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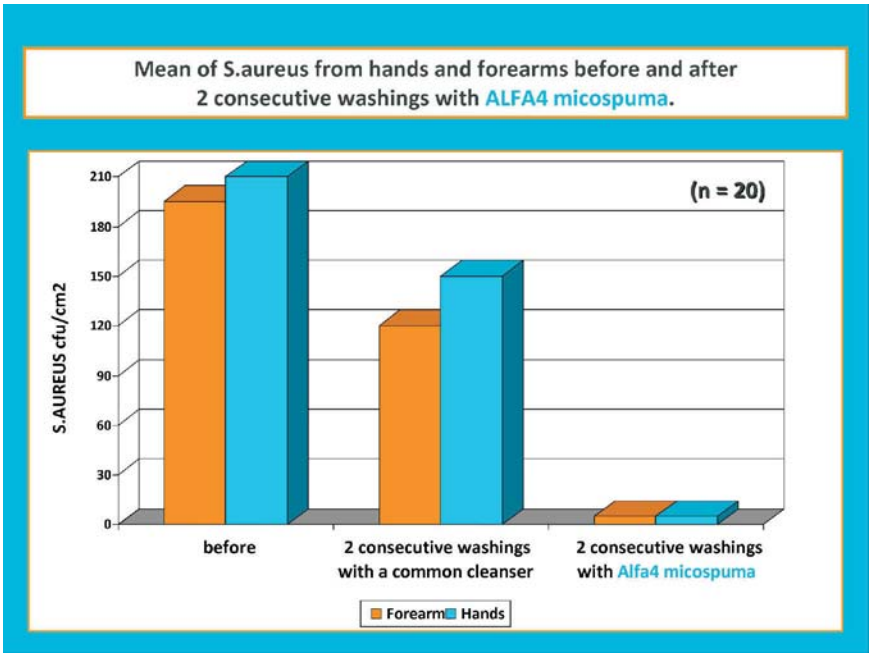

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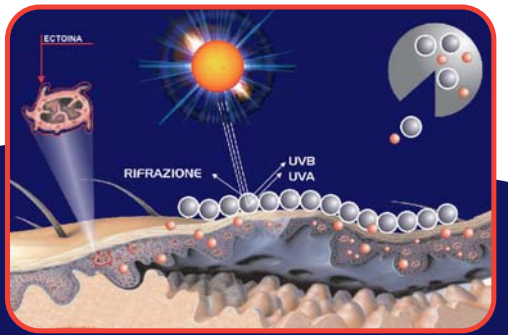

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Trimestrale di Dermatologia Cosmetologica
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Contents

Original Laboratory Studies

Book Reviews

New Insights on Anti-Aging Activity of Chitin Nanofibril-Hyaluronan Block Copolymers Entrapping Active Ingredients: *In Vitro and In Vivo Study*

Pierfrancesco Morganti[1], Marco Palombo[2], Giuseppe Fabrizi[3], Francesco Guarneri[4], Fabiano Svolacchia[5], Antonio Cardillo[6], Paola Del Ciotto[6], Francesco Carezzi[6], Gianluca Morganti[6]

[1] Prof of Skin Pharmacology, Dermatology Depart., 2nd University of Naples, Italy;
Visiting Professor, Dermatology Depart., China Medical University, Shenyang, China;
Head of R&D, Centre of Nanoscience, Mavi Sud, s. r. l, Italy

[2] Department of Plastic, Reconstructive and Aesthetic Surgery, CTO Hospital, Rome, Italy

[3] Head of the Dermatological Department, University of Parma, Italy

[4] Dermatological Department, University of Messina, Italy

[6] R&D, Centre of Nanoscience, Mavi Sud, s. r. l, Italy

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Key words: *Chitin nanofibril; Hyaluronan; Block copolymer; Skin aging; In-office treatment; Intrinsic aging; Extrinsic aging; Rejuvenation treatment;*

Summary

Background: Some observations suggest a similar molecular mechanism for genetic aging and photo-aging: the same proteins mediating cellular division and senescence, appear to mediate DNA damage, after UV irradiation or oxidative stress. Consequently collagen-fibers are disorganized or cross-linked and elastic fibers appear damaged, as well as redox signaling are amplified ,and chaperon proteins, assisting the folding of macromolecular structures as HSP47, are no more sufficiently synthesized. Thus, fine lines and wrinkles appear leading to formation of black spots, sagging, and loose skin.

Aims: It was designed to control *in vitro* and *in vivo* the antiaging activity of cosmetic formulations based on the use of Chitin nanofibril-Hyaluronan (CN-HA) block copolymeric nanoparticles, entrapping different active ingredients, to verify their effectiveness and safeness as rejuvenation treatment biologically active, capable to support and increase the in-office procedures of Plastic Surgeons and Dermatologists.

Methods: *In vitro* synthesis of collagen I, III, IV, and HSP47, as well as the release of IL-8 and Metalloproteinase-1, were controlled in fibroblast cultures by immunocytochemical methods, while the anti-collagenase activity and the relative citotoxicity were verified by colorimetric methods, on cultures of both keratinocytes and fibroblasts.

In vivo. Skin hydration and TEWL were controlled by the 3C-system, while the whitening activity was measured by the Chromameter C 300.
Results and Conclusions: According to our previous published studies, the obtained positive results confirm on one hand the capacity the block-copolymers CN-HA have to easily entrap and modulate the efficacy of different active ingredients ,increasing their delivery and effectiveness at level of the skin layers. On the other hand they seem to support the possibility for designing and formulating innovative anti-aging cosmetics, useful to optimize the in-office rejuvenation treatments of Plastic Surgeons and Dermatologists.

Riassunto

Introduzione: Diversi studi pongono in evidenza come meccanismi molecolari analoghi siano alla base sia dell'invecchiamento genetico che del foto-invecchiamento: le stesse proteine-segnale che mediano la divisione cellulare e la senescenza, sembrano mediare anche i danni al DNA, provocati dallo stress ossidativo e dagli UV. Come conseguenza le fibre di collageno appaiono disorganizzate o irrigidite da legami crociati, mentre le fibre elastiche risultano danneggiate, le reazioni di ossido riduzione si moltiplicano e le cosiddette shock proteine che regolano l'avvolgimento delle macromolecole, come la proteina HSP47, non vengono più sintetizzate con regolarità. Così appaiono rughe sottili e profonde, iperpigmentazioni; la cute si rilassa ed invecchia precocemente.
Scopi: Con questo studio si è voluta controllare l'attività anti invecchiamento di formulazioni cosmetiche basate sull'uso di block copolimeri CN-HA (Chitina nanofibrille - Acido ialuronico) che inglobavano diversi ingredienti attivi, per verificarne l'efficacia e la sicurezza nell'uso. Quale trattamento di ringiovanimento biologicamente corretto, queste formulazioni sono state ideate per essere utilizzate come supporto efficace in grado di migliorare e prolungare nel tempo le diverse procedure invasive e non invasive utilizzate negli studi di Chirurghi plastici e Dermatologi.
Metodi: *In vitro*. Su culture di fibroblasti è stata controllata la capacità di sintesi del collagene I, III, IV, oltre che dell'HSP47, utilizzando metodiche citochimiche, mentre il rilascio della citochina IL-8 e della metalloproteina-1, oltre che il controllo della eventuale citotossicità dei diversi ingredienti utilizzati, sono stati verificati mediante metodi colorimetrici.
In vivo. Mediante uno studio preliminare sono state controllate in doppio ceco l'idratazione cutanea e la TEWL, utilizzando il 3C-System, mentre le iperpigmentazioni sono state valutate con il Chromameter 300.
Risultati: In accordo con le nostre precedenti esperienze, questo studio ha confermato *in vitro* la capacità dei copolimeri CN-HA nell'inglobare facilmente i diversi principi attivi utilizzati, modulandone l'efficacia e incrementadone la penetrazione attraverso gli strati cutanei. D'altra parte è stato anche posto in evidenza *in vivo* come queste particolari nanoparticelle possano essere utilizzate per formulare prodotti anti invecchiamento innovativi, utili per esaltare e prolungare nel tempo i risultati ottenuti con le diverse metodiche di ringiovanimento adottate negli studi specializzati di Chirurghi Plastici e Dermatologi.

INTRODUCTION

Some observations suggest a common molecular mechanism for the chronological genetic aging and the environment-connected photoaging: the same signaling proteins, that mediate senescence and cell division, appear to mediate DNA damage, after UV irradiation or oxidative stress (1,2). The exposure to either UV or other aggressive agents, generating respectively reactive oxygen, nitrogen, or iron species (ROS, RNS, and RIS), results in premature entry into the senescent state (3). Thus in photoaged skin (extrinsic aging) collagen fibrils appear disorganized, abnormally cross-linked and elastin-containing material (4), as well as in genetic aging (intrinsic aging) the decline in signaling molecules and receptors induce fibroblast senescence and alteration in the synthesis and maturation of both collagen and scaffold stress proteins, as HSP47 (Fig.1) (5-7). In any way both intrinsic and extrinsic aging, inducing an high production of free radicals with a consequent generation of mt-DNA mutations, lead to chronic oxidative stress. Moreover, also if sunlight may be sometime beneficial, even a single minimum erythema dose (1 MED) can damages the skin matrix for ever. Thus while UVB irradiation, directly absorbed by cellular DNA, leads to formation of DNA lesions with a defective antigen presentation and formation, most of the adverse effects of UVA seem to be the result of an oxidative damage primarily inducing skin' lipid peroxidation, dimer formation, and cancer (Fig.2) (6, 8-10). Lipid peroxidation is, in fact, a well known consequence of the oxidative, stress affecting skin lipids, whether on the surface or in deeper layers.It is a chain reaction initiated by the singlet oxygen mainly produced by UVA, as well as by the superoxide anion.

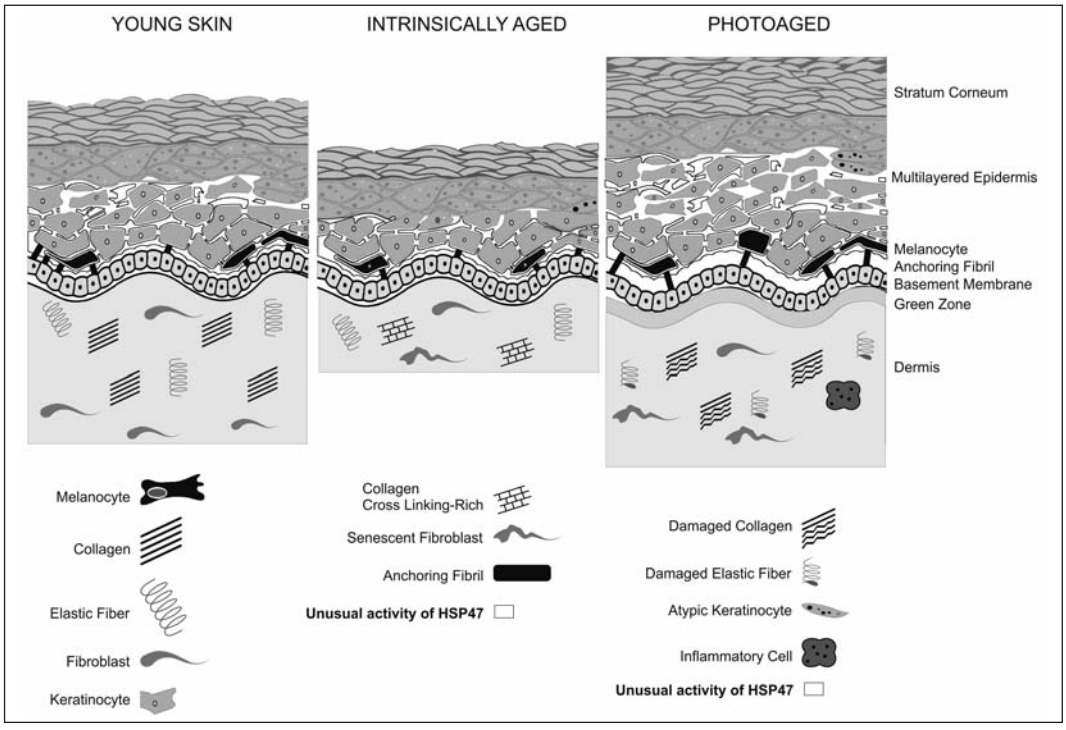


Fig. 1 *Reduced synthesis of HSP47 has been underlined in intrinsically skin: aged and photo-aged.*

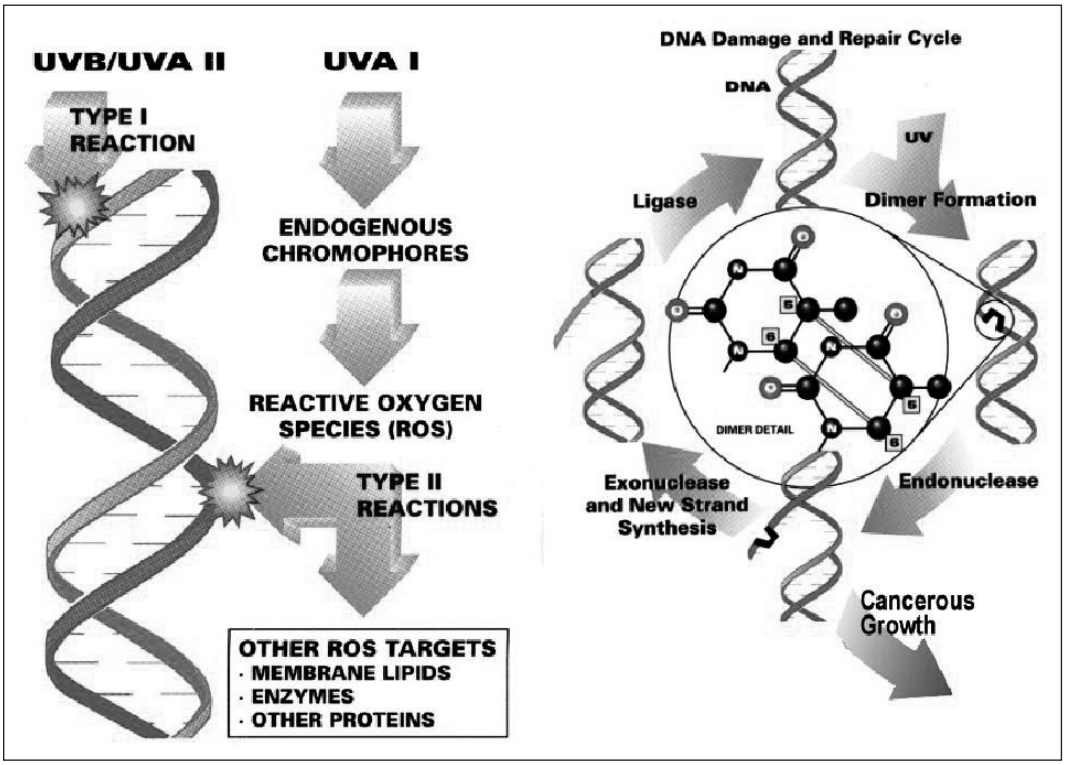


Fig. 2 *Direct and indirect activity of UVB/UVA rays on skin DNA.*

In addition, UV rays induce the secretion of cytokines (as Tumor Necrosis Factor-alpha and Interleukin-8) that, leading to propagation of intracellular signaling, interfere with the synthesis of procollagen I and III, stimulating the production of matrix-degrading enzymes, such as metalloproteinases (6, 11-13). As a consequence of the excessive formation of ROS it happens that, collagen I and III at level of dermis, and collagen IV at level of basal lamina, underline a reduced synthesis, while the skin antioxidants and the immunocompetent cells show a reduced presence and activity. As a result the epidermis appears atrophic for a decreased keratinocytes turnover, while seborrheic keratoses underlined by black spots became evident, with an higher induction of abnormal signaling events (14-16). Thus final lines and wrinkles appear gradually and the mechanical force of gravity, pulling down facial skin, lead to formation of a sagging and loose skin (5, 12-18).

For all these reasons the administration of antioxidants and immune modulating compounds are believed to be useful for the skin, removing respectively both free radical in excess and inflammatory agents. In the same way the topical use of innovative ingredients should be capable to repair an altered skin barrier with the potential of protecting the skin from UV aggression, preserving /repairing the integrity of collagens and other components of extracellular matrix (ECM).

AIMS

The aim of this study was to control *in vitro* the activity, safeness, and efficacy of Chitin-Hyaluronan (CN-HA) nanoparticles entrapping

different ingredients, and verify *in vivo* on skin surface of women affected by photoaging, the effectiveness of the designed nanoparticles, when enclosed into different innovative cosmetic formulations. The basic scope has been to give the plastic surgeon a professional-rejuvenation cosmetic treatment that, being biologically effective at level of the skin cells, should be capable to support and increase the efficacy of the in-office usual procedures (dermabrasion, injectable materials, lipotransfer or skin resurfacing ablative or not ablative), and to accelerate the skin regeneration, maintaining its homeostasis for a long period of time, also before and after more drastic surgical methods, such as blepharoplasty, rytidectomy, rhinoplasty, and solid implants in the chin, upper cheek areas, etc.

MATERIALS AND METHODS

Study Design

The goal of plastic surgeon, and dermatologist, in treating the picture of inflamm-aging, is to reestablish the youthful skin appearance for the longest time possible.

At this purpose, four different products have been designed and formulated for providing the surgeon with the necessary procedure and products to accomplish this goal. Thus, we try to control *in vitro*, on cultures of normal and aged keratinocytes and fibroblasts, the activity of different CN-HA block copolymeric nanoparticles verifying some parameters, such as their antioxidant properties, the possibility to modulate the IL-8 and MMP-1 release, the effectiveness at level of the relative synthesis of collagen I, II, III together with the right recovery of chaperon protein HSP47, and their capacity to regulate the grade of collagen degradation as result of the collagenase activity.

All the nanoparticles, inserted into different nanoemulsions were also controlled *in vivo* by a preliminary double blind multicenter dermatological study to verify the effectiveness of the global cosmetic treatment on the face and neck of women affected by photoaging.

Materials

All the block copolymer nanoparticles: such as Chitin-Hyaluronan nanoparticles (CN); CN-HA-gelatin-gly; CN-HA-arg-desamido collagen; CN-HA-MEB; CN-HA-ectoin-betaglucan; CN-HA-PCA-peptides; CN-HA-PCA-lys; CN-HA-Zn-TiO2-lutein; CN-HA-vit C-panthenol-peptides; CN-HA-arg-gly and the related vehicles used, were supplied by Mavi Sud, Aprilia (LT), Italy.

Formulations designed

Eye Contour Cream[1]:

Aqua (Water), Buxus Chinensis (Jojoba Extract), Petrolatum, Sodium PCA, Cetyl PEG/PPG-10/1 Dimethicone, Glycerin, Desamido Collagen, Tocopheryl Acetate, Sodium Chloride, Arginine PCA, Caprylic/Capric Triglyceride, Imidazolidinyl Urea, Methylparaben, Titanium Dioxide, Chitin (Nano-Fibrils), Parfum (Fragrance), Gelatin, Glycine, Alumina, Polyhydroxystearic Acid, Propylparaben, Sodium Hyaluronate, Silica.

Day Cream[2]:

Aqua (Water), Glycerin, Cyclopentasiloxane, Prunus Dulcis (Sweet Almond Oil), Paraffinum Liquidum (Mineral Oil), Butyrospermum Parkii (Shea Butter), Buxus Chinensis (Jojoba Oil), Sodium PCA, Zinc Oxide, Dimethicone, Lecithin, Palmitic Acid, Titanium Dioxide, C12-16 Alcohols, Silica, Alumina, Glyceryl Stearate, Dimethicone Crosspolymer, Cyclohexasiloxane, PEG-100 Stearate, Phenoxyethanol, Parfum (Fragrance), Tocopheryl Acetate, Sodium Ascorbyl Phosphate, Gelatin, Imidazolidinyl Urea, Acrylates Dimethicone Acrylate Ethylhexyl

[1] *Trade Name: QM Contorno Occhi®, Mavi sud s.r.l., Italy*
[2] *Trade Name: QM Giorno®, Mavi sud s.r.l., Italy*

Acrylate, Ectoin, Cetyl Alcohol, Glycine, Sodium Hyaluronate, Sodium Carboxymethyl Betaglucan, Polyacrylamide, PEG-75 Stearate, Methylparaben, Ceteth-20, Xanthan Gum, Laureth-7, C13-14 Isoparaffin, Steareth-20, Propylparaben, Chitin (Nano-Fibrils), Lauroyl Lysine, Disodium EDTA, Melatonin, Xanthophyll (Lutein).

Night Cream[3]:

Aqua (Water), Glycerin, Butyrospermum Parkii (Shea Butter), Paraffinum Liquidum (Mineral Oil), Sodium PCA, Prunus Dulcis (Sweet Almond Extract), Olea Europaea (Olive Oil), Dimethicone, Lecithin, Cyclotetrasiloxano, Palmitic Acid, C12-16 Alcohols, Glyceryl Stearate, PEG-100 Stearate, Parfum (Fragrance), Cyclopentasiloxano, Gelatin, Imidazolidinyl Urea, Hydrolyzed Wheat Gluten, Ectoin, Cetyl Alcohol, Glycine, Sodium Hyaluronate, Sodium Carboxymethyl Betaglucan, Polyacrylamide, PEG-75 Stearate, Methylparaben, Ceteth-20, Xanthan Gum, Laureth-7, C13-14 Isoparaffin, Tocopheryl Acetate, Steareth-20, Disodium EDTA, Propylparaben, Elaeis Guinensis (Palm) Oil, Tocotrienols, Tocopherol, Chitin (Nano-Fibrils), Melatonin.

Stearate, PEG-100 Stearate, Parfum (Fragrance), Cyclopentasiloxano, Gelatin, Imidazolidinyl Urea, Hydrolyzed Wheat Gluten, Ectoin, Cetyl Alcohol, Glycine, Sodium Hyaluronate, Sodium Carboxymethyl Betaglucan, Polyacrylamide, PEG-75 Stearate, Methylparaben, Ceteth-20, Xanthan Gum, Laureth-7, C13-14 Isoparaffin, Tocopheryl Acetate, Steareth-20, Disodium EDTA, Propylparaben, Elaeis Guinensis (Palm) Oil, Tocotrienols, Tocopherol, Chitin (Nano-Fibrils), Melatonin.

