprised between 1 mg/Kg and 100 mg/Kg with respect to the total weight of the composition, preferably between 4 mg/Kg and 80 mg/Kg.

20. The use according to any one of the claims 16 to 19, wherein said at least one antiseptic and salt reconstituent is present in a quantity comprised between 0.1% (w/v) and 50% (w/v) with respect to the total volume of the composition, preferably between 1% (w/v) and 30% (w/v).

21. The use according to any one of the claims 16 to 20, wherein said composition also comprises at least one among a physiologically acceptable vehicle, an amino acid, a sugar, a vitamin, a glucosaminoglycan, a peptide, a supplement.

22. The use according to any one of the claims 16 to 21, wherein said composition is formulated as a cream, unguent, ointment, powder, patch, impregnated membrane, solution, emulsion, suspension, vesicular dispersion, lotion, gel, spray.

INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2009/053287

A. CLASSIFICATION OF SUBJECT MATTER
INV. A61P17/00 A61P17/02 A61P17/12 A61P17/16 A61K31/00
A61K31/167 A61K31/7036 A61K45/06 A61K8/42 A61K8/60
A61K8/66 A61K8/98 A61K35/64 A61Q19/08 A61K38/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
A61K A61Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)
EPO-Internal, EMBASE, BIOSIS, WPI Data

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2004/076671 A1 (TIPPETT ALETHA [US]) 22 April 2004 (2004-04-22) paragraphs [0010], [0028], [0029], [0031], [0033], [0036], [0044], [0045], [0047]; claims 1,3,4,13,18,29	1-4,6,7, 10-14
X	WO 01/66066 A (AUSTRALIAN IMP ERS LTD [US]) 13 September 2001 (2001-09-13) page 1, line 5 - line 11 page 1, line 18 - line 21 page 2, line 21 - line 33 page 3, line 10 page 4, line 21 - line 27 examples 5,6 claims	1,4,5, 8-22

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[X] Further documents are listed in the continuation of Box C.

[X] See patent family annex.

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"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
30 November 2009	07/12/2009

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INTERNATIONAL SEARCH REPORT

International application No
PCT/IB2009/053287

C(Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	US 2006/233783 A1 (GOMEZ TORRES HAROLD A [CO]) 19 October 2006 (2006-10-19) paragraphs [0001], [0044], [0066], [0076], [0078], [0081], [0082], [0086], [0088] - [0093], [0142] - [0144]; claims 1-4,11	1,3,4, 10-14
X	US 4 748 022 A (BUSCIGLIO JOHN A [US]) 31 May 1988 (1988-05-31) claims column 1, line 10 - line 15 column 4, line 27 - line 38 column 5, line 12 - line 40	1,3,5,7, 9-14
X	WO 02/076411 A (OWOC DORIAN [PL]) 3 October 2002 (2002-10-03) page 2, line 61 - line 81 page 2, line 90 - page 3, line 119 page 7, line 301 - line 324 claims examples 3,5,7,8,16	1,2,5, 9-14
X	DE 199 04 801 A1 (KLOSSNER AXEL [DE]) 10 August 2000 (2000-08-10) example 1 column 3, line 20 - line 27 column 1, line 5 - line 29 column 1, line 38 - line 40 claims 1-5,9	1,2,5,6, 9-14
X	WO 2004/022034 A (DERMAPHYT LTD [PL]; MASLANKY JERZY [PL]) 18 March 2004 (2004-03-18) claims 1,6 page 4, line 11 - line 21 page 5, line 9 - line 12 page 7, line 19 - line 26 page 8, line 3 - line 6 page 9, line 4 - line 26 page 11, line 26 page 12, line 10 - line 11 page 16, line 23 - line 29 page 17, line 19 - line 25	1,2,5, 9-14
X	US 2008/044459 A1 (LIVINGSTON JAMES A [US]) 21 February 2008 (2008-02-21) paragraphs [0057], [0075], [0083], [0087], [0109], [0125], [0130], [0135], [0141], [0142], [0241], [0262]	1,2,4,8, 10-14
X	WO 2007/076446 A (COLGATE PALMOLIVE CO [US]; FETISSOVA NATALY [RU]; BLANVALET CLAUDE [BE) 5 July 2007 (2007-07-05) paragraphs [0020], [0033] - [0035], [0039], [0053], [0054], [0062] claims 1,2,14,20-23	1,4,5, 8-11,13

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No
PCT/IB2009/053287

Patent document cited in search report	Publication date	Patent family member(s)	Publication date
US 2004076671 A1	22-04-2004	US 2007110797 A1	17-05-2007
		US 2007104770 A1	10-05-2007
WO 0166066 A	13-09-2001	AU 5002301 A	17-09-2001
		AU 2001250023 B2	18-08-2005
		CA 2401391 A1	13-09-2001
		EP 1265584 A1	18-12-2002
		US 6416769 B1	09-07-2002
US 2006233783 A1	19-10-2006	AU 2003219351 A1	01-11-2004
		WO 2004089406 A1	21-10-2004
US 4748022 A	31-05-1988	CA 1320909 C	03-08-1993
		WO 8910750 A1	16-11-1989
WO 02076411 A	03-10-2002	AU 2002241416 A1	08-10-2002
DE 19904801 A1	10-08-2000	NONE	
WO 2004022034 A	18-03-2004	AU 2002368219 A1	29-03-2004
		PL 355298 A1	09-02-2004
US 2008044459 A1	21-02-2008	NONE	
WO 2007076446 A	05-07-2007	AR 058645 A1	13-02-2008
		AU 2006330510 A1	05-07-2007
		CA 2631768 A1	05-07-2007
		CN 101340886 A	07-01-2009
		EP 1973515 A1	01-10-2008
		JP 2009521508 T	04-06-2009