Serum[4]:

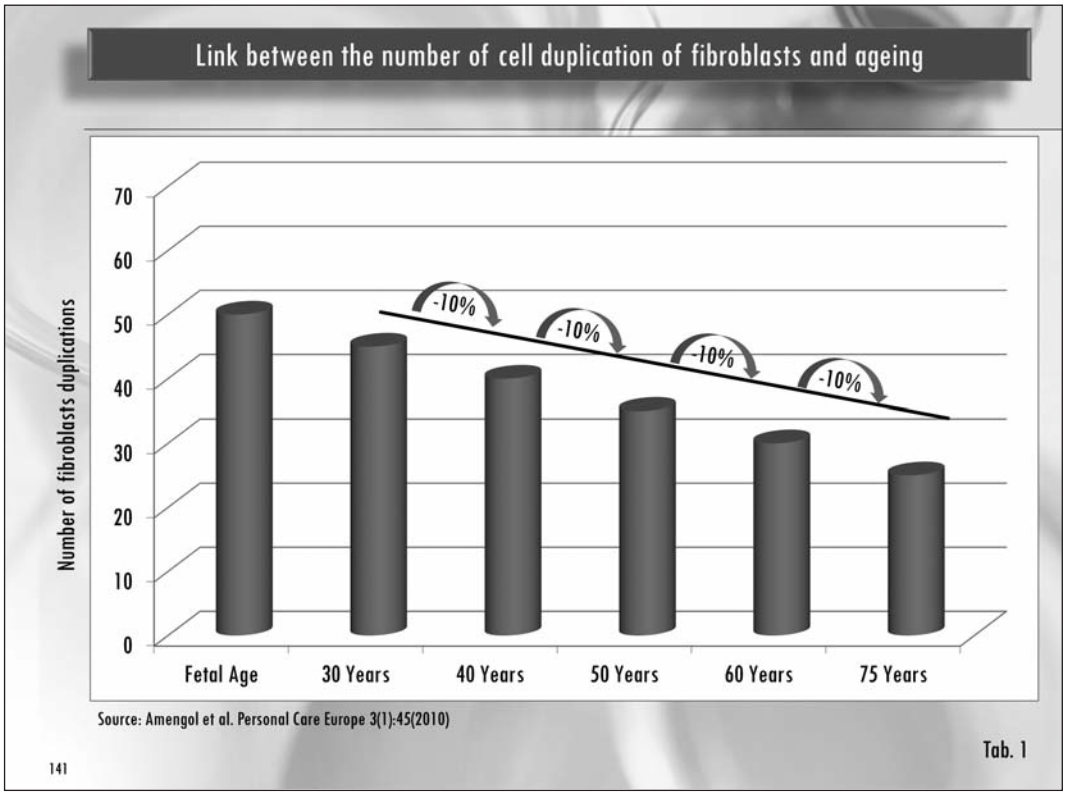
Soluble Collagen, Propylene Glycol, Aqua (Water), Lactic Acid, Glycerin, Pentylene Glycol, Propanediol, Jojoba Wax PEG-120 Esters, Glycine, Arginine, PEG-200 Hydrogenated Castor Oil, Panthenol, Parfum (Fragrance), Sodium Ascorbyl Phosphate.

In vitro studies
Skin and role of collagen

The skin is a dynamic organ with a long memory that shows the most obvious signs of aging. In direct contact with the environment, it undergoes aging also as a consequence of environmental aggression. Thus, while UV rays can damage irreversibly the dermal matrix, as aging progresses, keratinocytes' turnover decrease lipid lamellae modify their composition, the epidermis' barrier losses part of its function for the degradation of intercellular tight junctions, fibroblasts modify their replicative and metabolic capacity (Table I). A decreased synthesis of collagen and ECM components underline an unbalanced protease activity and an altered cellular response at level of elastin, also mediated and accomplished by an enhanced secretion of pro-inflammatory cytokines (19-23). As a consequence the skin becomes more vulnerable and many skin disorders appear in elderly patients, including pruritus, seborrheic dermatitis and xerosis, as previously described (24). It seems useful to remember that dermis is a fibroelastic tissue composed of collagen and elastic fibers with an interfibrillar gel of glycosaminoglycans-dominated from the presence of hyaluronic acid-, salts, and water. Collagen type I, synthesized from fibroblasts, is the major collagen in the dermis with type III collagen constituting approximately 15%, while type V and VI are present in lesser amounts.

Among the bundles of collagen there is a network of elastic fibers, embedded in a gel of glucosaminoglycans.

[3] *Trade Name: QM Notte®, Mavi sud s.r.l., Italy*
[4] *Trade Name: QM Gocce®, Mavi sud s.r.l., Italy*



At the interface between epidermis and dermis is located the basal lamina rich of collagen type IV which, functioning as an anchorage protein, ensures the cohesive function of the dermal-epidermal junction.

What seems important to underline is the biosynthetic pathway of collagen that involves the pro-collagen polypeptide synthesis in a multistep process, requiring the participation of several enzymes and chaperons, as the so called Heat Shock Proteins (HSPs). These chaperon proteins, expressed for proteasomal degradation, are also implicated in assembling the different alpha-peptide- chains for the formation of the collagen' triple helix. HSPs, in fact, carry old proteins to the cell's recycling bin (proteosome) and help newly synthesized proteins fold properly (25). The related activities are also part of a cell's own repair system, called cellular stress response or heat -shock response.

Among the stress proteins (called also heat shock proteins), HSP47 seems to have an important role during the triple helix formation, influencing the collagen fibre maturation (6, 26).

In any way, HSP47, known also as SERPIN NH1 and localized to the endoplasmatic reticulum lumen (Fig. 3), is a classical protease inhibitor. HSP functioning as lock and key molecule, binding to and blocking access to protease active site, plays also a straightforward but critical role in the function of the adaptive immune system (27). It may act, in fact, as molecule that alert cells to the presence of protease activity (28).

In response to cellular stresses, such as infection, heat, shock, oxidative damage, and inflammaging, the so called heat shock proteins (HSPs) are expressed and identified as misfolded or unfolded proteins.

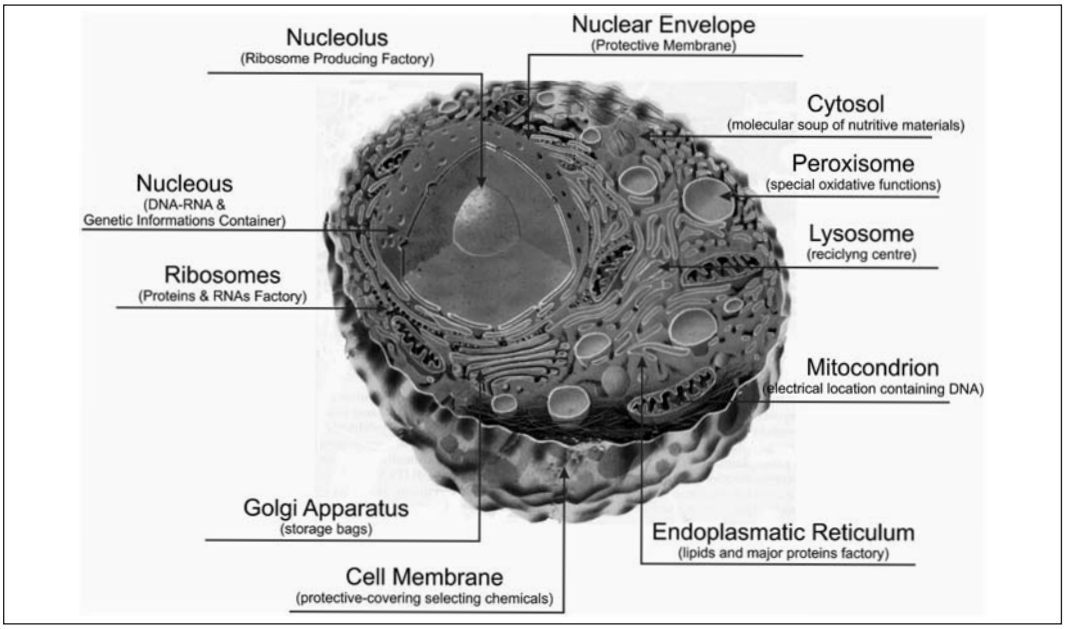


Fig. 3 *View of a cell where HSPs are located at level of endoplasmatic reticulum.*

Thus, for example, HSP27 and HSP70 chaperon proteins have been implicated in increasing the activity of the ubiquitin-proteosome system, though they are not direct participants in the process (29).

Cell culture

Human keratinocytes and fibroblasts were used for the *in vitro* assays. Cells, isolated from skin samples taken from the volunteer donors coming from the in study groups, were cultivated in 9 BM medium (Cambrex MD, USA) with 10%fetal bovine serum at 37° C and 5% CO2, according to our previous experience (30). Before starting, viability, citotoxicity and proliferation of both fibroblasts and keratinocytes added with the active ingredients in balanced mixtures were determined by the MTT-test. Cells were used from the second to fifth passage. To 3 cultures of fibroblasts or keratinocytes were added respectively a 10ng/ml of the following nanoparticles (NP):

1-CN-HA nanoparticles alone (basic control)
2-CN-HA nanoparticles entrapping Gelatin-Glycine
3-CN-HA nanoparticles entrapping Arginine-Hyaluronan-Desamido Collagen
4-CN-HA nanoparticles entrapping Melatonin-Vit E-Betaglucan (MEB)
5-CN-HA nanoparticles entrapping Ectoin-Betaglucan
6-CN-HA nanoparticles entrapping PCA-Peptides
7-CN-HA nanoparticles entrapping PCA-Lysine
8-CN-HA nanoparticles entrapping ZnO-TiO_2-Lutein
9-CN-HA nanoparticles entrapping Vit C-Peptides-Panthenol
10-CN-HA nanoparticles entrapping Arginine-Glycine.

Keratinocytes culture and Pro-inflammatory cytokine release

The interaction among cells, ECM network, and biological signals, on one hand are crucial for

the normal skin structure, function and degeneration. On the other hand, the cells die in the presence of low oxygen, nutrient concentrations, hight level of ROS, and cytokines. Cytokines and growth factors are produced in abundance by keratinocytes as autocrine regulators of barrier homeostasis. They regulate many biological processes, including inflammatory and immune responses. An imbalance between pro- and anti-inflammatory cytokines can result, in fact, in inflammatory diseases. Tumor necrosis factor alpha (TNF-α) is among the first cytokine to show an increase in mRNA, followed by interleukine 1α (IL-1 α) and interleukine -8 (IL-8). Moreover, the Nuclear Factor-kappa B (NF-kB), pro-inflammatory regulator of cytokine expression, is involved in cellular responses to different internal and external stimuli (as cytokines, ROS, UV aggressions, etc), inducing an inflammatory response by the secretion of immunoregolatory proteins as the same cytokines.
The NF-kB, activated by the aging process also, upon stimulation activates the secretion of the pro-inflammatory IL-8, inhibiting the TNF-α secretion (31).

IL-8 release

At cell confluence, the in study nanoparticles were introduced in fresh culture medium at 10ng/ ml with TNF-α at 100 ng/mL. The positive control was hydrocortisone at 1μM.
After a 24 hours incubation at 37°C and 5% of CO_2, the quantity of IL-8 was evaluated by ELISA on culture supernatant. The obtained results are shown in (Fig. 4).

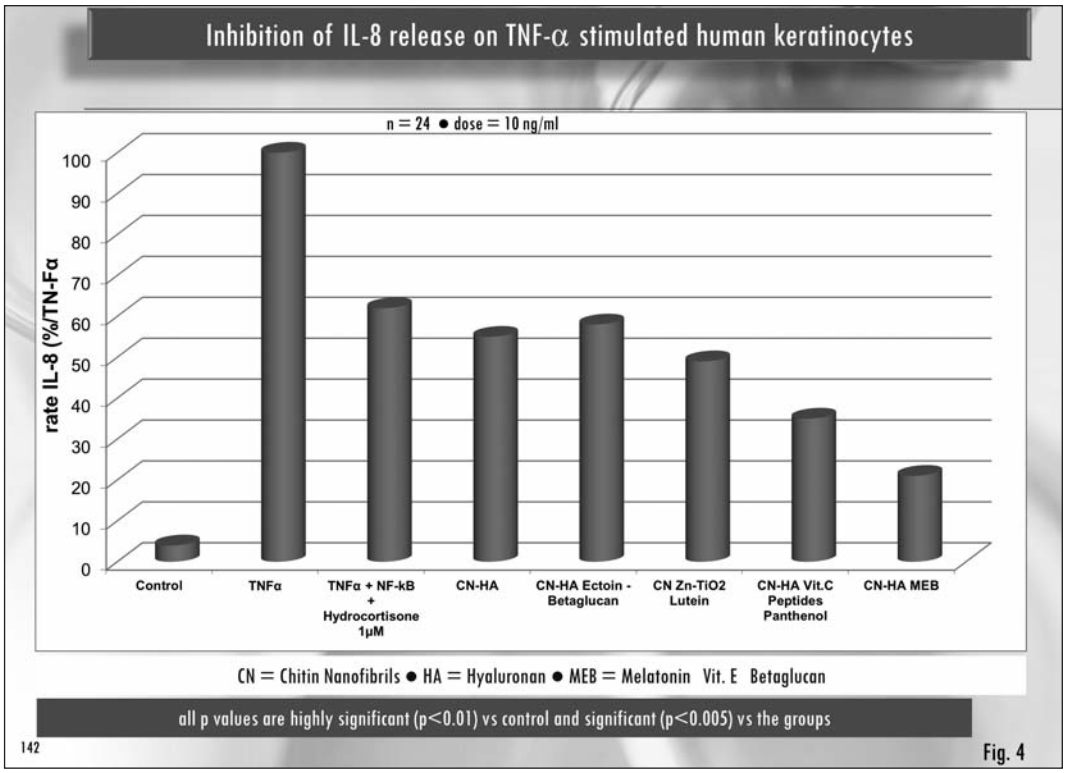


Fig. 4

Fibroblasts culture and collagen synthesis

Fibroblasts were cultivated in presence of the different nanoparticles and controlled by histochemical methods to verify variation in the synthesis respectively of, collagen I (by ELISA), collagen III (by immunocytochemistry), and collagen IV (by Elisa), pre-treated by the in study active nanoparticles.

The quantity of the chaperon HSP47 was also controlled by the use of Western Blot Test and evaluated by chemiluminescence (32).

The recovered results, expressed as the percentage of fluorescence in culture, are shown in (figures 5-8).

Antioxidant Activity

In normal healthy skin, there is a balance between the formation of oxidizing chemical species and their effective removal by protective antioxidants.

When redox imbalance occurs the so called oxidative stress appears, manifested by a lipid peroxidation of cell membrane, leading to many chronic diseases (33).

Peroxidation of membrane polyunsaturated fatty acids (PUFA) -particularly linoleic acid- produces, in fact, a plethora of reactive primary peroxides that, causing the cell death, trigger for new cell growth (34).

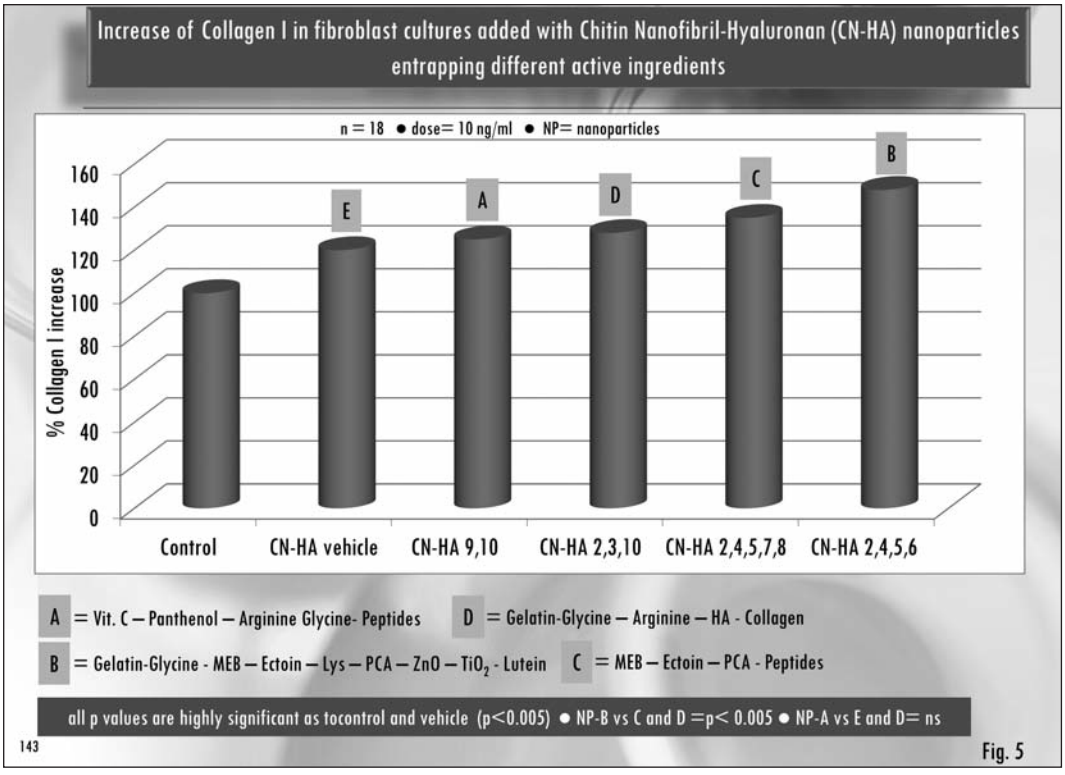


Fig. 5

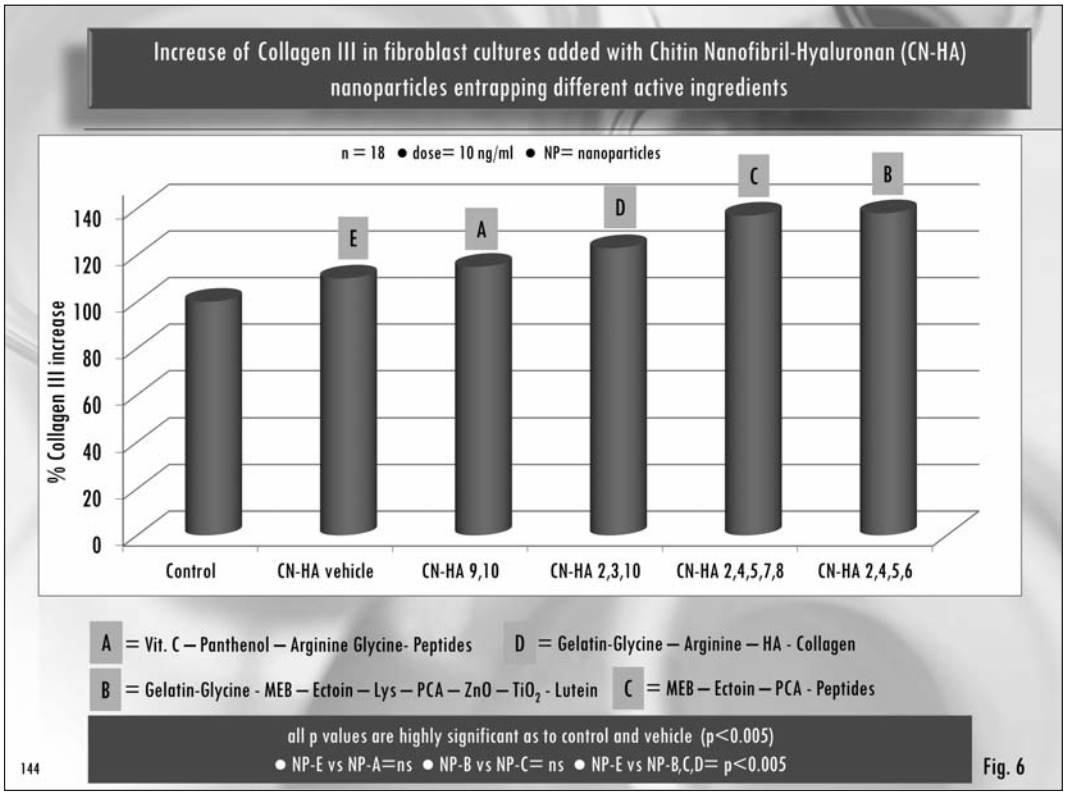


Fig. 6

At this purpose it has been shown that ROS, RNR and RIS might be used from the cells as *signal messenger and trigger* molecules (35, 36): as a rule, low levels of these free radicals activate cellular process, whilst higher levels turn them off.

However, oxidative and other chemical-physical stress may modify not only PUFA, but also carbohydrates, proteins, and complex macromolecules as DNA and RNA.

Thus, the aging process is accelerated by a degradation of the ECM components, and metabolic disturbances may appear by interference with gene expression. In this way, oxidative stress contribute to the onset of wrinkles, decreasing skin elasticity and reducing skin hydration. For all these reasons a combination of antioxidant compounds as vitamins C, E, and melatonin are used topically and/or by oral route to inhibit lipid peroxidation and prevent the phototoxic damages (37, 38), while immune protectant compounds, as betaglucan and ectoin are used to increase the immune reply to the environmental aggressions (Figures 5-7).

The total antioxidant activity of the different block copolymeric nanoparticles entrapping respectively Melatonin-Vit E-Betaglucan, Ectoin-Betaglucan or Vit C-Peptides-Panthenol was evaluated *in vitro* by the total peroxyl-radical-trapping parameter (TRAP), using the techniques described by Cao (39) and Wang (40) modified by our group and used together with selective standards such as vitamins (vitamin E and vitamin C) and phenolic acids (caffeic acid).

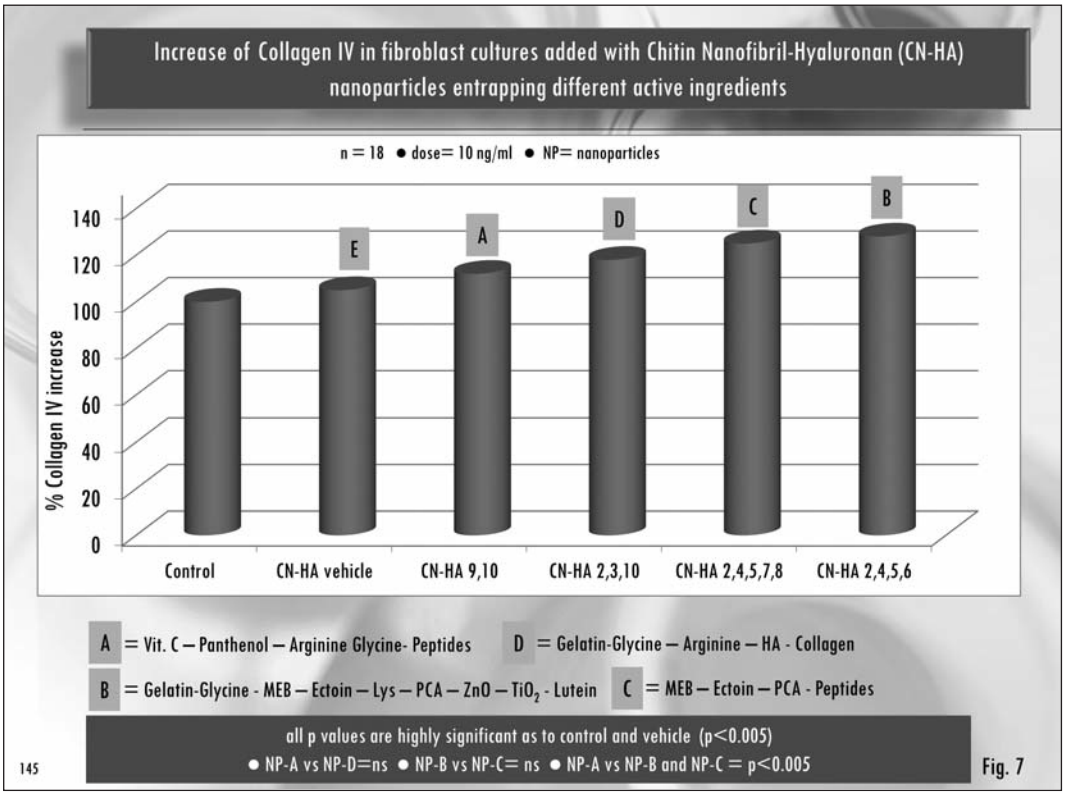


Fig. 7

The obtained results are shown on (Fig. 9).

The protective effect was assessed on linoleic acid treated by different nanoparticles or the vehicle, to control the lipid peroxidation of linoleic acid. The method is based on the peroxidation of linoleic acid by OH° and the formation of malondialdehyde (MDA) as final product.

Linoleic acid (10 mM) was dissolved in 1 ml of methanol, dried under nitrogen and redissolved in 2 ml of phosphate buffer according to Niki (41). Lipid peroxidation of linoleic acid treated with the described nanoparticles was induced by adding 10 µl of fetal bovine serum (FBS) for 15 min at 37°C.

The control consisted of linoleic acid peroxidated with 10 µl of AMVN for 15 min at 37°C without nanoparticles. The formation of MDA was detected by fluorimetric method according to Ursini et al. (42). Results have been expressed an µmol MDA/g of nanoparticles. The obtained data are reported on (Fig. 10).

Metalloproteinase release

ROS, implicated in skin aging, induces a reduction of collagen synthesis and a contemporary increased secretion of metalloprotein (MMP) enzymes, capable to degrade all the main constituents of ECM, both in keratinocyte and fibroblast cultures (43). Time-dependent degradation of extracellular matrix (ECM) following the cell death is, in fact, mainly regulated by matrix metalloproteinases, which are well known to function in the extracellular environment of single cells, degrading both matrix and non-matrix proteins as well.

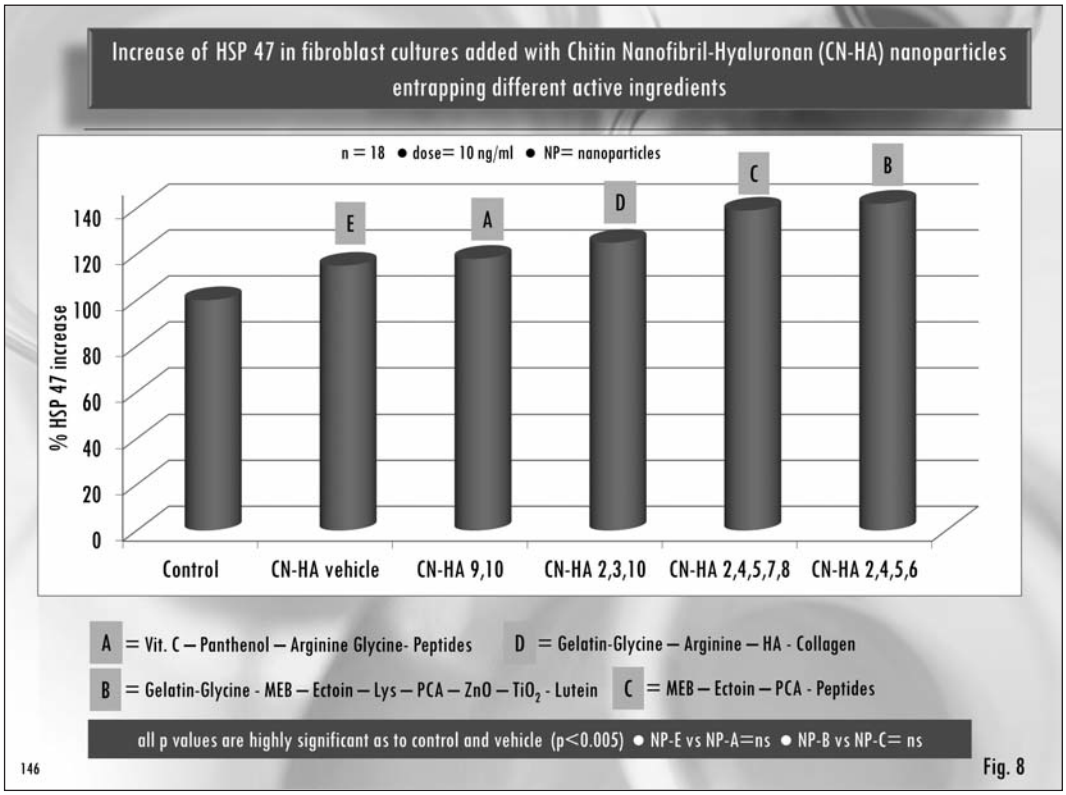


Fig. 8

An important mechanism regulating the aging process occurs via a balance between synthesis of new collagen, particularly type I and IV, and its degradation by metalloproteinase, as MMP1, that weakens the skin collagen scaffold.

Normally, to obtain an accelerate senescence of fibroblasts their culture are treated by H_2O_2, used to induce formation of ROS. Thus, Human fibroblasts were seeded in sterile 250 mL Flasks with DMEM medium and 10%FBS.

After 24 h incubation, the medium with and without 600μM of H_2O_2 and cells were incubated for further 2 h, and soon after the medium was removed and replaced with fresh medium.

Maintaining the culture for further 144 h and substituting the medium after 70 h, both aged and normal fibroblasts were detached with trypsin, seeded in 96-well microplates and cultured for 24 h. After this period of incubation, the culture medium was replaced with DMEM medium, containing either Transforming Growth Factor (TGF-β) (10μg/mL) or the nanoparticles in study at the dose of 10μg/mL and incubated for 72h, or left as control. The MMP1 evaluations, tested in triplicate, were performed by an ELISA kit.

The obtained results are reported on (Fig. 11).

Collagenase activity

As known, hydroxyproline represents about 10% of the global aminoacid content in collagen. By the determination of its quantity in fibroblast culture added with collagenase enzyme it is possible to establish indirectly the efficacy of the nanoparticles used as inhibitors of this enzymatic reaction.

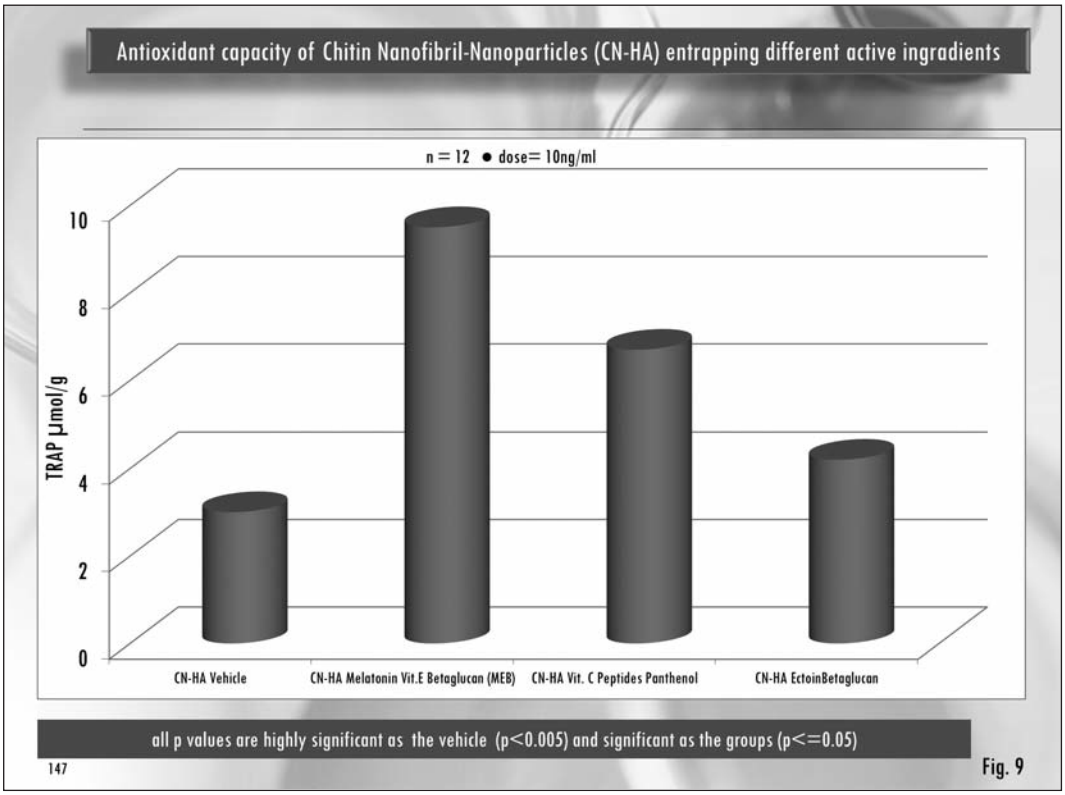


Fig. 9

Therefore, fibroblast cultures were incubated with collagenase enzyme (Sigma-Aldrich, Milano) and with all the nanoparticles under study. After hydrolysis and oxygenation, the red color obtained through the liberated hydroxyproline and the Erhlich's solution added was quantified by a spectrophotometer reading at 560 nm for each culture, according to Edwards and O' Brien and our previous study (44, 45). The obtained results are reported on (Fig. 12).

Citotoxicity assay in vitro

A comparison of cytotoxicity was performed on the test cells (keratinocytes and fibroblasts) with *in vitro* proliferation using the MTT method (46) modified and previously used from our group (47).

Briefly cells were plated in plates at a density of $1x10^4$ cells in 200 µL of complete medium, and incubated for 24 h to allow the cells to attach. The cells were them exposed to serial concentration of all the nanoparticles in study at 37° C for 48 h. At the end of incubation 20 µL of the MTT solution was added, with incubation at 37° C for another 4 h, and the medium was then replaced with 100 µL of dimethyl sulfoxide to dissolve MTT formazan crystals.

The plates were shaken for 10 minutes and adsorbance was measured at 570 nm using a microplate reader (BioRad, Model 680, Hercules, Ca, Usa). The obtained results made in triplicate are shown on (Fig.13 and 14).

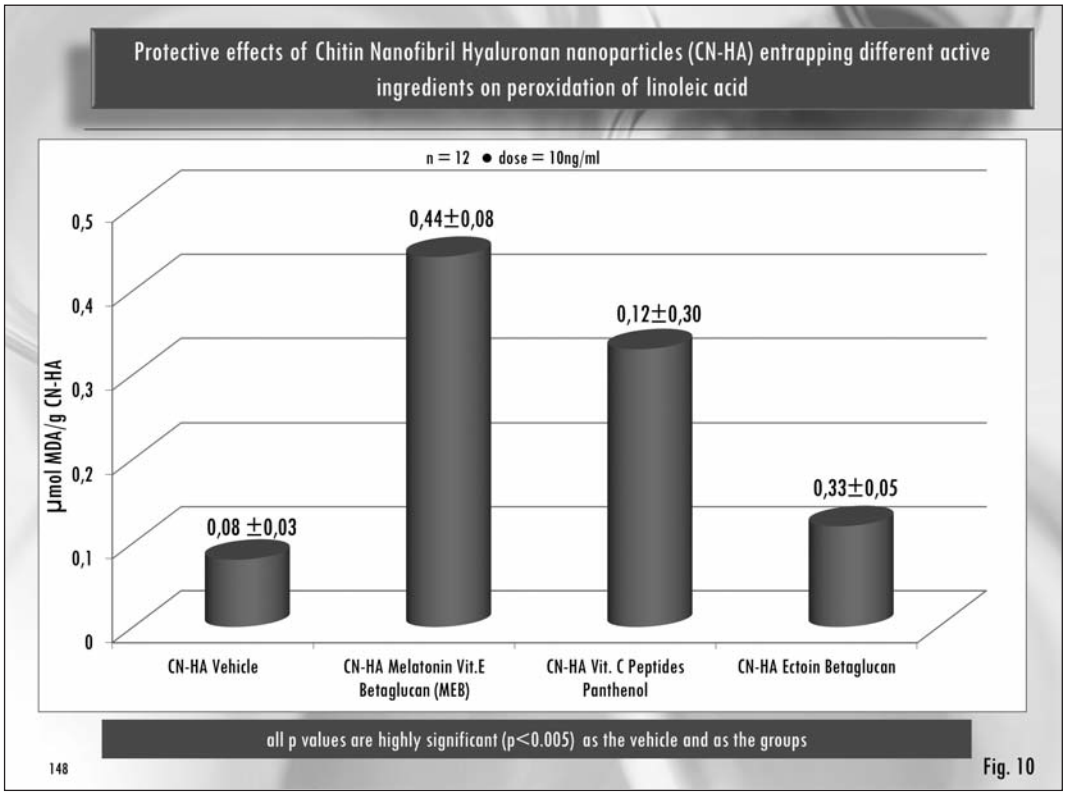


Fig. 10

Study Criteria in vivo

A multicenter randomized, vehicle-controlled preliminary study, for a period of 60 days at different plastic surgery /dermatological departments, on 60 Healthy women (mean age +/- 48 years) exhibiting signs of photoaging, was conducted to evaluate the safety, tolerability and efficacy of an innovative cosmetic treatment for face, neck and hands based on the combined use of different active ingredients encapsulated into block copolymers of CH-HA micro/nanoparticles obtained according to our methods reported elsewhere (47-50). The only criterion to entry in the study, conducted according the Declaration of Helsinki revised in Seoul, was the presence of one or more signs of photo-aging affecting the face and neck, such as fine wrinkling around the eyes, crease lines around the mouth and cheeks, telangiectasia, wrinkling and spots on the forehead of face and back of the hands, etc, corresponding to degrees 3-5 of the photodigital scale described by Larnier et all (51) and previously used by our group (52, 53).

According to our previous studies (47-50), these natural polymers are capable to bind each to others by ionic bonds to form block polymeric structures, because of the different electrical charges covering their molecules. Chitin nanofibrils (CN) being prevalently electropositive, while Yaluronan electronegative. During and according to the process of manufacturing, the block copolymers may entrap and /or encapsulate different active ingredient to form nanoparticles, successively englobed into micro/nano emulsions (47-49). Due also to our previous stu-

dies that have shown a boosting activity of the CN-HA nanoparticles, as well as an antiaging effectiveness, when entrapped by MEB and other active ingredients (30, 45,), we have designed a preliminary *in vivo* study to verify the possibility to have a global daily skin treatment by the combined use of different formulations, appositely studied for the plastic surgeons' necessities.

This is also because the different active ingredients, entrapped into the CN-HA nanoparticles, need to be enclosed into different cosmetic carriers to obtain the best results for effectiveness and safety, when applied on the skin. At this purpose four different formulations, composed of different active nanoparticles and carriers, were designed and formulated as reported on Table II. The selected subjects were instructed to apply each day respectively QM-daily cream on the face and neck in the morning and QM-night before retired in bed, while QM-eye cream had to be applied around the eyes two times a day and QM-concentrated serum (2/3 drops) two times a day, but three times a week only.

Naturally, nor the dermatologist neither the subjects had the possibility to know the differences between the active and the carrier (control) products, because all the assigned products (Active and Carrier) had the same packagings, classified by numbers 1 to 4. By this preliminary study only skin hydration, TEWL and aged spots were controlled as objective evaluations, as well as other subjective evaluations were performed from an expert dermatologist and from the same subjects (Data not reported).

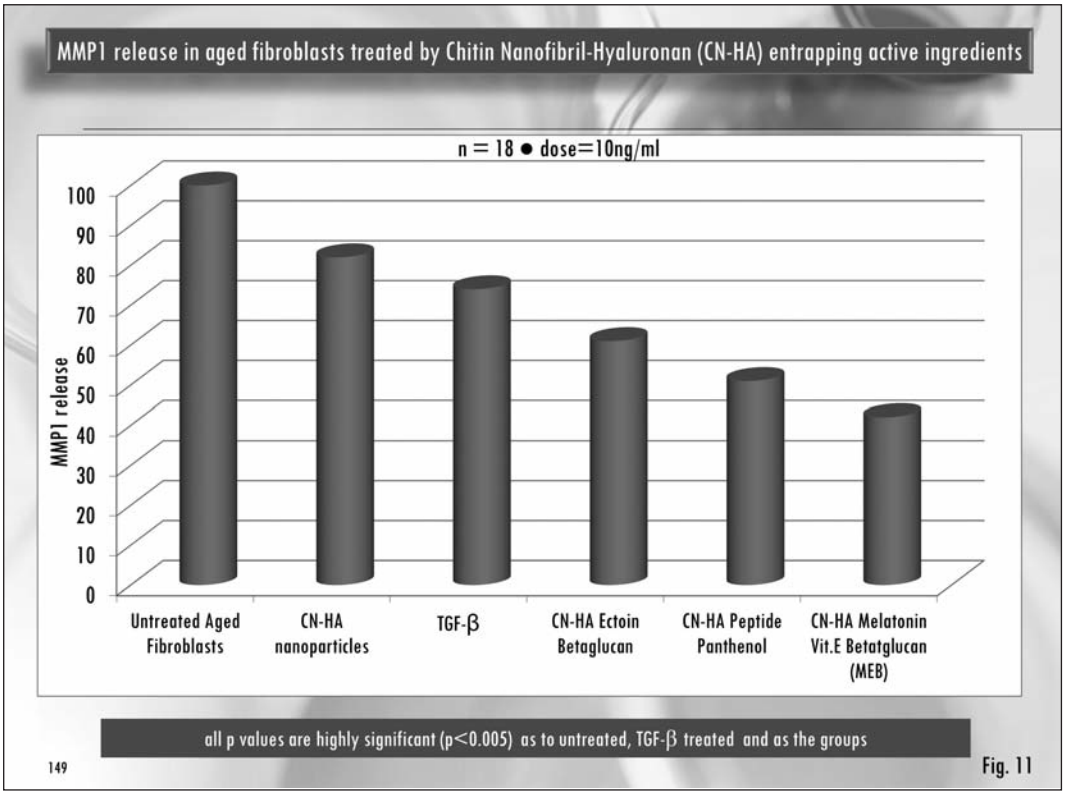


Fig. II

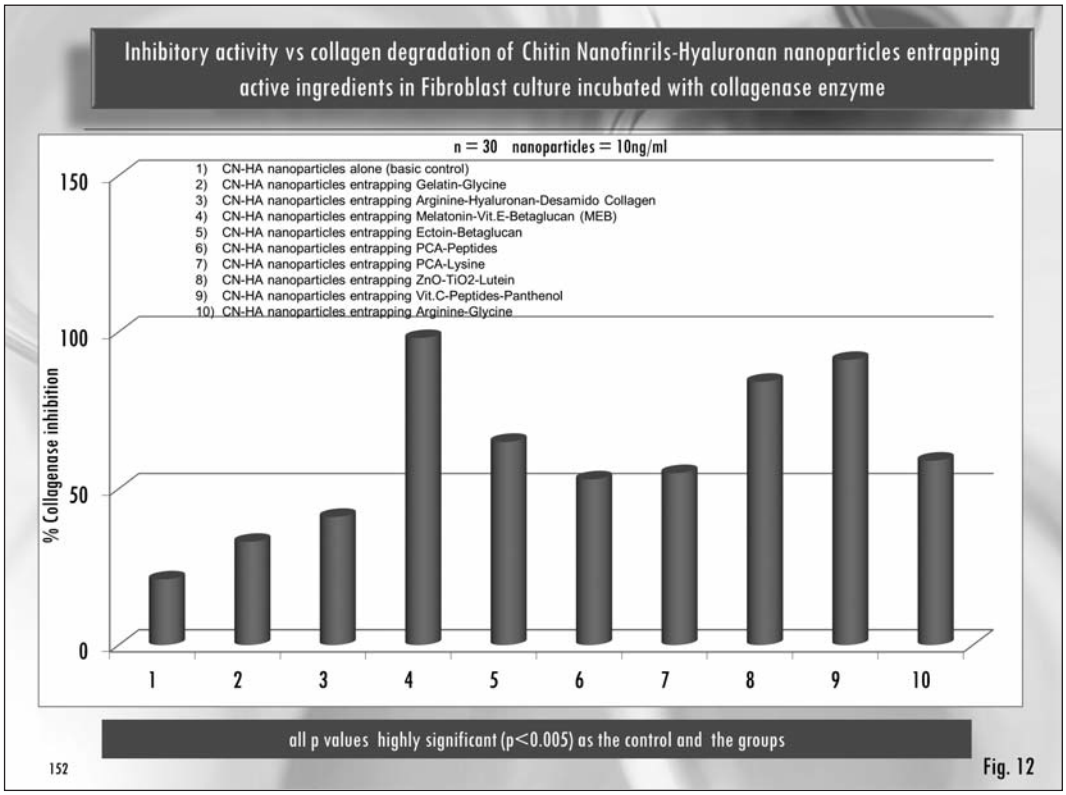


Fig. 12

Skin Hydration and TEWL measurements

Skin hydration and TEWL were controlled by the 3C system (Dermotec Rome, Italy), used from many years from our group (54). Skin hydration is based on the measurement of dielectric constant of water controlled by the skin capacitance, while TEWL is measured indirectly by two pairs of sensors making use of the Fick's diffusion law. All the data, controlled at day 30 and day 60 were contemporary analyzed by a microprocessor. The obtained results are reported on (figures 15 and 16).

Depigmenting activity measurement

According to Elsner (55) to control the depigmenting activity of the global treatment, the intensity of the color was measured at day 30 and at day 60 on different skin areas by the Minolta Chromameter CR 300, a light-weight and compact tristimulus color analyzer for measuring reflected object color. The obtained mean results are reported on (Fig. 17).

Statistical analysis

The results are expressed as mean ± SD from at least three independent experiments. Statistical analysis was performed applying the Student's t test and differences were assumed to be statistically significant when $p < 0.05$.

RESULTS AND COMMENTS

As previously reported skin aging and photoaging induce fibroblast senescence with a consequential alteration in the synthesis and maturation of both collagen and the scaffold chaperon proteins. In photoaged skin, collagen fibrils are disorganized and abnormal elastin-containing materials accumulates.

Further biochemical studies have revealed that in photoaged skin levels of type I and type III collagen precursors and crosslinks are reduced, whereas elastin levels are increased (6, 57, 58).

As reported from different authors (59-62) the chaperon protein HSP47 seems to be especially responsible of linking the alpha helix of pro-collagen to form the final triple-helix characterizing collagen. This activity seems to be confirmed from this study by which a production increase of collagen I, III and IV, as well as of HSP.47 was obtained, at level of aged fibroblast' cultures by the use of our nanoparticles, as reported in figures 5-8. At this purpose it is interesting to underline as the nanoparticles entrapping Collagen-peptides or Melatonin-vitE-Betaglucan (MEB) (complex of antioxidant and immunomodulant compounds), previously used from our group (45, 63, 70), have shown the best activity in increasing the collagen synthesis.

Chitin-Nanofibrils-Hyaluronan nanoparticles entrapping different active ingredients used for the in study formulations

FORMULATION					
PRODUCTS	**Vehicle**	**QM serum**	**QM eye cream**	**QM daily cream**	**QM night cream**
CLASSIFICATION	**NP-CN-HA-E**	**NP-A**	**NP-D**	**NP-B**	**NP-C**
Active ingredients entrapped	CN-HA Nanoparticles alone	• CN-HA • Vit.C-Peptides-Panthenol • Arginine-Glycine	• CN-HA • Gelatin-Glycine • Arginine-Hyaluronan desamido collagen • Arginine-Glycine	• CN-HA • Gelatin-Glycine • Melatonin-Vit.E – Betaglucan (MEB) • Ectoin-Betaglucan • PCA-Lysine • ZnO-TiO2-Lutein	• CN-HA • Gelatin-Glycine • Melatonin-Vit.E-Betaglucan (MEB) • Ectoin-Betaglucan • PCA-Peptides

ABBREVIATIONS: **NP**= nanoparticles • **CN**= Chitin Nanofibrils • **HA**= Hyaluronan

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Tab. II

However, it is also interesting to underline how this activity seems to be monitored from the vehicle used, i. e. the block copolymer Chitin Nanofibril (CN) – Hyaluronan (HA). Probably this ionic complex of natural ingredients as previously supposed (63-65), mimicking the ECM native microenvironment, leads to collagen and glycoprotein formation. Component of ECM network are comprised, in fact, of five classes of macromolecules with different functions, normally decreasing during the aging process.
They are: collagens, elastic fibers, proteoglycans, hyaluronan, adhesive glycoproteins, and soluble macromolecules, such as growth factors, chemokines and cytokines (66). As consequence, this network, represented by the complex of hyaluronan and chitin -polymer of glucosamine and acetyl glucosamine-, creating a favorable environment together with the active ingredients entrapped, facilitates the cell migration and function, sustaining the synthesis of both ECM fibers and soluble macromolecules.
Topical use of antioxidant compounds seems, in fact, useful for the skin' removal of free radicals in excess, preventing also the consequential increased production of inflammatory citokines and MMPs. Thus the antioxidant capacity of the in-study nanoparticles was evaluated *in vitro* by two different methodologies: the TRAP method to evaluate the overall antioxidant activity of the nanoparticles and the malondialdehyde formation as final product of the peroxidation of linoleic acid, added to the nanoparticles' emulsions.

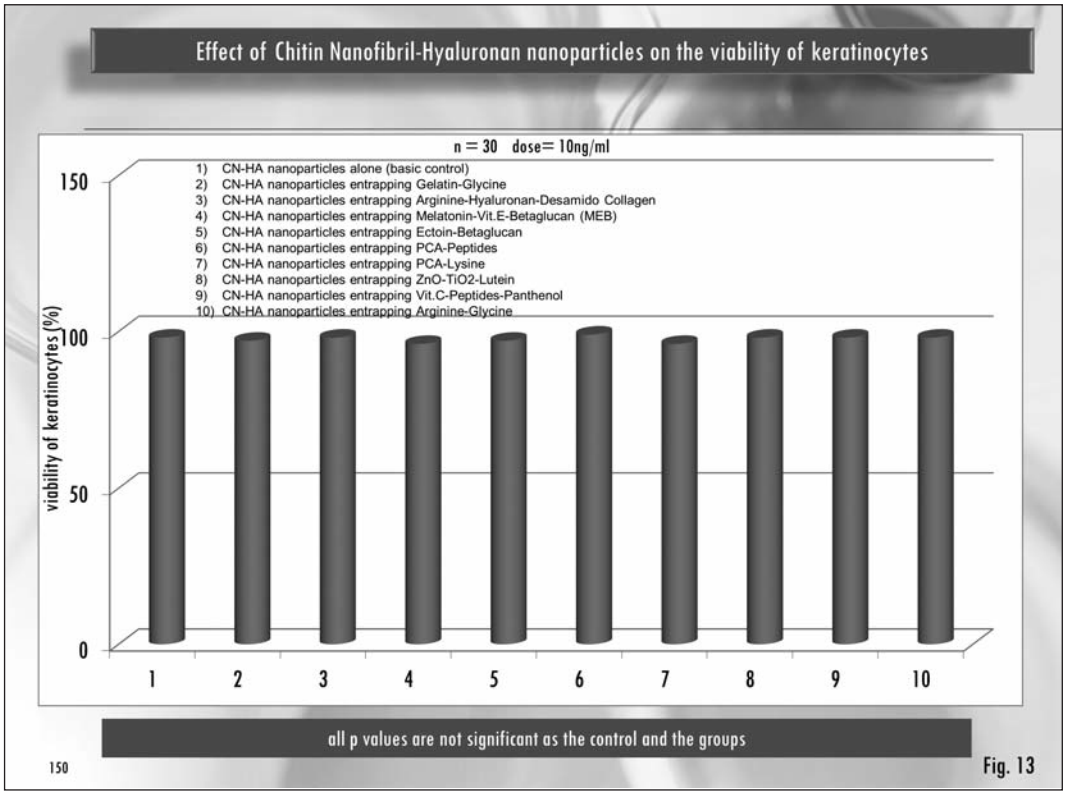

Fig. 13

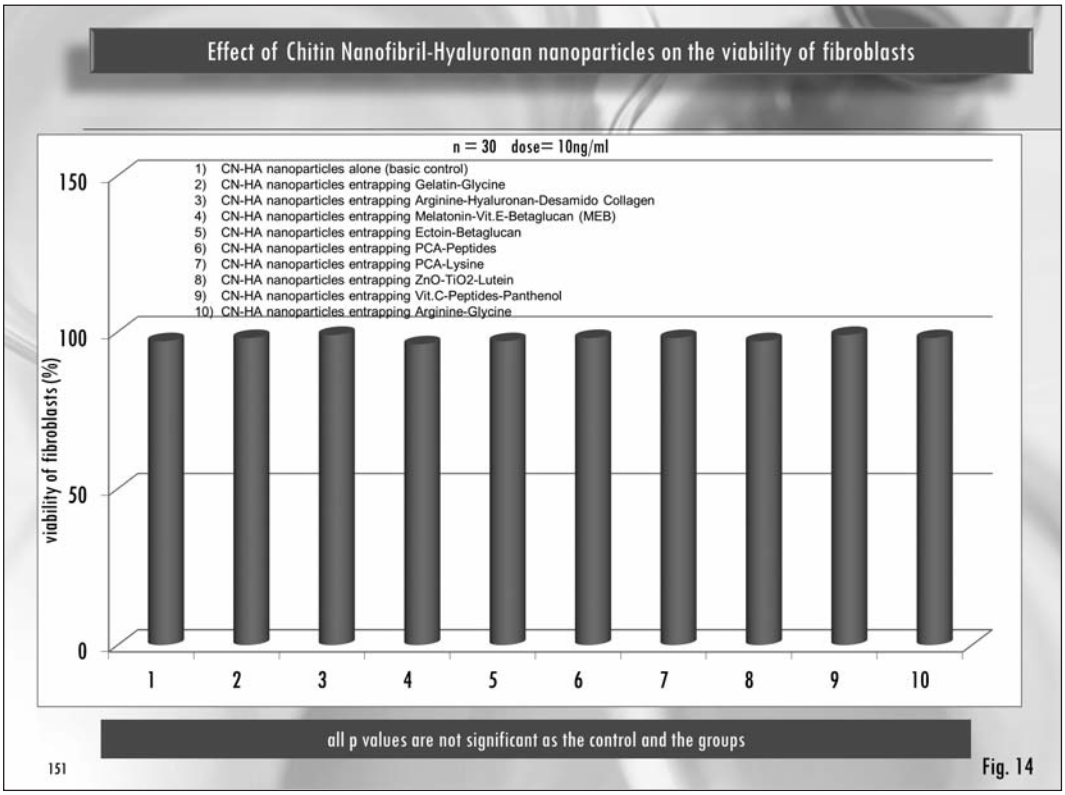

Fig. 14

All the experimentations were done in triplicate and verified versus antioxidant compounds at note activity.

As reported in figures 9 and 10, the CN-HA vehicle has shown antioxidant activity, notably increased when this carrier entrapped the proper and right quantity of antioxidant and immunomodulant compounds, such as vitamins E/C and betaglucan/ectoin. The best and highest activity was obtained by the use of MEB, with both the methodologies as reported on Fig. 9 and 10. The effectiveness of this complex has based, in fact, on the interesting antioxidant activity of melatonin that, for its binomial hydrosoluble/liposoluble character, has a double mean of action outside and inside the cell, while vitamin E is active exclusively at level of cell membrane. Moreover CN, as polymer of glucosamine and acetyl glucosamine, should have an adaptogen efficacy boosting the cell metabolism for its content in glucose, contemporary stimulating its defensive capacity against the oxidative stress (63-65).

At this purpose it is also interesting underline the other parameters controlled, such as IL-8 and the MMP-1 released as a consequence of inflammation. Inflammation, as natural biological response to injury or infection, is known to be also a pivotal mechanism in photoaging, for the UVB-induced immunosuppression and the high quantity of free radicals produced with a consequential overproduction of cytokines (56).

Inhibition of the cell signaling pathways, initiating the overproduction of the proinflammatory cytokines, may serves as a key mechanism in the

control of inflammation, also during the aging processes (10). Moreover, the enzymes MMPs, capable to degrade all the main constituents of ECM, increase notably during skin aging and under the aggression of free radilcals. In particular MMP1 is responsible for fragmenting type I collagen. During the aging process there are, in fact, alterations in connective tissue structure, reflecting a decreased synthesis of collagen with an increased production of metalloproteinases (67).

Thus, ingredients that should suppress the expression of these inflammatory mediators, as these innovative block copolymeric nanoparticles seem to do, may attract significant interest also as potential cosmeceuticals and therapeutics for the treatment of inflammatory diseases (68).

On figure 4 it is possible to see, in fact, that all the nanoparticles in study are capable to slow down the increased release of IL-8, stimulated on keratinocytes cultures by the activity of TNF-α. Surprisingly it is interesting to underline that CN-HA, in our experimental condition, seems to posses the same efficacy of hydrocortisone in reducing the rate of IL-8 production, also if used in a lower concentration. On the other hand the activity of CN-HA-MEB has shown to be 3 times more effective than hydrocortisone, while the other active ingredients entrapped have shown same but lower efficacy.

Moreover, CN-HA-MED was also two times more effective than TGF-β in reducing the MMP-1 release, as shown in figure 11.

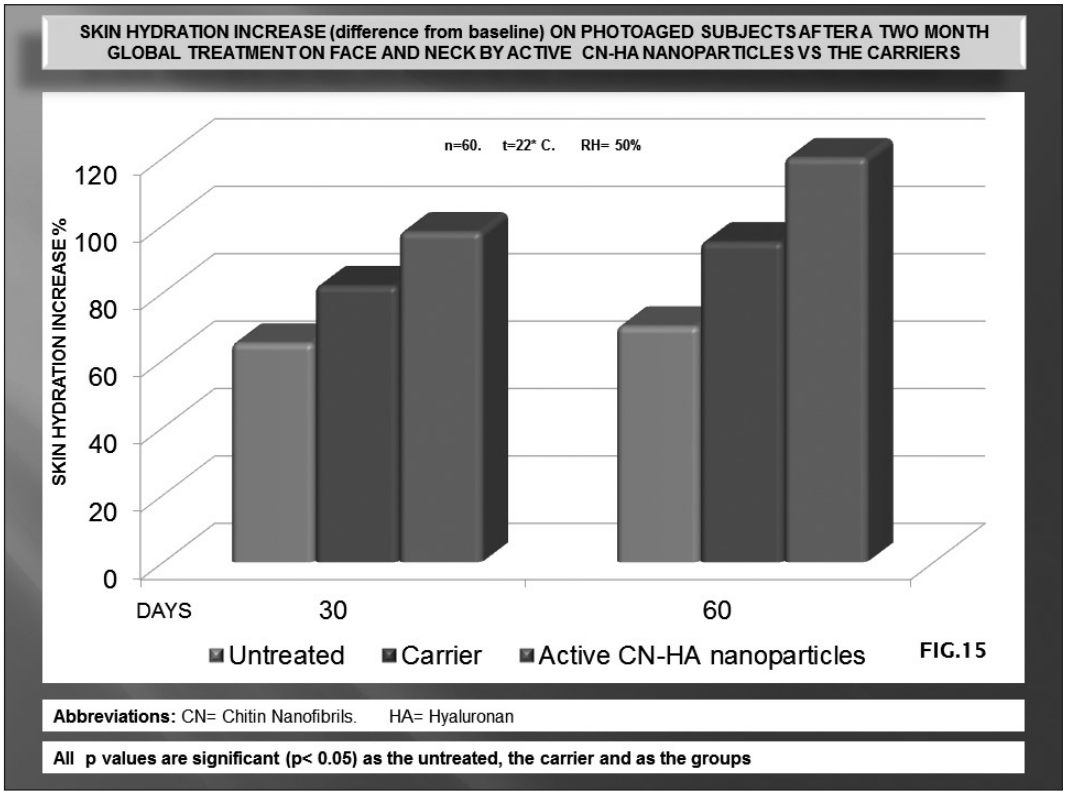

Fig. 15

On the contrary of our expectations, by this parameter the block copolymer CN-HA has underlined a lower activity to reduce the MMP-1 activity, compared to hydrocortisone and the other active nanoparticles used. However, the results reported on figure 12 reinforce the protective activity these nanoparticles seems to have against the enzymes capable to catabolize the ECM fibers, as collagenase. As shown on fibroblast cultures, the more effective activity was obtained again by the nanoarticles CN-HA-MEB, capable to inhibit of about 100% the activity of this enzyme.

The less effective were the CN-HA block copolymers entrapping respectively vitamin-E-Peptides-Panthenol or the complex of sunscreens composed of TiO2-ZnO-Lutein.

Moreover, it is interesting to underline that all the in-study nanoparticles have shown to be perfectly biocompatible, being non toxic on the viability of both the cell cultures of keratinocytes and fibroblasts used, as shown on figures 13 and 14.

The first results obtained *in vivo* by the parameters selected, have confirmed the *in vitro* one, reported also on our previous studies (30, 69-71). Skin hydration has increased soon after the first month of treatment (Fig. 15), while both TEWL (Fig. 16) and the skin spots (Fig. 17) were sensibly reduced, evidencing an interesting skin whitening activity accomplished by a reduction of the wrinkling depth, according also to the opinion of both the subjects treated and the dermatologists involved in the study.

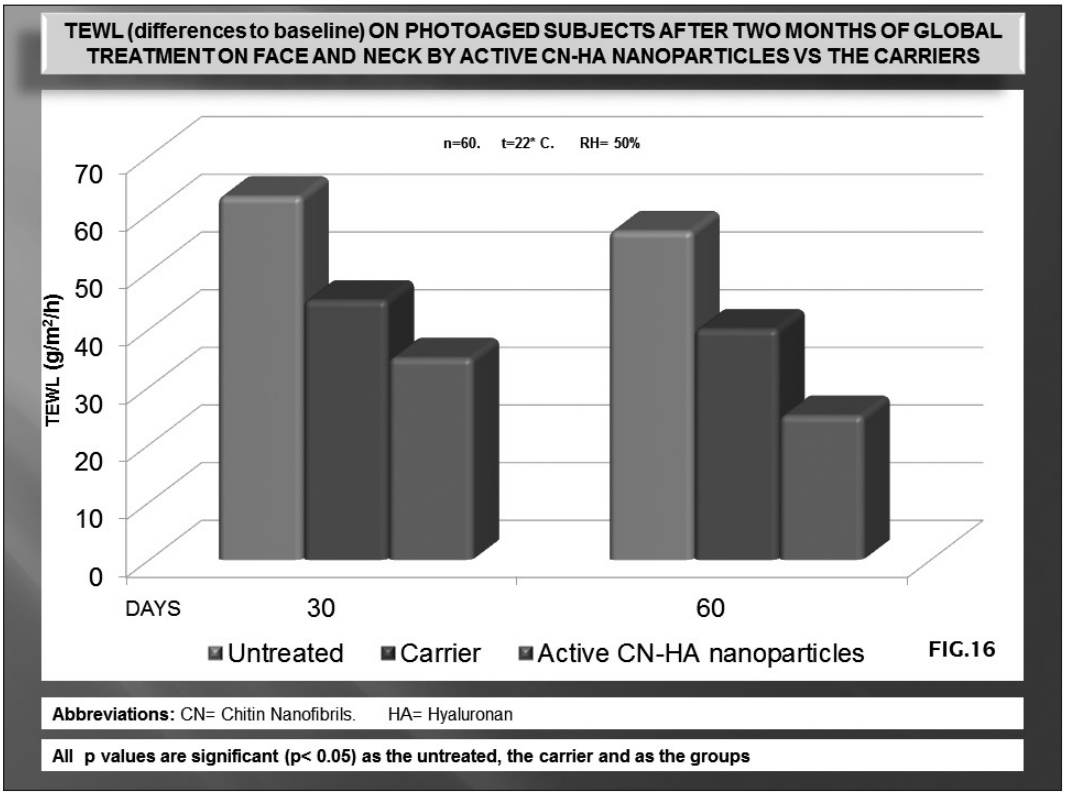

Fig. 16

The global amelioration was more evident at the second month of treatment as shown on figures 15-17.

In conclusion the CN-HA block polymeric nanoparticles made from biodegradable and natural polymers have to be preferred over other colloidal carrier systems, like micelles owing to their higher stability and flexibility in tailoring the ingredient load and its release rate. From the obtained data it has been shown that CN-HA nanoparticles should be potential candidates for delivering active payloads, such as MEB (Melatonin, vit E and Betaglucan) as antioxidant compounds, or TiO2 and ZnO as inorganic sunscreens.

It is interesting to underline, in fact, that these nanoparticles may be produced positively or negatively charged on their surface, according with the productive process selected (47-50).

It has also been shown, in fact, that the nanoparticles, whose periphery is covered by positive surface charges, show an ability to disturb the lamellar layers of the stratum corneum, enabling a better diffusion of the entrapped active ingredients through the skin.

On the contrary, when the lamellar surface is covered by negative charges the active ingredients seems to remain at level of the more external corneocytes, as necessary, for example, for the sunscreen compounds (47-50).

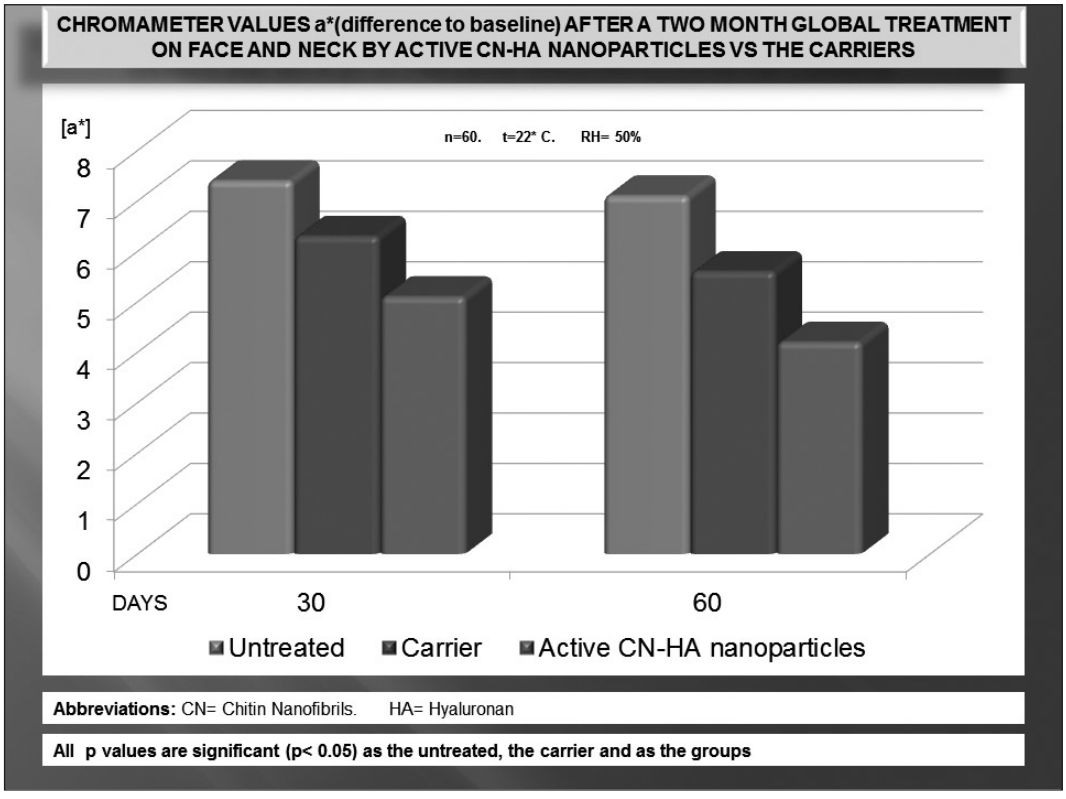

Fig. 17

In any way these block polymeric nanoparticles remained stable in time, when entrapped into the w/o/w multiple nanoemulsion used for the antiaging formulations designed for this study (Data not reported).

Considering these first obtained results we are continuing to control *in vivo* the same formulations by the use of other clinical antiaging methodologies. Moreover, our group are developing other innovative carriers by the use of same physicochemical approach, to deliver the active ingredients through the epidermis, activating also the cellular protein-messages and maintaining the skin homeostasis with respect of the environment.

Chitin Nanofibrils produced by the use of the fishery waste and Hyaluronan produced by enzymatic processes are, in fact, not only natural raw materials highly biocompatible and respective of the environment (71-74), but seem also capable to modulate the skin's intercellular signal transduction, acting by the NICE-TCM approach (71, 75-79).

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Author Address:

Pierfrancesco Morganti Ph.D.
Mavi sud S.r.l.
Viale dell'Industria 1
04011 Aprilia (LT) - ITALY
Fax: +39 06 92 81 523
Email: morganti@mavicosmetics.it

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A randomized, placebo-controlled double-blind parallel group study in the Treatment of Aging Symptoms of the skin using Topical and Oral Treatments

J. Wadstein, M.D., Ph.D; E. Thom, Ph.D.
ETC Research and Development, Oslo, Norway

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Key words: *Microencapsulated retinyl palmitate; Marine complex[2]; Aging skin; Randomized; Placebo; Double-blind; Skin elasticity; Skin thickness; Skin support system[1];*

Summary

The present placebo-controlled double-blind study was carried out in 57 nonsmoking female subjects with aging symptoms of the skin.
The present skin support system[1] is a new anti-aging treatment regime, combining topical and oral treatments.
The topical treatment is based on a proprietary microencapsulated retinyl palmitate in the form of a cream. The oral treatment is a tablet containing a proprietary marine complex[2], vitamins, and minerals. The cream as well as the tablets are manufactured by Pharma Medico, Denmark.
The active treatment was given twice daily to 29 subjects as cream and tablets, while 28 subjects received placebo cream and tablets. The study had a duration of 4 months. The two treatment groups were clinically comparable at the start of the intervention with respect to the main parameters, age and grade of aging symptoms of the skin.
Objective measurements, skin thickness and elasticity, as well as subjective observations of clinical parameters were used for evaluation of the effect. In addition the participants made self-evaluations of the effect, using visual analogue scales (VASs).
The results showed a significant improvement[1,2] in skin quality, in objective as well as in subjective parameters.
Compared to previous studies conducted with the active components[1,2] of the cream and tablets separately, the present combination gave an additive effect and is thus an attractive and well-tolerated treatment for the aging skin.

[1] *Trademark: Nourella® Skin Support System*
[2] *Trademark: Vercilex®*

Riassunto

È stato condotto il presente studio in doppio ceco verso il placebo su 57 donne non fumatrici che mostravano segni di prematuro invecchiamento. Tutti i soggetti sono stati sottoposti ad un nuovo regime anti-invecchiamento denominato Support System[1] che prevede l'azione combinata di un trattamento topico ed uno sistemico.

Il trattamento topico era basato sull'uso di una crema contenente palmitato di retinile microincapsulato, mentre il trattamento sistemico sull'uso di capsule contenenti un complesso marino associato a minerali.

Sia il trattamento attivo che il placebo sono stati effettuati due volte al giorno per la durata di 4 mesi rispettivamente su 29 e 28 soggetti.

I due gruppi sottoposti a sperimentazione presentavano stessa età e analoghi segni di invecchiamento paragonabili tra di loro.

La valutazione di spessore ed elasticità della cute è stata effettuata sia mediante metodi obiettivi sia mediante osservazioni soggettive dei parametri clinici. Inoltre i soggetti hanno fatto autovalutazioni dei risultati usando anche la scala visiva (VASs).

I risultati ottenuti hanno mostrato un miglioramento significativo della qualità della cute che è risultato più evidente rispetto ai precedenti studi condotti su soggetti che applicavano o solo la crema o assumevano solamente l'integratore alimentare.

INTRODUCTION

Several treatment options are available for the anti-aging treatment of the skin. Prescription drugs, cosmeceutics and cosmetic agents as well as food supplements are claimed to have anti-aging effects on the skin.

However, for most of these agents, at least for the ones which are scientifically acceptable, there is a lack of documentation for the efficacy and safety.

Marketing highly priced preparations without documented effect is unethical towards the consumers, and have therefore also been heavily criticized and even stopped by authorities in some countries.

One exception, however, is retinoic acid, which has been convincingly documented to have an effect on aging symptoms of the skin in a number of well-designed clinical studies (1-4). Retinoic acid is, in the majority of the countries in the world, a prescription drug and thus not legal to advertise directly to the consumer or sell without a prescription from a physician.

The investigational preparations used in this treatment concept (Nourella® Skin Support System) are a combination of a topical formulation (cream) and a systemic treatment (tablet). The concept is that the treatment should work both ways - from the outside in as well as from the inside out - in the treatment of the aging skin. The two treatment concepts from Pharma Medico Group have previously been studied separately but no formal studies have, to our knowledge, been carried out or published with the combination.

The main active ingredient in the cream is a proprietary microencapsulated retinyl palmitate from Pharma Medico which has been investigated in several clinical studies with respect to efficacy and tolerability in the treatment of the aging skin (5-7). As the treatment with retinoic acid has been demonstrated to give side-effects in some subjects (4-10%) in the form of soreness and redness of the skin (2), retinyl palmitate appears to be a good alternative as a cosmetic agent. Pharma Medico has chosen to microencapsulate retinyl palmitate in a polysaccharide (beta-cyclodextrin) in order to increase the penetration of the product (8). Encapsulation has been shown to increase the penetration of retinyl palmitate in in-vitro model tests (9) as well as in a clinical study (5). A good skin penetration is important for the clinical effect of retinyl palmitate (10).

Retinyl palmitate, however, is easily oxidized on the skin surface if not protected (11) and can give toxic degradation bi-products (12, 13). This can be avoided through the specific microencapsulation used in the cream tested in this study.

The main active ingredient in the tablet is a proprietary marine extract[2] which is extracted from fish and a number of publications are available with different compositions of marine ingredients and vitamins and minerals (14-17). As is the case with the monotherapy with conjugated retinyl palmitate, significant results are obtained with oral treatments with preparations containing proteoglycans, like in the present marine extract[2], in the anti-aging treatment of the skin.

Based on the abovementioned clinical experiences it was decided to carry out a controlled study with a combination of topical and systemic treatments on the effect of aging of the skin, in order to investigate the efficacy and tolerability of the preparations in females with aging symptoms of the skin, and further investigate if this creates a synergistic effect. To our knowledge only very few combination studies of the type outlined above have been carried out (18).

MATERIALS AND METHODS

The study was carried out as a randomized placebo-controlled double-blind study in 60 non smoking females aged 32-70 years.

[2] *Trademark: Vercilex®*

Thirty of the subjects received the active preparations, while 30 were randomized to receive placebos. Blinding of the treatments were achieved by using identical appearing, smelling and tasting active cream/tablets and placebo cream/tablets. The total treatment period was 4 months and the participants participated in follow-up controls after 1, 2, and 4 months.

The active cream contained the proprietary microencapsulated retinyl palmitate. The concentration of retinyl palmitate was 0.4% while the concentration of beta-cyclodextrin was 0.8%. The placebo cream used was the vehicle (cream base) without complex bound retinyl palmitate.

The participants were asked to use the cream on the face twice daily (once in the morning and once in the evening) and were instructed on how to apply it.

The participants were asked to take two tablets per day (one in the morning and one in the evening) together with food and to swallow them with water. Each tablet contained 260 mg of the marine extract in addition to 30 mg Vitamin C plus additives.

The two main ingredients in the placebo tablets were oxidized starch (E1404) and dextrin (E1400) and additives like in the active tablet.

All participants received verbal and written information about the aim of the study before they gave their informed consent to participate.

The aging symptoms of the skin were evaluated clinically by use of a five point scale. 0= Absent, 1=Very modest, 2=Modest, 3=Moderate, 4=Pronounced.

The following symptoms were scored: fine wrinkles, coarse wrinkles, tactile roughness and teleangiectasia. On the follow–up visits after 1, 2 and 4 months, the effect of the treatment was evaluated by using VASs (Visual Analogue Scales) with a length of 10 cm with defined endpoints "No change" and "Pronounced change" for the four parameters listed above. An overall rating of the extrinsic aging was also performed using a three point scale; 0=Modest, 1=Moderate, and 2=Pronounced. In addition, a global evaluation of the effect of the treatment was made also by using VASs with the defined endpoints as above.

In addition, the participants themselves performed self-evaluation by using VASs with defined endpoints "No change" and "Very pronounced change" upon their return to the follow-up visits.

Objective measurements of skin thickness and skin elasticity were performed initially and at each of the follow-up visits using two measuring sites; right and left side of the face (lateral angle of the eyes). Each measurement was done in duplicate and the mean value was registered. In the following the mean values of the measurements on the right and left of the face are listed.

At baseline and after 1, 2 and 4 months, measurements of skin elasticity, thickness and hydration of the product application area (cream) was taken by a trained technician(s) using Derma Lab® with a suction cup, DermaScan® C, respectively (Cortex Technology, Hadsund, Denmark).

A DermaLab® with a suction cup was used to evaluate skin elasticity. The suction probe, which is placed on the test site, is capable of producing a vacuum up to 65 kPa. Within the suction chamber there are two light beams set at fixed distances from the skin surface.

The measuring aperture is 10 mm in diameter and the probe itself has an ultra low weight of approximately 7 g for minimum skin bias. The probe is secured to the measuring site using an adhesive ring. When the suction pump is activated it creates a vacuum that draws the skin into the chamber. The pressure required to draw the skin to the point where it blocks the lower light beam is recorded. The pump remains on, and the skin continues to be drawn into the chamber to the point that it will eventually block the upper light beam as well. The skin is then allowed to relax for 10 seconds before the vacuum resumes

for a total of five cycles. The geometry of the suction cup standard probe is such that the 10 mm diameter section of the skin being sampled is extended approximately 2% and 12% when lifted to these respective levels. Since we know both the stress and strain at these two points we can also compute the "stiffness" ratio.

Non-invasive measurements of skin thickness were made on all subjects' mid-region of the volar aspects of the forearms at the designated time-points. A DermaScan® C (Cortex Technology, Hadsund, Denmark) was utilized. This system consists of a hand-held probe that contains an ultrasonic transducer, which is interfaced to a specially configured computer. The probe has a built-in closed water-path and is capable of scanning within a 22.4 mm x 22.4 mm area of the skin surface. To ensure good transmission of the ultrasound signals, a water compatible ultrasound gel was used as a skin contact medium. The scanner processes four frames per second and displays the results as a live B-mode image on an interactive monitor. In the present study, a standard 20 MHz transducer with a bandwidth of 15 MHz, a focal distance of 30 mm and a 6dB focal length of approximately 13 mm was employed. Using this transducer, the axial system resolution is 50 μm, the lateral system resolution 350 μm, and the typical usable tissue penetration is 10 mm. The field of view in depth can be set in four steps going from a minimum of 1.7 mm full screen to a maximum of 13.4 mm full screen. All four fields of view can be panned live to a maximum scanning depth of 30 mm. The B-mode images are presented in a 256 shade pseudo color scale using the factory grey scale-color assignments. The scales are directly related to the A-scan amplitude, which corresponds to the returning echo. The amplitudes displayed are proportional to the recorded amplitudes up to the value 200 within 0 to 256 scale. Above this value, the amplitudes are displayed compressed. The images are computed from 224 A-scan lines recorded along the transducer movement, each A-scan having 256 sampling points. The live images can be instantly frozen and then saved as exportable image files for subsequent processing. Any A-scan line can be displayed and its position marked on top of the frozen image for closer analysis and measurement of distances. Average measurements were made using a total image boundary threshold method. Gain settings were selected that are most likely to produce the best visualization of the skin.

In order to avoid inter-observer variability all subjective skin measurements were carried out by the same trained person.

Ethics

The study protocol, the patient information sheet, and the informed consent forms were sent to the Regional Ethic Committee (REK) prior to starting the study. REK had no objections against starting the study.

Statistical methods

The change from the baseline elasticity, thickness and hydration parameters was compared between treatments using a paired t test or Wilcoxon signed - rank test, as appropriate. The VAS subjective assessments were analyzed similarly. The changes from baseline were tested within each treatment using the paired t test. Data after 1, 2 and 4 months was analyzed independently.

RESULTS

A total of 57 subjects concluded the study according to the protocol, 29 of these subjects received the active treatment with cream and tablets while 28 received placebo. The one drop-out in the active group was due to skin rash while the

two in the placebo group did not arrive for the follow-up control after 2 months' treatment, and were thus excluded from the statistical evaluation. The overall tolerability was good. None of the persons on placebo reported any side-effects. All the subjects concluding the study had a compliance of least 80% of the recommended dose in order to be included in the statistical analyses. Compliance was checked by counting the returned tablets and by weighing the returned cream packages.

In Table I the demographic as well as the global rating of the aging symptoms initially in the two treatment groups are shown. All the included subjects were females. As can be seen from Table I the two groups of subjects were clinically comparable at the start of the study.

The results from the global clinical evaluation are shown in Table II. Following a 4-month treatment period no significant differences in the global evaluation could be detected in the persons receiving placebo, while a significant effect could be seen in the group receiving the active preparation. It is worthwhile to underline that this effect was more pronounced after 4 months than after 2 months as can be seen from the table, which indicates a synergistic effect setting in at some point.

Objective skin measurements

In Table III, the measurements of the skin thickness and elasticity are presented, respectively. When comparing the two treatment groups with regard to change in skin thickness and skin elasticity there are highly significant differences between the group receiving the placebo treatment and the group receiving the active treatment.

Participant's assessment of effect

The self-evaluation of the effect of the treatment by the subjects revealed a significant difference in favour of the active group after a treatment period of 4 months, as shown in Table IV.

Of the 29 subjects in the active group, 23 (79. 31%) felt that they had a visible improvement of the skin quality after self-evaluation, while only three of the placebo-treated, subjects felt they had a slight effect.

TABLE I

Demographic data of the participants at entry in the two treatment groups (SD in parentheses)

Group	Age (yrs)	The grade of aging symptoms (No. of patients)		
		Modest	Moderate	Pronounced
Active	48.5 (7.2)	9	17	3
Placebo	47.2 (8.3)	7	19	2

TABLE II

Global assessment of the skin aging symptoms as a function of the treatment duration using VAS (SD in parentheses)

Group	After 2 months (cm)	After 4 months (cm)
Active	4.5 (1.8)	8.3 (3.0)
Placebo	0.8 (0.9)	0.6 (0.7)

Skin aging as function of age

In Table V we have listed measurements of skin thickness as well as elasticity on a population of Nordic females (19). Altogether the population consists of 70 females divided into age brackets of decades from the age of 20 to the age of 80 with 10 females in each of the brackets. As can be seen from the table there is a significant decrease in thickness as well as elasticity as a function of age. The results from our study can easily be compared with the data in Table V and Table III, and the objectively measured parameters shows an objective reversing of age in the skin equivalent to 20 years.

The majority of the subjects (28 subjects (97%)) in the active group would like to continue with the treatment at the end of the study period, while only one of the subjects in the placebo group would like to continue.

TABLE III

Mean skin thickness and elasticity index of 57 females treated with active treatment or placebo treatment for a period of 4 months (SD in parentheses)

	Active		Placebo	
	Skin thickness(mm)	**Elasticity(%)**	**Skin thickness(mm)**	**Elasticity(%)**
Initially	0.92 (0.13)	48 (8.4)	0.95 (0.17)	47 (7.2)
8 weeks	1.02 (0.15)	51 (8.6)	0.92 (0.13)	46 (7.0)
16 weeks	1.31 (0.17)	65 (8.7)	0.93 (0.15)	46 (6.9)

TABLE IV

The results of the participants' self-evaluation of the effect of the active treatment and placebo as function of treatment duration on VAS (SD in parentheses)

Group	After 2 months (cm)	After 4 months (cm)
Active	5.2 (1.9)	8.1 (3.1)
Placebo	0.3 (0.6)	0.2 (0.8)
p-value	≤0.001	≤0.0001

TABLE V

Age-related values of skin thickness and skin elasticity in Nordic females as function age (SD in parentheses)

Age	Skin thickness (mm)	Skin elasticity (%)
20 (n=10)	1.49 (0.15)	77 (7.1)
30 (n=10)	1.47 (0.20)	74 (7.0)
40 (n=10)	1.23 (0.21)	62 (5.4)
50 (n=10)	1.10 (0.14)	54 (4.5)
60 (n=10)	0.96 (0.13)	47 (3.1)
70 (n=10)	0.79 (0.12)	45 (4.5)
80 (n=10)	0.71 (0.13)	43 (4.7)

Tolerability

The overall tolerability was good. None of the participants in either of the two groups stopped treatment due to side effects, or reported any side effects.

DISCUSSION

The results from this study demonstrate that a combination of topical and oral treatments, as used in this study, have an additive effect on the skin parameters as compared to the treatments given separately.

We have previously published our experiences with the use of complex bound retinyl palmitate as a single treatment of the aging skin (5-7), and numerous studies have also been published on the use of monotherapy with marine extract based treatments.

We are convinced that the microencapsulated retinyl palmitate as used in the active preparation tested, has two main advantages as compared to plain retinyl palmitate when used on the skin surface. Firstly the complex binding will improve the skin penetration of Vitamin A ester (5, 8, 9) and secondly, the complex binding will protect the ester from oxidation and creation of toxic decomposition products on the skin surface.

Several preparations are available on the market where marine extracts are one of the main ingredients, however, only this specific skin treatment system[1] contains the proprietary marine extract[2] - and favourable results have been documented.

The results from this study demonstrate that improvements in the two main parameters of skin thickness and skin elasticity are significant, and the age cycle of the skin is reversed to an exceptional degree.

Recently it has been documented that other cosmetic preparations can have a significant anti-aging effect on the skin (20, 21).

The excellent tolerability and efficacy of the present skin support system[1] treatment, makes it an attractive, highly effective and safe treatment possibility for the treatment of age and sun damaged skin.

[1] *Trademark: Nourella® Skin Support System*
[2] *Trademark: Vercilex®*

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Author Address:

Erling Thom
ETC Research and Development
Bjoernveien 45A
0774 Oslo Norway
Email: erlingthom@etc.as

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Molecular docking studies of L-cysteine (a non-essential sulfur containing aminoacid) as a potent Tyrosinase inhibitor

N. Radhakrishnan[1], S. Ashok[2], V. Kavitha[1], G. Rameshkumar[2], A. Gnanamani[1]

[1] Microbiology Division, Central Leather Research Institute (CSIR-New Delhi), Adyar, Chennai, India

[2] AU-KBC Research Centre, MIT Campus of Anna University, Chromepet, Chennai, India

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Summary

L-cysteine enhances resistance (anti-static) and stimulates the growth of hair and nails. In other-words, it is regard as hair and nails revitalizing agent. In addition, it is required for the biosynthesis of trypanothione, coenzyme -A, hypotaurine, taurine as well as ubiquitous iron-sulfur (Fe-S) clusters, which are involved in electron transfer, redox regulation, nitrogen fixation, and sensing for regulatory processes. In the present study, we calculated molecular physicochemical and drug-likeness properties of L-cysteine using molinspiration online tool. In addition, we evaluated L-cysteine docking behavior with the copper-bound *Streptomyces castaneoglobisporus* tyrosinase and investigated its putative binding residues using Autodock 4.0. The study results reveal that L-cysteine complies very well with thumb rule of five. With reference to docking studies, L-cysteine exhibits lowest binding energy of -3.15 kcal/mol and its putative binding residues were Arginine, Glutamic acid and Tryptophan (at 55th, 182th & 184th position) respectively. Thus, our present molecular docking studies could contribute for the further, development of tyrosinase inhibitors for the prevention of hyper pigmentation.

Riassunto

La L-cistina incrementando la resistenza e stimolando la crescita sia dei capelli che delle unghie, può essere considerato un agente rivitalizzante per entrambi questi annessi cutanei. Inoltre, affinchè si verifichi la biosintesi del tripanotione sintetasi è necessaria la presenza del coenzima-A, dell'ipotaurina, della taurina e del complesso ferro-zolfo (Fe-S) coinvolti nel trasferimento degli elettroni, nella regolazione del sistema ossido-riduttivo ed in altri processi di sintesi.
Con il presente studio si sono volute evidenziare sia le caratteristiche chimico-fisiche e molecolari che le attività terapeutiche della L-cistina utilizzando il mezzo informatico denominato *molinspiration* che si basa sui modelli in silicio.
Inoltre, attraverso il modello molecolare denominato *Autodock* si è cercato di valutare il legame che lega il rame alla L-cisteina nell'attività svolta dalla tirosinasi.
In riferimento agli studi effettuati con l'Autodock, la L-cisteina rivela un legame energetico molto basso pari a -3.15kcal/mol, mentre i legami Arginina-Acido glutammico e Triptofano sono stati rispettivamente pari a 55^{th}, 182^{th} e 184^{th}.
Dati i risultati ottenuti, riteniamo che il nostro studio possa contribuire allo sviluppo di inibitori della tirosinasi necessaria per prevenire la comparsa delle iperpigmentazioni cutanee.

INTRODUCTION

L-Cysteine (a sulfur-containing amino acid), which is wide spread in virtually all living organisms from bacteria to higher eukaryotes, and plays a vital role in the various cellular processes including stability, structure, regulation of catalytic activity (enzymes), and posttranslational modification for various proteins. It also exhibits antioxidant properties and it serve as building block for the biosynthesis of glutathione, which is found in humans as well as other organisms. In addition, it is also required for the synthesis of trypanothione, coenzyme -A, hypotaurine, taurine as well as ubiquitous iron-sulfur (Fe-S) clusters, which are involved in electron transfer, redox regulation, nitrogen fixation, and sensing for regulatory processes(11).

L-Cysteine has wide industrial applications (in various fields such as pharmaceutical, food and cosmetic). Chen and Chavin (1) reported, inhibition of human melanoma tyrosinase by L-cysteine-HCl using L- (U-^{14}C) tyrosine as substrate. Kermasha et al (3) reported, inhibition of mushroom tyrosinase by both L-cysteine and DL-cysteine using catechin as substrate. Vieira and Fatibello-Filho (13) reported, inhibition of sweet potato root polyphenol oxidase (PPO) by L-cysteine using flow injection system. Gacche et al (2) reported, reversible inhibition of apple polyphenoloxidase (PPO) by L-cysteine. Recently, we also reported inhibition of mushroom tyrosinase by L-cysteine(11). Hence, in the present study, we calculated molecular physicochemical and drug-likeness properties of L-cysteine using molinspiration online tool. In addition, we evaluated L-cysteine docking behavior with the copper-bound *Streptomyces castaneoglobisporus* tyrosinase and investigated its putative binding residues using Autodock 4.0.

MATERIALS AND METHODS

Target protein Identification and preparation

The three dimensional structure of the copper-bound *Streptomyces castaneoglobisporus* tyrosinase (PDB id: 1WX2) was obtained from the RCBS Protein data bank [PDB] (http://www.rcsb.org/pdb). The protein was pre-processed separately by deleting the ligand as well as the crystallographically observed water molecules (water without Hydrogen bonds).

Ligand preparation

Chemical structures of ligands such as L-Cysteine [CID no: 5862] was retrieved from Pubchem compound database (10).

Molecular descriptors calculation

Molinspiration (7) was used to calculate (molecular physicochemical & drug-likeness properties) thirteen descriptors such as logP, polar surface area, molecular weight, number of atoms, number of O or N, number of OH or NH, number of rotatable bonds, volume, drug likeness (includes GPCR ligand, ion channel modulator, kinase inhibitor and nuclear receptor ligand) and number of violations to Lipinski's rule for ligand (L-cysteine) taken for the analysis (5).

Docking setup

Docking was performed using Autodock 4. Autodock combines energy evaluation through precalculated grids of affinity potential employing various search algorithms to find the suitable binding position for a ligand on a given pro-

tein (8). Kollman united atom charges and polar hydrogens were added to the protein PDB using Autodock tools (8). All rotatable bonds in the ligand were kept free to allow for flexible docking. Grid size was set to 40 x 40 x 40 grid points (x, y and z), with spacing between grid points kept at 0.375 Å. The Lamarckian genetic algorithm was chosen to search for the best conformers. Standard docking protocol was applied. One hundred independent docking runs were carried out for ligand (L-cysteine) generated using genetic algorithm searches.

RESULTS

In the present study, LogP value of L-cysteine was observed as -2.199. The molecular weight of the L-cysteine was calculated as 121.161 g/mol. Hence, L-cysteine in the present study complies very well with all the five rules and exhibited nil violation as shown in the Table I.

With reference to docking studies, L-cysteine exhibits lowest binding energy (LBE) of -3.15 kcal/mol as shown in the Table II and its putative binding residues were Arginine, Glutamic acid and Tryptophan (at 55th, 182th & 184th position) respectively as shown in the Figure 1 and Table II.

TABLE I

Represents the molecular physicochemical and the drug-Likeness properties analysis of L-cysteine using molinspiration software

Molecular Physicochemical properties	L-Cysteine
Log A (Octanol-Water partition coefficient)	-2.714
TPSA (Polar surface area)	-63.322
natoms (Number of non hydrogen atoms)	7.0
MW (Molecular weight)	121.161
nON (Number of hydrogen bond acceptors [O and N atoms])	3
nOH NH (Number of hydrogen bond donors [OH and NH groups])	3
nviolations (Number of Rule of 5 violations)	0
nrotb (Number of rotatable bonds)	2
Volume (Molecular volume)	102.215
Drug likeness (or) Bioactivity score	
GPCR ligand	-2.93
Ion channel modulator	-2.83
Kinase inhibitor	-3.66
Nuclear receptor ligand	-3.44
Protease inhibitor	-1.49
Enzyme inhibitor	-1.49

TABLE II

Represents the SMILES, Lowest and mean binding energy of L-cysteine

Ligand	SMILES*	Lowest binding energy (kcal/mol)	Mean binding energy (kcal/mol)
L-Cysteine	C([C@@H](C(=O)O)N)S	-3.15	-2.42

*- Simplified Molecular Input Line Entry System (SMILES).

TABLE III

Interaction sites, bond sites and bond distance, between tyrosinase (Streptomyces castaneoglobisporus) and L-cysteine

Ligand	Interaction sites	Bond	Bond distance (A°)
L-cysteine	55th residue Arg	Arg 55-NH_2···O(L-cysteine)	3.6
	55th residue Arg	Arg 55-NH_2···O(L-cysteine)	3.3
	55th residue Arg	Arg 55-NH_2···O(L-cysteine)	3.1
	182th residue Glu	Glu182-OE2···H(L-cysteine)	2.1
	184th residue Trp	Trp184-NE1···O (L-cysteine)	2.5

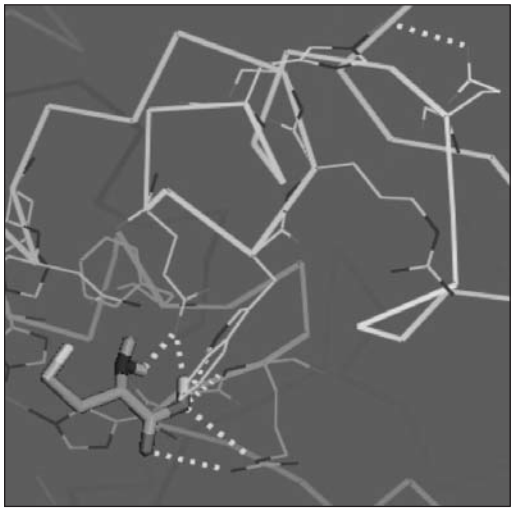

Fig. 1 *Represents the docking results of L-cysteine with tyrosinase (Streptomyces castaneoglobisporus).*

DISCUSSION

Molecular physicochemical and the Drug-likeness are the two properties that are significant for considering a compound to become a successful drug. The rule formulated by Lipinski et al (4) considered as the thumb rule, where the rule describes molecular properties significant for a drug's pharmacokinetics in the human body. It is also important for drug development, where a pharmacologically active lead structure is optimized step-wise for increased activity and selectivity, as well as drug-like properties as described by Lipinski's rule.

LogP (Octanol-water partition coefficient) is used as significant tool in both quantitative structure activity relationship (QSAR) studies and rational drug design as a measure of molecular hydrophobicity. LogP value less than 5 will be preferred for drug likeness property. Hence L-cysteine, compiles with this rule number one. The preferred range of molecular weight for drug likeness property was in range between160 to 480 g/ mol as reported by Tambunan and Wulandari (12). Hence L-cysteine, compiles with this rule number two. With regard to the preferred number of N, O (hydrogen bond

acceptors) and OH & NH (hydrogen bond donors) 10 and or less than 10 and 5 and or less than 5,which compliance with the rule number three and four respectively. Further, the preferred number of rotatable bonds (rotb) is 15 and or less than 15, which compliance with the rule number five. Hence, L-cysteine in the present study agrees very well with the five rules and exhibited nil violation. Regulation of the enzymatic activity of tyrosinase has been the important focus of investigation due to its potential applications in medicine, agriculture and cosmetics. Indeed, understanding and inhibiting tyrosinase would be a significant in medicine due to its clear role in Parkinson's disease (14), melanoma (9) and hyper pigmentation (6). Hence, our present study results provide new insight in understanding the L-cysteine, as a potent tyrosinase inhibitor.

To our knowledge, this is the first kind of report on molecular docking analysis of L-cysteine with tyrosinase (*Streptomyces castaneoglobisporus*).

CONCLUSION

Thus, our present molecular docking studies could contribute for the further development of tyrosinase inhibitors and the prevention of hyper pigmentation.

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Author Address:

Dr. A. Gnanamani
Microbiology Division
Central Leather Research Institute (CSIR-New Delhi)
Adyar, Chennai 20
Tamil Nadu, India
Fax : 91-44-24912150
Email: gnanamani3@gmail.com

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Biomedical Science is a multidisciplinary field, involving material, mechanical, and medical engineering, clinical medicine, molecular cell biology, bioethics, regulatory affairs, business, etc, all interacting each other and contributing to ameliorate the human health.

This book, organized in 11 Chapters and 1 Appendix, covers various applications on human use of these biomaterials, emphasizing the need for a clinical and engineering integration approach. The development of technology for future medical applications is, in fact, a growing segment of our economy, and many companies, universities, and laboratories are striving to get products into this field, to be accepted for use by the medical community.

The rapid prototyping of innovative technology solutions into products for a wide range of customers is, therefore, an essential business requirement for success in the technology and development marketplace. At this purpose, many technologies, appliable to the medical area, are available today from different industries and from different applications, punctually reported on the book. However, the ultimate design of a biomedical product would be its ability to orchestrate desirable effects and then degrade without leaving undesirable metabolities.

After some introductory remarks reported on **Capter 1**, **Chapter 2** is dedicated to explain the meaning of hemocompatibility, defined as a material that induces an unacceptable adverse reaction when placed in contact with blood for a specific time. It should be noted that any foreign material may cause some kind of reactions, whether local and/or systemic, controllable or not.

The adverse reactions include the formation of a local blood clot, and possible shedding of this clot, which will undesirably travel elsewhere as an embolus having devastating effects, such as stroke. Thus the issue of contacting time with blood may be a particularly important fader to consider, regarding biomaterials and their respective medical technologies. The patient's needs of addressing biomaterials analysis and control have been reported and discussed, underlining the diverse nature patient variability, and the necessity of the implementation of human clinical trials with long-term follow-ups, in several clinical states. As a consequence, the necessity of an enhanced characterization of the blood-biomaterial interactions in autoimmune models have lead to the development of an enhanced surface modification of some biomaterials.

Chapter 3 is completely focused on the *Medical Application of Micro-Electro-Mechanical Systems (MEMS) Technology*. The micromechanical devices are integrated with electronics to develop these MEMS-high-performance closed-loop controlled micro-electro-mechanical systems.

Today, in fact, the miniaturization of electromechanical systems offers unique opportunities for scientific and technological progress in the medical science. Because of the exceptional performance and cost/benefits, the commercial market of these microsensor devices are growing at rapid rate. It is interesting to underline the real potential of MEMS because their integrated circuits can be thought of as the *brains* of the system.

They augment the decision-making capability with *eyes* and *arms* to allow microsystems to sense and control the environment. The lower cost of these miniaturized electromechanical systems also allows them to be easily and massively deployed and more easily maintained and replaced as needed. Thus many the benefits of MEMS technology for medical applications.

First their integrated circuit-like processes enable the integration of *multiple* and *diverse functionalities* onto a single microchip. Second, integrated circuit fabrication techniques coupled with the tremendous advantages of silicon and many other thin-film materials in mechanical applications allows the reliability of miniaturized electromechanical systems to be exceptionally high. Third the per-unit device cost of the systems can be radically reduced. Fourth, the miniaturization enables many benefits, including increased portability, lower power consumption, and the ability to place sensors in a smaller amount of space and without any increase in weight. Fifth, the ability to make the signal path smaller allows to improve the overall performance of these electromechanical systems. In conclusion, also if the challenges to obtain the right biocompatibility and biostability of all the materials used for the MEMS are stringent, and not easy to solve, this technology provides new and unique capabilities for the development of smart products for many applications, including medicine and biology.

Chapter 4 describes the barriers associated with various delivery routes, specifically tissue, cellular, and molecular nanoparticles delivery approaches across tissue barriers are, therefore, discussed along with their systemic biodistribution and interactions with the biological environment.

Any therapeutic nanocarrier requires, in fact, high-throughput optimization of many physicochemical parameters, including surface hydrophilicity, surface charge, surface functional groups, particle size, core materials, linker composition, nanoparticle shape, and targeting ligand density, for optimization of therapeutic efficacy, reduced toxicity, and pharmacokinetic parameters. Certainly, non-degradable nanoparticles that accumulate intracellularly are likely to have a number of toxic effects and, therefore, need to be designed and targeted for rapid excretion.

Thus the necessity to a better understanding of the nanoparticles' composition and the microenvironments where they should accumulate, is of critical importance to the development of more effective nanoscale systems for therapy and diagnosis.

Polymeric nanoarticle delivery systems have the potential to significantly impact, for example, the treatment of cancer and other fundamental human pathologies. Their physicochemical properties, in fact, can be engineered at the molecular level, and their shape, size, and electrical charges can be controlled, as well as the surface density of the targeting ligand can be optimized for specific applications.

Tissue engineering aim to restore and regenerate the lost tissue structure and function by delivering the right cues to guide tissue regeneration.

Collaboration between clinicians and engineers is very important for advance in the field of biomaterials science. Thus, every nanoparticle or nano device must be manufactured with appropriate/functional biomaterials for optimized long-life performance, when used for or implanted

in the human body. They can be functionalized at their surface with molecular moieties (i.e. for example antibodies), adding extreme versatility for various therapeutic applications, thus allowing the creation of different therapeutic uses. This versatility is, therefore, an attractive technology to increase the portfolio of pharmaceutical products by performing minor modifications. It is to remember, in fact, that these nanostructured products/devices may have the dimension as or lower than the human cell. This is the reason why nanotechnology could be defined as a group enabling technologies capable of developing structures, systems, and devices in the range scale between 1 and 100 nanometers(nm). In order to have an idea of this scale, the diameter of a hair is about 80, 000 nm, the size of a red cell is in the order of 7000 nm, and a bacterium is about 1000 nm long.

Chapter 5 is focused on biomaterial to be used for implant in dentistry for improving stability and longevity of implant-based clinical treatments. The new biomaterials selected for construction of dental implant body sections, controlled by biomechanical principles, minimize, in fact, the biodegradation and fracture phenomena, exhibiting an interesting bone integration. The evolution until today of surgical implants and materials in dentistry is amply reported, analyzed, and discussed in this chapter.

Different problems are reported on **Chapter 6** where a variety of biomaterials used in the treatment of different disorders of the central nervous system (CNS) are discussed. Regardless of the material's stability over time, it must be deemed biocompatible before it may be implanted intracranially.

Biocompatibility with tissue outside CNS does not always predict biocompatibility with brain and spinal cord tissue, nor does short-term biocompatibility predict long-term tolerance implantation. Understanding a material biocompatibility requires, therefore, an intimate understanding of the brain's immune system and inflammatory cascade. Properties of different materials such ad naturally occurring or synthetic polymers are reported and focused in this chapter, including the studies of toxicity and integration with the host tissue.

Biomaterials in Obstetrics and Gynecology is the topic of **Chapter 7**, where the medical and surgical conditions throughout the woman' s health are discussed together with her quality of life. Decreased fertility is a known characteristic of affluent Western Societies, as consequence of a family planning measures and a desire to postpone childbearing. Thus the use of many *barrier methods*, such as condom, diaphragm and intrauterine devices made by different biomaterials. In this chapter is focused and reported the role of biomaterals used in obstetrics and gynecology during all the woman' s life time, not only for contraception but also to improve her quality of life.

Appropriate selection of cells and biomaterials is the key factor in the construction of viable and clincally relevant engineered tissue for myocardial regeneration. This is the topic reported on **Chapter 8**, where the various applications of biomaterials for such purpose are discussed. Owing to limited regenerative capacity of the adult mammalian hearth, any significant myocardial cell loss is mostly irreversible and can lead to heath failure.

Appropriate selection of cells and biomaterials is the key factor in the construction of viable tissue for myocardial regeneration. Thus the necessity to create performed cardiac construct or injectable-based biomaterial systems with scaffolding capacities is expected to have notable impact on cell-delivery efficiency. At this purpose, vascularization has emerged as a prerequisite for designing large tissue in vitro and for enhancing graft survival following implantation by providing a robust source of oxygen and nutrient supply, as well as intracellular signaling critical to further tissue development. Vascularization tissue and biological signals play an important role for musculoskeletal tissue engi-

neering research also, together with heterogeneity host-tissue integration, overcoming immunogenicity, and improved mechanical properties. This the topic discussed on **Chapter 9**.

The musculoskeletal system represents, in fact, the major structure of the body, and enables the whole body to move and maintain its form and function in load-bearing conditions. All tissues in this system contain a similar type of hierarchy that defines the diverse tissue structure and functions in human body.

The interactions between cells, matrix networks, and biological signals are crucial for the normal tissue structure and function, and disruptions in these processes often lead to musculoskeletal diseases and/or tissue degeneration.

Loss of the tissue, caused by traumatic injury, aging, or disease is common and a guided regeneration is required. Thus, tissue engineering aims to restore and regenerate the lost tissue structure and function by delivering the right cues to guide tissue regeneration. At this purpose, most tissue engineering strategies, composed of single or a combination of three key components -scaffolds, cells, and instructive signals- are designed and optimized for specific needs of various tissues.

The scaffold, that serves as a temporary matrix to support the tissue regeneration, may also be considered as a reservoir system that releases biological signals. These signals, coordinating many physiological necessities, play an important role during the normal tissue development process and mediate the tissue remodeling during injury.

To reach this goal, properly designed scaffold architectures are developed to trigger the different cellular fates for biological functions of specific organs. This requires not only controlling the biochemical and physical properties of scaffold materials, but also developing microstructures within the scaffolds of dimension comparable to the cells' size. These microstructures take important roles in sustaining cell proliferation and facilitating the diffusion of nutrients, wastes, and signaling molecules in a tissue-engineering scaffold.

A better understanding of cell-niche interactions as well as appropriate engineered models will be crucial for developing functional tissue substitutes to repair musculoskeletal tissue injures.

With the advent of new technologies, the science of biomaterials has evolved rapidly. This evolution has brought about several consequences, including a more intimate contact of materials with the human body, the increasing concerns about the toxicological and environmental fate of these micro/nano structures and their social and ethical consequences.

The mapping of the innovative technologies together with their risk/benefits for human uses are analyzed on **Chapters 10** and **11**, where the in progress new perspectives are reported.

To visualize the future development of the medical market for biomaterials, has been the principal scope of this book, that provided the lecturer with a comprehensive list of the today more important applications in the medical field.

A large room has been reserved to the discussion on the risk/benefits of all the biomaterials used, as well as an overview on the clinical trials designing has been reported together with the market needs and the relative regulatory challenges.

The book, written from well known engineers and physicians expert in the field of the biomedical science, provides a clear picture of the innovative engineered materials used up today without forgetting the patient's needs. Thus it may be considered an important key stone for all physicians and chemical engineers involved in this fascinating field of engineered-medicine.

The importance of the problems reported and clearly explained, and the in deep discussion of the

treated topics, gives the lecturer the necessary and right informations useful for both the Chemical and Medical communities which are involved or wish to better understand the progress and future perspectives of the innovative materials used in the biomedical field, differently engineered for trying to solve many patient's needs.

P. Morganti
Editor-in-Chief

Gold Nanoparticles for Physics, Chemistry and Biology

by C. Louis and O. Pluchery

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The gold played a great fascination in every century and its use in the form of nanoparticles is related to the history of red-coloured glass born probably in Egypt and Mesopotamia in 1400-1300 BCE. However, the first milestone in the history of gold ruby glass is represented from the Roman Lycurgus cap that, dated to the fourth century has revealed today by electronic microscopy, the presence of minute amounts of gold (about 40 ppm) and silver (about 300 ppm) of 5-100 nm in diameter. It is interesting to underline that gold nanoparticles (AuNPs) exhibit a rich array of interesting and important catalytic properties, which has sparked a huge interest in gold-based systems in several interdisciplinary areas.

All the topics of interest on this unusual metal are reported in this book, organized in 13 chapters with an introductory Preface.

Gold possesses a unique combination of physical and chemical properties in both the macroscopic and microscopic states; on the macroscopic scale it is know for its unique yellow color, as well as for its chemical stability and high redox potential. On the nanoscale, its unusual electronic configuration, made by high ratio of surface atoms to bulk atoms, results in particular electromagnetic properties and quantum effects. Thus AuNPs, for example, as colloidal nanoparticles (CNP) have resilient properties compared to other inorganic nanoparticles, such as unique plasmon-resonance, optical properties and a bioconjugation surface for molecular probe. These unique properties have drawn attention with a view to developing a totally different method in the field of nanomedicine.

Colloidal AuNPs has been used as carrier of antitumoral drugs, antibodies, and other drugs for selective killing of diseased cells and microbes, not for their own antimicrobial activity, but for the more significant antimicrobial effect showed when coupled with antibiotics. In other fields AuNPs have shown an interesting role as catalysts for important reactions such as those occurring in the exhaust of internal combustion engines or in electrocatalytic and photocatalytic processes. Naturally the size of the gold particles to be prepared on supports depends on the further use of the materials. For catalysis, very small particles are needed, generally smaller than 5 nm, while for physics or biology larger particles can be required.AuNPs can also be deposited on organic supports or embedded in organic materials leading to hybrid inorganic-organic materials for applications in life science.However, nanometer-sized gold nanoparticles are of fundamental and practical interest, as their chemical, electronic, and optical properties can potentially be exploited for different applications.

These properties are, in fact, extremely dependent upon their size and shape. At this purpose, numerous approaches to synthetically and sistematically control morphology electrical charge and surface composition have been developed in recent years, as well as many methods to prepare gold nanostructures, including electrochemical, gas and liquid phase methods. All these methods are necessary to exploit and better understand the unique properties and structures of these nanoparticles for the many and possible practical applications.

The structure and geometry of the surface atoms of gold in liquid phase, generally referred to as a *monolayer protected cluster*, is different from that of central atoms, and forms surface facets and edges that dominate catalytic activity. The surface protecting ligands are anchored on the surface atoms, stabilizing their structure, and sometimes provide surface functionality that influences the chemical nature of the particles in solution. In any way gold nanoparticles are classified into types according to their size and size-dependent properties: size ranges of less than 2 nm, 2-10 nm, and -0-300 nm.

Particles smaller than 2 nm in diameter are called gold clusters, which consist of a few ten to a few hundreds of gold atoms. Because of their outstanding properties, gold nanoparticles can find applications in fields in a wide range of areas, including chemical or biological sensors, imaging, medical materials, catalysis for organic chemical synthesis, and energy generation.

Consequently, easy, rapid, and large-scale synthesis, and diverse and stable surface treatments of these nanoparticles are still required, and this is the reason why a better understanding of the unique properties of hold surfaces in the nanometer size regime are currently being performed. Thus, the convergence of nanotechnology, health, and optics based on the extraordinary optical properties of AuNPs supporting Localized Surface Plasmon (LPS) Resonances. For AuNPs such resonances occur in the visible range of the spectrum and their features are determined by the particle geometry and environment. Upon suitable illumination matching the resonance conditions, the light is efficiently coupled to, the nanoparticles. Part of the coupled light is efficiently scattered: (1) in the near field.

Leading to an enhanced field at the particle surface, and (2) to the far field, the NP acting as an efficient optical antenna. The remaining part of the energy is absorbed and dissipated into heat, creating an increase of the metal temperature. Thus AuNPs offer a set of new opportunities to improve diagnosis and therapy of diseases such as cancer. They, in fact, may be used for carrying anti-tumour drugs. These metal-nanoparticles can be transported into the cancer cells being encapsulated in advance with molecules having a high affinity for the proteins, expressed only by the tumour. In conclusion AuNPs have shown to possess unique and useful chemical and physical properties. This is the reason why they are used in many fields, such as diagnostic assays, as specialized stains for electronic microscopy, as the colouring agent in niche glassware and ceramics, in high-end decorative inks, and in some electronics as sinter inks.

Many are the other potential applications in progress in the high-technology domain of AuNPs with high added values, so that the value of the gold they contain is insignificant as a proportion of the final cost.Finally, also if the lethality of AuNPs per se is relatively low and pure metallic gold is non-toxic and non-irritating when it is ingested, research into nanotoxicology and particularly nano-ecotoxicology is still at an early stage, and more data is needed before gold nanoparticles can be used safety for diagnostic or therapeutic purpose.

As conclusive remarks, also if the fascination with gold is a story which spans millenia, this interesting book reporting a great quantity of news on the process methods, chemical and physical pro-

perties an uses of this metal in the nanustructured forms opens an interesting window of interest not only for graduated students in chemistry and physics, but also for the Medical and Chemical Community, wishing to better understanding the potentiality of the nanostructures in general, and the gold nanoparticles in particular.

P. Morganti
Editor-in-Chief

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Trimestrale di Dermatologia Cosmetologica
Quarterly Review of Cosmetic Dermatology

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Efficacy of a Cosmetic Caffeine Shampoo in Androgenetic Alopecia management. II Note

T. Sisto MD, C. Bussoletti, MD, L. Celleno, MD
Department of Dermatology, Catholic University of the Sacred Heart, Largo Gemelli 8, 00168 Rome, Italy

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Summary

Androgenetic alopecia (AGA) is a common, progressive, patterned loss of visible scalp hair. The severity and frequency are greater in men. It causes psychological distress and negative effects on the quality of life and thus the prospect of treating AGA is mandatory. Dihydrotestosterone (DHT) is responsible for hair loss in AGA. At present only two medical treatments, minoxidil and finasteride, are licensed for the treatment of male balding. Caffeine was identified as a stimulator of human hair growth. We report the results of a randomised, controlled, double-blind, parallel group study to assess, in a panel of healthy men suffering from AGA, the skin compatibility of a shampoo containing caffeine and to assess its anti hair loss efficacy and its cosmetic qualities, after application for 6 consecutive months versus placebo. Our data support the beneficial effects of topical application of caffeine in AGA, suggesting it as a cosmetic growth-stimulating agent suitable for AGA treatment.

Riassunto

L'alopecia androgenetica (AGA) è una condizione comune, caratterizzata dalla progressiva perdita di capelli. La gravità e la frequenza sono maggiori negli uomini. È fonte di stress psicologico e causa effetti negativi sulla qualità della vita, quindi è auspicabile la ricerca di un trattamento efficace. Il diidrotestosterone (DHT) è responsabile della perdita dei capelli osservata nell'AGA. Al momento solo il minoxidil e la finasteride sono autorizzati per il trattamento della calvizie maschile.
La caffeina è stata identificata come stimolatore della crescita dei capelli umani.
Riportiamo i risultati di uno studio randomizzato, controllato, in doppio cieco, a gruppi paralleli per valutare, in un gruppo di uomini sani che soffrono di AGA, la compatibilità cutanea di uno shampoo che contiene caffeina e per valutare la sua efficacia contro la caduta dei capelli e le sue qualità cosmetiche, dopo l'applicazione per 6 mesi consecutivi, rispetto al placebo.
I nostri dati supportano gli effetti benefici dell'applicazione topica di caffeina nell'AGA, suggerendola come agente cosmetico in grado di stimolare la crescita dei capelli per il trattamento dell'AGA.

INTRODUCTION

The term androgenetic alopecia (AGA) describes a form of scalp-hair loss in which there is a decline in production of hair, which may eventually lead to balding. It affects both sexes and all ethnic groups although the severity and frequency are greater in men and there are racial differences in prevalence. Male AGA is a common androgen-dependent trait that can start at any age after puberty. In the majority of men balding is patterned, in which the two major components are fronto-temporal recession and loss of hair over the vertex. Hair become shorter and may, although not always, become finer in caliber. Ultimately, this may lead to complete hair loss except at the lateral and posterior margins of the scalp where hair is retained. In elderly men, hair may also be lost in these parts of the scalp. Hamilton classified male balding into several stages (1) and the revision of his classification by Norwood is still widely used (2). The predisposition to male balding is predominantly due to genetic factors. AGA is probably a polygenic trait, although the male hormone testosterone plays an important role, maybe independent of genetic predisposition. Male balding is especially an androgen-dependent trait.

Dihydrotestosterone (DHT), the 5α-reduced metabolite of testosterone, catalyzed by the enzyme 5α-reductase, is responsible for hair loss. This hormone binds to androgenic receptors (AR), and the specific bond triggers cellular processes which reduce the anagen phase of the hair cycle. It is now commonly accepted that male AGA is associated with an increase in 5α-reductase activity leading to an increase in local production of DHT. The mechanism by which the local DHT increase leads to hair follicle loss is not clearly demonstrated. Inhibition of cell proliferation in the dermal papilla and a vascular process based on the inhibition in local production of vascular endothelial growth factor (VEGF) have been proposed. The increase in 5 α-reductase activity is genetic and depends on androgen receptor polymorphism (3). The follicular changes in androgenetic alopecia comprise a gradual reduction in the duration of anagen, a prolongation of the "latent phase" of the hair cycle and progressive miniaturization of hair follicles (4). Miniaturization may eventually lead to deletion of hair follicles. Population frequency and severity of AGA increase with age. It commonly begins by 20 years of age and affects nearly 50% of men by the age of 50 years. Some authors have suggested that scalp hair loss in elderly men may develop independently of androgens (senescent alopecia) but this remains to be verified (5). At present, only two medical treatments, minoxidil and finasteride are licensed for the treatment of male balding. Both drugs will stimulate some regrowth of hair in some men but are perhaps better regarded as preventative treatments. Neither will regrow hair on completely bald scalp and continued treatment is necessary to maintain the response. Recently, certain newer advances have shown caffeine to have beneficial effects in patients suffering from AGA (6, 7, 8, 9). We report the results of a randomised, controlled, double-blind, parallel group study to assess, in a panel of healthy men suffering from AGA, the skin compatibility of a shampoo containing caffeine and to assess its anti hair loss efficacy and its cosmetic qualities, after application under the normal conditions of use for 6 consecutive months versus placebo.

MATERIALS AND METHODS

The study has been performed in 66 male subjects: 33 for the Verum group[1] and 33 for Placebo group (2), matched for age, hair pull test and Hamilton-Norwood Score. The individual typological characteristics of the test subjects are reported in Table I.

[1] *Trademark: Alpecin® Caffeine Shampoo C1*
[2] *Trademark: Alpecin® Medicinal Shampoo Concentrate*

TABLE I

Individual typological characteristics of the test subjects.

		Group		
		Placebo	Verum	Total
Age (years old)	Mean	37,5	35,5	36,5
	Standard Deviation	8,3	7,1	7,7
	Median	38,0	36,0	36,0
	Minimum	23	24	23
	Maximum	54	55	55
	N	33	33	66
Number of hair from the "Hair Pull Test"	Mean	20,9	21,5	21,2
	Standard Deviation	2,2	2,5	2,4
	Median	20,0	21,0	21,0
	Minimum	18	18	18
	Maximum	26	27	27
	N	33	33	66

		Group					
		Placebo		Verum		Total	
		N	%	N	%	N	%
Hamilton-Norwood	II	3	9,1%	1	3,0%	4	6,1%
	II A	10	30,3%	11	33,3%	21	31,8%
	III	7	21,2%	13	39,4%	20	30,3%
	III A	3	9,1%	4	12,1%	7	10,6%
	III vertex	4	12,1%	1	3,0%	5	7,6%
	IV	5	15,2%	2	6,1%	7	10,6%
	IV A	1	3,0%	1	3,0%	2	3,0%

The informed consent form was personal and previous to the start of the study.

Inclusion criteria were: age from 18 to 55, male, phototype (Fitzpatrick) from I to IV, androgenetic alopecia in the stages of Hamilton-Norwood 2-4, a hair count of the hair pull test of at minimum 18 hair fibers, no use any hair restorer (tablet, capsule, tonic nor shampoo) since the last 6 months, good health conditions, no significant skin disease located on scalp, able to compromise the evaluation of skin tolerance of the investigational product or being possibly aggravated by the application of the investigational product, such as dermographism, recurrent herpes, pityriasis versicolor, psoriasis, important pigmentary disorders (multiple lentigines, numerous or congenital nevi), UV light induced dermatitis, urticaria, no history of organ removal (kidney, lung, spleen, axillary lymph nodes) or organ transplant, no current treatment able to interfere with the interpretation of the study results, such as immunosuppressive drugs, anti-inflammatory products, antihistamine products, antibiotics.

Exclusion criteria were: different causes of alopecia [alopecia areata, psychosomatic alopecia i.e. Trichotillomania, hair loss due to medication (immunologics, chemo-therapy, etc.)], unhealthy condition of the scalp (widely spread, highly expressed eczema, high grade of dandruff), regular use of hair dye, bleaching products or products for permanent wave, present or past history of hypo/hyperthyroidism or iron deficiency (at the present or in the past), known allergies to the same type of products as the investigational products (cosmetic hygiene and care products) or other products (drugs, food), personal history of atopy, treatment, prior to the study, able to interfere with the interpretation of the study results (topical or systemic medication with anti-inflammatory or antihistamine products within the 6 months, antibiotics within the 4 weeks, medication for malignancy).

Investigational products were: a shampoo con-

taining caffeine (Verum group) and a shampoo containing placebo (Placebo group). The packaging of the two shampoos was identical. The samples, in neutral packaging, were identified by the sponsor with a progressive number from 1 to 66 according to a predefined randomization list and not known by the investigating centre.

The experimental conditions adopted (experimental areas, quantity of product applied, frequency and duration of the applications) reproduced the normal conditions of use advocated: the shampoo has been applied at home by test subjects, once per day for 6 months, in a quantity of 7ml on hair and scalp, kept on the scalp for 2 minutes and then rinsed off.

The constraints for the test subjects during the study were: no application of any other hair products than the tested one, no intensive sun or UVA exposure (UV lamps), no colouring, hair bleaching, permanent wave during the study, no additional vitamin supply (vitamins B or H) or products which could have an effect on hair, no hair loss treatment by oral route, no medical treatments likely to induce an alopecia (antimitotics, anticoagulants, antithyroid agents, anticonvulsants, beta-blockers, hypocholesterolemic drugs, retinoids), no application of hair lacquer or gel, no violent brushing and repeated massage of the scalp, no change of diet.

After 3 and 6 consecutive months of treatment, the volunteers answered a proband questionnaire to rate the efficacy of the investigational product and the scalp conditions. Checking of the skin compatibility (local tolerance) was based on a skin examination of the experimental area, before and after product use, by the investigator. This examination had to be performed, visually, under standard daylight source, at baseline then after 3 and 6 consecutive months of treatment. On the basis of this examination, the investigator filled in a dermatological questionnaire to rate the efficacy of the investigational product and the scalp conditions.

Statistics

The primary hypothesis was that the contentment of the volunteers with the Verum was significantly higher compared to the Placebo. This hypothesis has been tested using the exact, two-sided fisher Test. The effect of the test products (group: Verum vs. Placebo) on the secondary efficacy variables has been tested using analysis of variance (ANOVA) with repeated measurements (baseline, after 3 months, and after 6 months). The hypothesis was that the interaction time x group was significant (indicating that the improvement in the Verum group was higher compared to the Placebo group). In addition, in each group it has been checked separately whether there was a significant effect on secondary efficacy parameters and scalp condition using the exact Friedman Test (one-way repeated measures analysis of variance by ranks). Furthermore, the Mann-Whitney Test has been used to check whether there was a significant difference between groups at the baseline. Statistical tests have been performed as two-sided tests with an alpha-level of 0.05.

RESULTS

The individual answers of the questionnaires concerning the targeted anamnesis of each volunteer are reported in Table II. After 3 and 6 consecutive months of product use at home, no intolerance reaction has been noted by the investigator and no sensation of discomfort has been described by the volunteers. The primary efficacy parameter was the contentment of the subjects with test product after 6 months of application. The results are shown in Table III. Compared to the Placebo (36.4%), the contentment of the subjects with the Verum (84.8%) was significantly higher after 6 months of application ($p<0.001$, two-sided, exact Fisher Test).

TABLE II

Anamnesis.

Since when do you suffer from increased hair loss?					
Question	**Answer**	**Test subjects**			
		Placebo		**Verum**	
		Nb	**%**	**Nb**	**%**
	Since 1-5years	11	33,3%	11	33,3%
	Since 6-10years	12	36,4%	16	48,5%
	Since 11-15years	4	12,1%	2	6,1%
	Since 16-20years	3	9,1%	1	3,0%
	Since more than 21years	3	9,1%	3	9,1%
Hair length?	Long	1	3,0%	1	3,0%
	Medium	5	15,2%	8	24,3%
	Short	27	81,8%	24	72,7%
	Total	33	100,0%	33	100,0%
Do you know about other cases of androgenetically pattern hair loss?	No	9	27,3%	4	12,1%
	Yes	24	72,7%	29	87,9%
	Total	33	100,0%	33	100%
Do you have problems with your scalp?	No	15	45,5%	11	33,3%
	Yes	18	54,5%	22	66,7%
	Total	33	100,0%	33	100,0%
Which kind of problems?	/ (=no problem)	15	45,5%	11	33,3%
	Greasy scalp	10	30,3%	10	30,3%
	Dandruff, itching, reddening	4	12,1%	1	3,0%
	Dandruff	1	3,0%	4	12,1%
	Dandruff, reddening	1	3,0%	0	0%
	Greasy scalp, reddening	1	3,0%	2	6,1%
	Itching, reddening	1	3,0%	0	0%
	Reddening	0	0%	2	6,1%
	Dandruff, dry and tension on scalp	0	0%	2	6,1%
	Greasy scalp, dandruff	0	0%	1	3,0%
	Total	33	100,0%	33	100,0%
How often do you feel that?	/ (=no problem)	15	45,5%	11	33,3%
	Permanently	3	9,1%	2	6,1%
	Often	5	15,1%	17	51,5%
	Rarely	10	30,3%	3	9,1%
	Total	33	100,0%	33	100,0%
Do you feel the increased hair loss permanently?	No	25	75,8%	22	66,7%
	Yes	8	24,2%	11	33,3%
	Total	33	100,0%	33	100,0%
Is there a typical season for your hair loss?	No	8	24,2%	12	36,4%
	Yes	25	75,8%	21	63,6%
	Total	33	100,0%	33	100,0%
Do you ever treat or medicate against this problem?	No	14	42,4%	19	57,6%
	Yes	19	57,6%	14	42,4%
	Total	33	100,0%	33	100,0%
If yes, have you been satisfied by that treatment?	/ (n.a.)	14	42,4%	19	57,6%
	No	8	24,2%	7	21,2%
	Yes	11	33,3%	7	21,2%
	Total	33	100,0%	33	100,0%
Can you tell the products having applied in the past?	/ (n.a.)	14	42,4%	19	57,6%
	Crescina	4	12,1%	7	21,2%
	Minoxidil	4	12,1%	1	3,0%
	Keramine H	4	12,1%	2	6,1%
	Dercos	4	12,1%	2	6,1%
	Bioscalin	1	3,0%	1	3,0%
	Aminexil	2	6,1%	1	3,0%
	Total	33	100,0%	33	100,0%

TABLE III

Contentment with the test product.

Are you satisfied with the product?		Test product			
		Placebo		Verum	
		N	%	N	%
After 3 months of application (T3)	no	31	93,9%	22	66,7%
	yes	**2**	**6,1%**	**11**	**33,3%**
After 6 months of application (T6)	no	21	63,6%	5	15,2%
	yes	**12**	**36,4%**	**28**	**84,8%**

This was already the case (6.1% vs. 33.3%) after 3 months of application of the test products (p=0.011, two-sided, exact Fisher Test).

Furthermore, the efficacy of the shampoos has been assessed by the volunteers using a questionnaire at baseline, after 3 and 6 months of application of the shampoo. Secondary efficacy variables were: intensity of hair loss, decrease or normalization of hair loss, number of hairs falling out while combing, thickness of the hair. After 6 months, the subjects have been asked if they would like to continue with the test product. According to the evaluation by the subjects, the intensity of hair loss significantly improved during application of the test product in the Verum group (p<0.001, exact Friedman Test) and in the Placebo group (p<0.001, exact Friedman Test). ANOVA demonstrated that the improvement in intensity of hair loss was more pronounced in the Verum group compared to the Placebo group (time x group interaction: p=0.002). After 3 and 6 months the decrease/normalization of hair loss was significantly more pronounced in the Verum group compared to the Placebo group (p<0.001, exact Mann-Whitney Test). The number of hair in the basin significantly decreased during application of the test product in the Verum group (p<0.001, exact Friedman Test) and in the Placebo group (p=0.049, exact Friedman Test). ANOVA demonstrated that the decrease was more pronounced in the Verum group compared to the Placebo group (time x group interaction: p=0.002). The strength and thickness of the hair significantly improved during application of the test product in the Verum group (p<0.001, exact Friedman Test), but not in the Placebo group (p=0.111, exact Friedman Test). ANOVA confirmed that the improvement was more pronounced in the Verum group compared to the Placebo group (time x group interaction: p<0.001). Compared to the Placebo (36.4%), significantly more subjects would like to continue with the Verum (84.8%) [p<0.001, two-sided, exact Fisher Test].

The efficacy of the shampoos has been assessed by the investigators using the dermatological questionnaire at baseline, after 3 and 6 months of application of the shampoo. The following efficacy variables have been assessed: strength of hair, progression of the balding, extent of the falling out of hair. The strength and the thickness of hair significantly improved during application of the test product in the Verum group (p<0.001, exact Friedman Test) and in the Placebo group (p=0.001, exact Friedman Test). However, ANOVA revealed that the improvement was more pronounced in the Verum group compared to the Placebo group (time x group interaction: p<0.001)(Table IV). The balding significantly improved during application of the test product in the Verum group (p<0.001, exact Friedman Test), but not in the Placebo group (p=0.333, exact Friedman Test). ANOVA confirmed these

results (p<0.001) (Table V). The extent of the falling out of hair significantly improved during application of the test product in the Verum group (p<0.001, exact Friedman Test) and in the Placebo group (p<0.001, exact Friedman Test). ANOVA confirmed that the improvement was more pronounced in the Verum group compared to the Placebo group (time x group interaction: p=0.002) (Table VI). Furthermore, the investigator was asked (multiple choice question) to evaluate the efficacy of the test product. According to the evaluation by the investigator, Verum reduced the premature hair loss in 24 subjects (72.7%), whereas this was the case only in 11 subjects (33.3%) who applied the Placebo. The investigator preferred significantly more often the Verum (72.7%) compared to the Placebo (33.3%) (p=0.003, two-sided, exact Fisher Test).

TABLE IV

Strength of the hair.

How do you rate the strength of the hair?			strong	medium	thin	very thin	total
Placebo	Baseline (T0)	N	0	8	14	11	33
		%	**,0%**	24,2%	42,4%	33,3%	100,0%
	After 3 months (T3)	N	0	8	15	10	33
		%	,0%	24,2%	45,5%	30,3%	100,0%
	After 6 months (T6)	N	1	9	17	6	33
		%	**3,0%**	27,3%	51,5%	18,2%	100,0%
Verum	Baseline (T0)	N	0	9	19	5	33
		%	**,0%**	27,3%	57,6%	15,2%	100,0%
	After 3 months (T3)	N	0	18	12	3	33
		%	,0%	54,5%	36,4%	9,1%	100,0%
	After 6 months (T6)	N	7	16	10	0	33
		%	**21,2%**	48,5%	30,3%	,0%	100,0%

TABLE V

Progression of the balding.

How do you rate the progression of balding?			very slight	slight	moderate	severe	total
Placebo	Baseline (T0)	N	3	17	12	1	33
		%	**9,1%**	51,5%	36,4%	3,0%	100,0%
	After 3 months (T3)	N	3	17	12	1	33
		%	9,1%	51,5%	36,4%	3,0%	100,0%
	After 6 months (T6)	N	5	15	12	1	33
		%	**15,2%**	45,5%	36,4%	3,0%	100,0%
Verum	Baseline (T0)	N	1	24	6	2	33
		%	**3,0%**	72,7%	18,2%	6,1%	100,0%
	After 3 months (T3)	N	8	18	5	2	33
		%	24,2%	54,5%	15,2%	6,1%	100,0%
	After 6 months (T6)	N	19	9	5	0	33
		%	**57,6%**	27,3%	15,2%	,0%	100,0%

TABLE VI

Extent of the falling out of hair.

How do you rate the extent of the out falling hair Please, comb the hair several times and make a semi-quantitative rating			very slight	slight	moderate	severe	total
Placebo	Baseline (T0)	N	5	19	7	2	33
		%	15,2%	57,6%	21,2%	6,1%	100,0%
	After 3 months (T3)	N	6	19	6	2	33
		%	18,2%	57,6%	18,2%	6,1%	100,0%
	After 6 months (T6)	N	9	20	4	0	33
		%	27,3%	60,6%	12,1%	,0%	100,0%
Verum	Baseline (T0)	N	5	15	9	4	33
		%	15,2%	45,5%	27,3%	12,1%	100,0%
	After 3 months (T3)	N	9	15	9	0	33
		%	27,3%	45,5%	27,3%	,0%	100,0%
	After 6 months (T6)	N	16	15	2	0	33
		%	48,5%	45,5%	6,1%	,0%	100,0%

DISCUSSION

AGA is a common, progressive, patterned loss of visible scalp hair. It causes psychological distress and negative effects on the quality of life and thus the prospect of treating AGA, a worldwide trichological problem, is mandatory.

Caffeine is a methylxanthine, a well-known substance, but its effect on human hair follicle growth is not yet defined. The proposed mechanism which would counteract DHT-induced miniaturization of the hair follicle include inhibition of phosphodiesterase by caffeine, which increases cAMP levels in cells and therefore promotes proliferation by stimulating cell metabolism[10]. The beneficial effects of topical application of caffeine in AGA can also be attributed to improvement in barrier function, follicular penetration, stimulation and promotion of hair growth. Thus it appears to be a useful adjuvant in the management of AGA.

Fischer and colleagues (6) used ex vivo hair follicles from balding areas of men with AGA and cultivated them in vitro with different concentrations of caffeine to study its stimulatory effects on the hair follicles. It has been shown that 0.001% of caffeine prevented the suppressive effect of testosterone on the cultured hair follicles.

The stimulatory effect of lower concentrations of caffeine may be partly due to the increased levels of cyclic AMP and partly due to a direct effect against apoptosis, which is induced in AGA.

Brandner et al. (7) proved by their double-blind placebo-controlled trial that caffeine application causes a substantial reduction in the transepidermal water loss in men compared to women, thus improving barrier function in men.

In the past, it has been assumed that the intercellular route was the only relevant penetration pathway for topically applied substances. Recent results on follicular penetration emphasize that the hair follicles represent a highly relevant and efficient penetration pathway and reservoir for topically applied substances.

A recent study, which assessed the follicular penetration of topical caffeine in hair follicles, proved hair follicles to be faster route of drug delivery for topically applied drugs (8).

An important requirement for the treatment of AGA is follicular drug delivery. Another recent

study assessed the follicular penetration of caffeine on topical application in a shampoo formulation for 2 min and showed that penetration via hair follicles was faster and higher compared with the interfollicular route and that hair follicles were the only pathway for faster caffeine absorption during the first 20 min after application[9].

Caffeine has been shown to counteract the inhibitory effect of testosterone in hair follicles and has been identified as a stimulator of human hair growth in vitro, a fact which may have important impact on clinical management of AGA.

According to the experimental conditions adopted in our study, both products in study had a very good skin compatibility, after application under normal conditions of use. Moreover, the shampoo containing caffeine showed a good cosmetic efficacy in the treatment of male androgenetic alopecia.

Compared to placebo at the end of the study the contentment of the volunteers with Verum was significantly higher. Precisely, 84,8% of the volunteers have been satisfied with Verum, while 36,4% of the volunteers have been satisfied with Placebo.

Moreover, the volunteers referred that the reduction in the intensity of hair loss, the decrease/normalization of the hair loss, the decrease of the number of hair in the basin, the improvement of the strength and thickness of the hair were significantly higher in the Verum group than in the Placebo group.

At the end of the study, significantly more subjects would like to continue with the Verum because it reduces the hair loss, than with the Placebo.

Also the investigator confirmed the good cosmetic efficacy of the shampoo containing caffeine. Particularly, it has been noted that the improvement in the strength of the hair loss and the reduction of the progression of the balding were significantly higher in the Verum group than in the Placebo group.

At the end of the study, the investigator preferred significantly more the Verum than the Placebo.

Our data support the beneficial effects of topical application of caffeine in AGA, suggesting that this substance may be a cosmetic growth-stimulating agent suitable for AGA treatment.

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Author Address:

Teresa Sisto
Department of Dermatology
Catholic University of the Sacred Heart
L.go Gemelli 8, 00168 Rome, Italy
E-mail: teresa-sisto@libero.it

What prevails in the market of herbal hair wash preparations: Myth or Truth?

M. Frederick[1], S. Ranganathan[2], S. Gokulshankar[3], M.S. Ranjith[4], S.R. Ashutosh[3], B.K. Mohanty[5]

[1] Skin Clinic, 20 Paper Mills Road, Perambur, Chennai, India

[2] R&D wing, ClinRise Derma Pvt., Ltd.,Anna Nagar, Chennai, India

[3] Faculty of Medicine, AIMST University, Malaysia

[4] Faculty of Medicine, Quest International University Perak, Malaysia

[5] Faculty of Medicine, UniKL-RCMP, Malaysia

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Summary

Hair is an important 'cosmetic apparatus' in man. Commercial herbal hair wash powder preparations are becoming popular in the recent years in India among consumers as they are marketed with the claims as 'traditional' and 'safe'. But some of the marketed commercial brands of herbal hair wash preparations include chemicals like SLS and silica.

The exact effect of such commercial herbal hair wash preparations in comparison with the traditional 'natural' preparations on hair cuticle is not studied in detail. The present study unleashes the hidden truth behind the 'so-called safe' commercial herbal preparations. The study is also significant as some of the commercial Ayurvedic/herbal Indian hair wash powders are exported to several countries and are becoming globally popular.

Riassunto

Il capello rappresenta un importante simbolo estetico sia per l'uomo che per la donna. Negli ultimi anni in India sono diventati popolari preparazioni in polvere per lavare i capelli, esaltati come shampoo tradizionali e salutistici. Alcuni dei marchi, pubblicizzati come prodotti naturali a base di erbe, includono nella loro formulazione silicio e sodio lauril solfato (SLS). L'effetto che questi shampo alle erbe svolgono a livello della cuticola dei capelli non è mai stato studiato nei confronti all'attività svolta dai prodotti tradizionali del commercio. Questo studio ha voluto verificare questo effetto anche perche' queste preparazioni sono dichiarate sicure nell'uso e vengono esportate in molte nazioni quali prodotti naturali di erbe che agirebbero secondo la tradizione Aiurvedica.

INTRODUCTION

Silky, long beautiful hair is impressive and enviable and importantly, adds to human psychology. Such hair, the beauty it adds and its care have been emphasized since time immemorial (1). It appears that the above psychological need has co-evolved with man. Shiny and lustrous hair is accepted as the index or indicator of youth and good health by people all over the world. In contrast, less hair or bald scalp has always built a low self-esteem in a person and they have always tried to hide this fact and attempted to build their confidence (2).

Use of 'wigs' to cover the bald scalp started as early as 14th century. This has been described as 'purchased beauty' by William Shakespeare in his famous work – The Merchant of Venice (3). The need for having beautiful hair is very complex, where the emotion, intelligence and self-esteem of man are tailored together and hence, man is always after such need to fulfill it. To this effect, there is a continuous flow of commercial products in the market to meet such needs with tall claims and promises (4). The market space is well understood by the industry and hence there is exploitation.

The continuous arrival and availability of multivarious hair care products in the market with eye catching advertisements/promises, signature the weakness of man and to keep his beauty well protected and preserved through use of these hair care products. Among the various hair care products, herbal hair wash products are gaining importance in the market as they are largely perceived to be safe and effective (5).

The questions that are commonly asked and require to be answered in this context are: How safe are these herbal hair wash (powder shampoo) products? Do they really help in maintaining 'beautiful hair' or harm the hair and scalp? Who should ask these questions or verify them carefully? - the buyer, the seller or the regulatory authority?

It is widely believed that all the traditional hair care preparations (herbal hair wash powders) are safe and effective (6). However, to what extent are these herbal hair wash powders 'truly' traditional and safe? Are all branded products in the market truly traditional as claimed? Are these hair wash powders strictly prepared according to the traditional methods or are they prepared differently like other synthetic products to meet the commercial requirements?

This study was conducted to answer the above questions on the real effect of various hair wash powders available in the Indian market. Both, commercial preparations and those made by the traditional methods or folklore dictum were used for the study and the observed findings are discussed. The study is also significant as some of the commercial Ayurvedic/herbal Indian hair wash powders are exported to several countries and are globally popular .

MATERIALS AND METHODS

Study materials

The following preparations were used in the study:

Matured shikakai (*Acacia concinna*) pods were collected and shade dried. The shade dried pods were powdered and sieved to 500 mesh size.

The fresh leaves and flowers of *Hibiscus rosa sinensis* (shoe flower plant) were collected and ground to paste form with water.

Similarly, green gram (*Vigna radiata*) was powdered & sieved to 500 mesh size.

Commercial herbal hair wash preparation containing SLS (Sodium Lauryl Sulphate) with or without silica powder and with or without conditioners procured from market.

Treatment procedure

Hair swatches (black hairs) weighing approximately 50g was used for studying the effect of various hair wash preparations. The hair swatches were divided into three equal groups of 6 swatches in each group. The length of the hair was 15 cm.

One set of 6 hair swatches were treated with coconut oil (1g each per swatch) while another set of 6 hair swatches were treated with sesame oil (1g each per swatch). The third group of 6 hair swatches did not receive oil treatment. The entire experiment was repeated twice and independently on separate hair swatches. All experiments were done simultaneously.

One gram of the hair wash powder/preparation was made into a paste with water and applied evenly on each of the hair swatches individually in the respective groups. The hair in each swatch was massaged 10 times and was rinsed well with distilled water. The hair swatches were dried with towel.

Ten hair strands from each swatch were collected at random and observed under a microscope. Ten fields were chosen for each hair and the appearances of the cuticular scales such as flat & smooth, protruding or spiky nature were recorded. Further, the prominence or conspicuousness of each characteristics and the extent of their prominence were recorded as 'normal', 'moderate' or 'worse' with symbols +, ++ or +++ respectively.

Coomassie blue stain was used to enhance the contrast during microscopic examination whenever required. The final observations were compared with the hair collected from the respective swatches before and after treatment to ascertain the effect or role of the treatment product.

RESULTS

TABLE I

Effect of different hair wash preparations on the hair swatches

Si No	Hair wash preparations	Oil pre-treatment/damage score		No oil pre-treatment
		Coconut oil	Sesame oil	
1.	Shikakai paste	+	+	+
2.	Shikakai with hibiscus paste	+	+	+
3.	Fresh hibiscus paste	+	+	+
4.	Green gram paste	+	+	+
5.	Commercial herbal hair wash powder*	+++	+++	+++
6.	Commercial herbal hair wash powder**	+++	+++	+++

*with Silica, SLS and Conditioner, **with SLS and Conditioner
+ denotes normal, ++ denotes moderate and +++ denotes worse

DISCUSSION

The problem of hair fragility and hair breakage growing globally may be due to the use of multivarious hair care/hair styling products. This problem is posing a major issue to the individual as well as challenge to the dermatologists all over the world (7).

The present study was aimed to establish the damaging or protecting effect of various herbal hair wash preparations on the hair cuticle. Many herbal hair wash powders claimed to be of traditional origin are sold rampantly in the market with tall promises of benefits. Interestingly most of such preparations are concocted with SLS (Sodium Lauryl Sulfate) as a foaming agent along with some conditioners. Further, silica powder is also generally used in the hair wash powders. It is known that silica powders are admixed in the formulation to damage the eggs of various insects to prevent insect infestation. The sharp edge of the silica is also known to disintegrate and dehydrate the eggs of various insects.

In the traditional or folklore hair wash powder preparations, no such chemicals are used. Whether use of various chemicals such as silica, SLS etc., in the herbal hair wash powders, still qualify these products to be called 'traditional' – Ayurvedic / Siddha, or not is beyond the scope of the present research. However, establishing the benefit of such preparations over strictly prepared traditional hair wash powders undoubtedly is very essential.

The powder of *Acacia concinna* pods, popularly known as 'skikakai powder' is widely used for washing hair (8) in many parts of India. The powder is made into paste and then used for hair wash.

In the present study, we found that the damage of the cuticle surface was relatively nil to normal when plain shikakai paste or fresh hibiscus or green gram paste was used for washing the hair swatches. The cuticular scales showed reasonably flat & smooth appearance rather than protrusions or spikiness.

The extent of damage of hair cuticle was very high with the commercial preparations with SLS and with or without silica. Cuticle damage was present throughout the hair and in addition, extensive spikes and protrusions were seen when such preparations were used to wash the hair. We strongly presume, that the surfactant SLS may have facilitated or predisposed the damage to hair surface by the abrasive effect of the preparation with silica.

To study the effect of particle size effecting damage to the hair as could have been in the commercial preparations especially with silica, coarse shikakai powder paste was prepared and used to wash the hair swatches. Interestingly, even this paste caused no further damage to the cuticle. The non abrasiveness of shikakai may be due to its quick ability to absorb / imbibe water and become soft. We strongly presume that in commercial preparations, the water absorbing property of the herbal powder could be affected by the interference of SLS and conditioners adding to the abrasive nature of the powder. The contribution of silica to the above effect cannot be underestimated. Further, SLS more competitively binds to the hair, softening / opening the surface of the hair and hence making it vulnerable to further damage.

Oiling of the hair did not significantly protect the hair or change the ill effects of the commercial hair wash powders.

Although the hair examination was done using a light microscope, we could still observe the effect of various herbal hair wash preparations on hair accurately.

The findings of this study, clearly shows the ill effects of the commercial preparations on the hair and such preparations should be carefully studied before being allowed into the market. The present study also accurately validates the

astute insight and wisdom of our ancient traditional medicine (Ayurveda and Siddha) in identifying and preparing safe products for our use. Safety and efficacy should prevail over commercial ends and means while marketing various products under the banner of Indian system of medicine.

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Author Address:

Dr. S. Gokulshankar
Microbiology Unit
Faculty of Medicine
AIMST University
Jalan Bedong, Semeling
Bedong 08100
Kedah, Malaysia
Email: gokkavi@gmail.com

Coloring the Cosmetic World using Pigments in Decorative Cosmetic Formulations

By Edwin B.Faulkner

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www.Alluredbooks.com
E-mail:books@allured.com

Most light sources produce a spectral color of various wavelenghts which the human eye cannot always distinguish from each other. In any way, it represents the visual perceptual property corresponding in humans to the categories called, red, bleu, yellow, green and others, also if the physiological quantifications of color do not fully explain the psychophysical perception of its appearance. What it is interesting to underline, however, is that the shades of colors we distinguish, for example in the birds' plumage, are often the consequence on the optical phenomenon of diffraction. Thus the perceived blu-green color of the butterfly *Papilio palinurus* is not made from a green or blu color, but is a consequence of the light reflected from its wing scales that, covered by a hollow surface made of micrometric dimples of chitin, act as a selective mirror. And the perceived color is of great interest of the Coloring Cosmetic World also, reported by this book and organized in 12 Chapters, an Introduction, two Appendices and Bibliographic references.

The visual color used in everyday life with its psychological significance is, in fact, the essential core of the fashion industry, which decorative cosmetics are a key element.

Color affects human moods and attitudes towards events so that, *black* tends to signify power and authority, *white* purity and innocence, *red* is associated with love, *bleu* and *green* evoke calmness and tranquillity, *yellow* is associated with sun and health, while *purple* is the color of royalty and *brown*, as color of earth, is connected with stability and reliability. It is, however, to be pointed out that color additives, incorporated into cosmetic products when bombarded with white light, adsorb some of its wavelengths reflecting others. Those reflected are the ones that are perceived by the human eye. Thus in the cosmetic field, specialty pigments that change or enhance the absorption colors are quickly entering into the market. All of these types of pigments are constructed of various layers or platy particles, with different indices of refraction that allow for *interference* with incident light, causing maximum reflection, refraction and transmission of incident light.

Mimicking the interior of the oyster shell, constructed of alternating layers of calcium carbonate and proteinaceus material, the most important class of effect pigments used today in decorative cosmetics is the oxide coated micas. They are composite mixtures using refined natural muscovite mica as a base onto which an absorption pigment, as titanium dioxide and/or iron oxide, is deposited. Depending on the particle size of mica and the type and thickness of color oxides used to coat its strata, a very wide variety of colors are obtained with interesting sparkling effects. Also if color shades are to be considered as important characteristics for the coloring cosmetics, their stability in time

into cosmetic products together with the uniform distribution from batch to batch are of fundamental importance also.
Dispersion is, in fact, the keystone for formulating decorative cosmetics connected with the important process necessary for incorporating pigments into emulsions or powders. This process "converts a *raw* pigment into a usable form that consists of wetting separating and distributing particles in vehicle-providing the best color and money values".
The variables that play a part in the effectiveness of wetting, necessary to replace the air that is trapped during the productive process, are the pigment-to-vehicle ratio, surface tension and time. Thus the necessity to use the right machinery and efficiency of the process method, considering also the energy employed, the time utilized, the viscosity of the wetted pigment, and the avoidance of the polychromatic dispersion. Moreover, due to differences in particle size, particle size distribution, and degree of agglomeration, the color values of a particular color additive can vary distinctly from batch to batch. Thus, the proper technical evaluation of colors before they are used in cosmetic production serve to minimize all the problems occurring during manufacturing.
At this purpose in order to save time and lab efforts, it would be best to prepare a large dispersion of a controlled standard preparation by an appropriate system and use it to test numerous batches of color additives as received, evaluating them by the same test methodologies. Like all other aspects of formulating decorative cosmetic products economics has to play an important role in the selection process. As more than one treatment type should serve the intended purpose, the formulator must review all of them to decide one(s), should fit into the cost structure of the finished product.
Least but not last, the color selection process has to begin with regulations. It is absolutely essential to ensure that any additive, selected for use in a decorative cosmetic or toiletry product, meets the regulatory requirements of the country where the product will be sold.
Colorants are, in fact, the most highly regulated chemicals used in cosmetic products, and great care must be taken to conform to respective regulations. The state of today regulations in USA, EU, Japan and China, are described and reported in this interesting book, enriched by the Appendix A, reporting the more in use Pigment Test Methods and the Appendix B, where the more used and interesting patents on pigments together with their uses are reported and described. A Glossary of Terms represents the final part of this book that may be of great interest and utility not only for the Cosmetic Chemists who intend enter into the fascinating sector of make-up and coloring cosmetics in general, but also for all the Chemical and Medical Community who should like to know the reasons of the knowledge and creativity necessary to formulate these categories of cosmetic products, having also a deeper look on the International actual regulations, governing this particular sector of the Cosmetic and Chemical Market.

P. Morganti
Editor-in-Chief

Allured's Flavor and Fragrance Materials (FFM) Buyers Guide 2013

2013. Pages 476 Softcover
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E-mail: customerservices@allured.com

This interesting Buyer's Guide reports the worldwide reference list of materials used in compounding flavors and fragrances, providing an alphabetical classification of the most recent materials, synonymis, and products, including FEMA, CAS, CoE, natural status and supplier information. The aim of the book is to survey suppliers to the industry for new, updated and correct information on fragrance materials present on the international market.

The reported fragrance material have been compiled from the list of materials that Research Institute of Fragrance Materials (RIFM) has investigated for safety in fragrance use, together with the list of the additional material submitted from the International Fragrance Association (IFRA) and the Flavor and Extract Manufacturers Association (FEMA), for which there are standards published. This report includes the current materials cited in the FEMA GRAS lists through the GRAS 26 list, the list of flavoring substances which can be used in food, and the materials in the publications of the Council of Europe.

One of the difficulties to obtain the right information on any fragrance substance, is to classify its real identity being generally reported by different names or terms. Thus the 2013 FFM Edition reports trade name, trivial name in common usage, and conventional chemical name of each fragrance, without regard to systems of nomenclature, for facilitating the reader' s consultation. Moreover, materials derived by distillation from natural materials are listed as oil or distillate, as well as material derived by solvent extraction from natural materials, as water, alcohol, hesane, methylene chloride and carbon dioxide, are specified. This book results surely interesting to know not only the many cross-references connecting fragrances and to understand the meaning necessary to classify particular flavoring substances deigned as natural, natural-identical, or organic, but also to find the right supplier for obtaining much more technical support before buying the raw material.

It is to remember that the right characterization of every flavor ingredient may be of support for Cosmetic Chemists and for Dermatologists in a period during which the discussion on the final list of allergizing/sensitizing fragrances is until now open and in progress among the dermatological community. On the other side Perfumes represent an important niche of the cosmetic market, also because people like and live the different fragrances as a mean to enrich their own emotions necessary for ameliorating the quality of life. Sense of smell, in fact, has a direct connection with the emotions more strong of that existing among the other sensorial systems. According to many other scientists, it is possible to underline that odors and fragrances are so important for human wellness to be

considered fundamental and not only hedonistic compounds.
In conclusion, this technical book has to be enclosed into the library not only of the Cosmetic Chemists and Cosmetic Industry, but also of Dermatologists, Plastic Surgeons, Psychologists, Marketing managers and all people interested and involved in the fascinating field of Perfumes and Fragrances.

P. Morganti
Editor-in-Chief

Antioxidants and the Skin

by Roger L. McMullen

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E-mail: books@allured.com

Skin is the largest and outmost organ of the body acting as a biological barrier to offer thermal insulation, prevent water loss, and protect the internal organs from the external environment and foreign substances. It is approximately 1.8 to 2 square meters, less than 2 mm thick and composed of several layers. The outmost layer, the epidermis, is further divided into stratum corneum, stratum granulosum, stratum spinosum, and stratum basale. Located at the interface between body and environment, the stratum corneum (SC), is composed of corneocytes embedded into lipidic lamellae and directly exposed to a prooxidative environment, including air pollutants, UV solar light, chemical oxidants, and microorganisms. To counteract oxidative injury of structural lipids and proteins, human skin is equipped with a network of enzymatic and non-enzymatic antioxidant systems.

This book consisting of 9 Chapters and V Appendices, reports an overview of information on the skin antioxidants and their use in the cosmetic field, including the techniques necessary for controlling and measuring their effectiveness, when used in antioxidant treatments.

The SC is comprised, in fact, of unique, highly lipophilic lamellae prevalently composed by lipids, such as ceramides, cholesterol and free fatty acids. And this structure plays a key role in determining the barrier integrity, essential to maintain the skin in healthy conditions, characterized by a balanced skin hydration with a normal cell turnover and desquamation This skin layer, considered in the past as a *dead area*, has been shown to be part of a highly respected structure composed by corneocytes, lipids enriched with many enzymes, antimicrobial and cellular signaling proteins, carrying out various and important specific roles. The outermost surface of the skin serves, in fact, as the first line of defense from the environment' aggressions. Colonies of flora, embedded on the skin surface, prevent foreign pathogens from gaining access to the body's internal elements. In addition sebum and SC lipids maintain a low pH, which is less favorable for the colonization of the so called *opportunistic* bacteria. The launch of signaling pro-inflammatory and anti inflammatory proteins, such as cytokines and chemokines, or the antimicrobial peptides as defensins, in the center of epithelial defense mechanisms ,are all topics reported on **Chapter 1**, where these multiple and at first glance independent and protective mechanisms are focused and discussed.

Oxygen is critical for all the biological systems and organisms, but its inappropriate metabolism may be cause of toxicity. This toxic effect is not due to molecular oxygen per se, but to reactive metabolites, the free radicals, generated as byproducts of the normal oxidative metabolism. The onset of free radicals occurs when they are produced in excess at level of the healthy cells, resulting in molecular damage to cellular components, such as lipids, proteins, and DNA. Thus, excessive production of

these reactive radical species (ROS) occurs in certain disease states or as a result of toxic insult by selected foreign compounds/drugs (xenobiotics).
No matter the origin, skin possesses a variety of systems to prevent and/or minimize this oxidative injury. The balance between cellular production and catabolism of oxidants and antioxidants is, therefore, critical for the maintenance of skin homeostasis. This is *why oxidative stress* is referred to as condition of imbalanced pro-oxidant/antioxidant equilibrium in favor of the former. In any way, skin antioxidant network consists of a complex defense system against an oxidative stress in which the function of one antioxidant often supplements or regenerate another antioxidant. Thus preventive antioxidants keep radicals from forming in the first place, while radical scavenging antioxidants prevent the initiation of free radical chain reactions or stop it from propagating. Nevertheless, when antioxidants are not able to prevent free radical damage, the skin is found in state of *oxidative stress*, leading to disease, cancer, or aging.
This topic is discussed on **Chapters 2** and **3**, where the key components of the skin antioxidant system are reported.
Effects of Solar Radiation on the Skin is the topic of **Chapter 4**. Sunlight is, in fact beneficent and life supporting, but we cannot ignore its cumulative negative effects manifested as skin aging and cancer of the exposed skin, such as basal and squamous cell carcinomas. Thus, to prevent or to ameliorate these light-induced reactions, effective sunscreens applied topically and photoprotective ingredients taken by oral route, are considered essential. Moreover, it is well documented that UV rays play a key role in the induction of signaling pathway in skin. This result in ligant-independent activation of cell surface receptors, such as growth factors and cytokines. Again, the activation of these receptors results in signal transduction cascades, which ultimately lead to the activation of a transcriptional factor. Thus, the upregulation or downregulation of particular gene is affected by external stress factors, such as UV irradiation, which activate intracellular pathways normally induced by intercellular messengers. UV-induced cellular signaling pathways, therefore, may lead to skin photoaging and cancers.
The epidermis has the capacity to synthesize a broad range of lipids and its biosynthetic capacity has been shown to rival that of liver, kidney and gastrointestinal tract in both rodents and primates.
Despite its apparent autonomy from exogenous regulatory influences, the epidermis does take up circulating lipids, including distinctive species of dietary origin such as plant sterols and free and unsaturated fatty acids, as well as fish-oil derivatives. Thus, the skin must obtain the essential fatty acids, as linoleic acid, from the circulation.
Most importantly, permeability barrier requirements regulate epidermal lipid metabolism. Thus, the importance to maintain in healthy state the skin lipid lamellae, also because their dysfunctional activity, due to lipid peroxidation caused by the ROS attack, can lead to pathological state and even carcinoma.
The skin lipids' control and measurements are focused on **Chapters 5, 6** and **7**, where **Chapter 8** reports the use of antioxidant-formulations in various skin treatments to maintain its healthy state.
The concept of formulation' strategies with antioxidants stems from the need to prevent oxidation within the formulation and also to deliver through the skin active and bioavailable antioxidants. The use of the right carrier system represents a real asset for the delivery of antioxidants to skin and include various types of emulsions, based on vesicular, or micro/nano particle systems.
Chapter 9 reports all the antioxidant compounds employed in personal care formulations. They are

described individually, reporting for each compound the empirical formula, molecular weight, nomenclature, chemical abstract service (CAS), registry number, and the simplified molecular-input line-entry system (SMILES) format.

Last part of the book is enriched with a complete list of updated references: **Appendix I**, composed of *Glossary of Terms*; **Appendix II**, that describes the B*iologically Important Molecular and Mechanisms* going from amino acids to hormones and neurotransmitters; **Appendix III**, focused on *Cellular Signaling in Skin*, where the ability of the cells to communicate with each other is described; and **Appendix IV**, in which the *Thermodynamic and Kinetic Factors that Contribute to Antioxidant Behavior* are discussed: the reactions between antioxidants and free radicals are governed, in fact, by thermodynamics and kinetics; least **Appendix V**, representing the closure of the book, in which all the antioxidants used in the more known personal care products present in the international market, are reported.

Antioxidants and the Skin, for all the information reported on the use and control of the activity of these important class of ingredients, represents a key book to be included in the library of researchers, cosmetic formulators, clinicians and academicians of both Chemical and Medical Community, and represents also a useful mean of study for students and marketing people, interested to understand in a deep way the reason of the antioxidants use in the field of Cosmetic Dermatology.

P. Morganti
Editor-in-Chief

Biomaterial and Stem Cells in Regenerative Medicine

By M. Ramalingam, S. Ramakrishna and S. Best

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Regenerative medicine based on the capacity to restore the structure and function of damaged skin, is estimated to have a worldwide market of US $ 500 billion, according to the US Department of Health and Human Services. To develop regenerative medicine technologies, the most promising emergent sources appear to be stem cells harvested from newborn discarded tissues, from amniotic fluid and tissue, placenta and UC stroma. These sources, in fact, may provide benefits similar to that of the umbilical cord' hematopoietic stem cells (UC-HSCs) particularly lack of immune-rejection and ability to bank cells prospectively.

This book, consisting of **25 Chapters**, describes the up today work performed to offer suitable cell scaffolds and substrates for tissue repair; moreover it addresses the range of different types of applications for which biomaterials and stem cells therapy can be utilized.

But what biomaterial means?

It is defined as a material or combination of materials, synthetic or natural in origin, which can be used to repair, replace or model tissues and organs *in vitro* and *in vivo*. The biomaterials can be classified according to their performance and criteria, such as polymers, metals, ceramics, and composites. Most of all, polymers representing the most significant class of biomaterials in medical application, have contributed to improve quality of life of millions of people, also for their capacity to support the correct structural architecture of many human tissues. Thus, the combination of polymer chemistry and cell biology has led to significant advances in identification and understanding of synthetic matrices which provide cellular support, aiding cellular differentiation and tissue formation.

Well-designed scaffolds together with synthetic biopolymers as cell carriers, could promote greater cellular engraftment, providing a template to guide the formation of new tissue. Solid scaffolds, such as poly(glycolic acid) (PGA) or hyaluronic acid-based provide, in fact, substrates on which cells can attach and proliferate, while liquid and gel scaffolds function to physically entrap the cells. This is the reason why hydrogels-mediated stem cell engineering has been proved to be effective in regenerating various tissue types, such as cartilage, adipose, bone, vascular, and nerves. The encapsulation of stem cells in hydrogels seems to be beneficial, in fact, to overcome teratocarcinoma formation and the unwanted immune responses.

In any way, scaffolding materials play a critical role in tissue engineering by serving as a synthetic extracellular matrix (ECM) to provide temporary mechanical support for the cells and to subject

them to conditions highly mimicking the native microenvironment that lead to tissue formation. The normal ECM network comprised of fibers, such as collagen, hyaluronan, and soluble macromolecules, such as growth factors, chemokines and cytokines provide, in fact, biophysical and biochemical cues to guide cell behavior within tissue.

For all these reasons, significant attention has paid to fabricate scaffolds with an interconnected pore shapes necessary to facilitate mass transfer and guide cellular organization, minimizing the dead volume. Moreover, the mechanical properties of this scaffold, including stiffness, act as a physical signal for cells, influencing important cellular behaviors such as adhesion, spreading, motility, survival, and differentiation. However one of the challenges of developing tissues, *in vitro* and *in vivo*, is imparting vascularization into their structures in order to maintain physiological growth of the cells into particular lineages, as well as to synthesize other cellular components required for tissue development.

In any way, the major consideration in tissue engineering is the pursuit of scaffolds that provide an architecture on which seeded cells are directed to proliferate and differentiate to form new tissues and organs. For this approach to be successful, stem cells need to be directed to the site of injury, proliferating and differentiating within a local microenvironment, provided by biomaterial scaffolds. However, the actual biomaterial scaffolds, while providing structural support for a new tissue formation, do not adequately mimic the complex interactions between host stem and progenitor cells and the ECM that promote functional tissue regeneration.

These are all the considerations reported and discussed on the **Chapters 1** to **4**. Therefore, future advances in tissue engineering and regenerative medicine will depend on the development of smart biomaterials that actively participate in the formation of functional tissues. However, tissue engineering technology has now advanced to a stage where generation of new tissue, to replace the damaged ones or growing artificial organs for use in organ transplantation, is now undergoing extensive clinical trials in many countries.

Thus, research on stem-cell-based tissue engineering has just begun in the cardiovascular area were combination of stem cell technology with research on native and artificial scaffolds are providing important answers to control unwanted cellular responses, preventing the mechanical failure. On periodontal-related diseases, the actual surgical membrane are based on biopolymers obtained by the electrospinning technology capable to regenerate both structure and function of the damaged periodontal tissues. In bone regeneration, bone mesenchimal stem cells (BMSs) migrate from the bone marrow or periosteum to the regeneration site as a result of expression of bone morphogenetic proteins (BMPs). BMP-2, in particular, plays a major role in initiating the cascade of chemotaxis differentiation of bone mesenchimal stem cells. Thus, one of the strategy for bone regeneration is to use combination of the RGD amino acid sequence, obtained from insoluble collagenous network combined with soluble fraction of the bone ECM grafted to scaffolds made by polylactide (PLA), polylactide-glycolide (PLGA) and polyethylen glycol necessary to improve cell adhesion and differentiation.

Severe loss of vision is another important disability caused by injury, disease, or aging of specific eye tissues. Also in this case there is need to optimize the properties of biomaterials to be used. In some cases, it is necessary to culture the cells on a substrate *in vitro* for subsequent transplantation back into eye, whereas in others, it may be possible to provide a scaffold for stem cells within the eye to grow and repopulate a damaged area. Thus, the use of substrates on which the stem cells may grow. These may be biologically derived with the disadvantage of potential limited supply and the

difficulty to process, control immunogenicity, and maintain sufficient mechanical properties. At this purpose a significant improvement of clinical treatment could be achieved by the use of synthetic materials which can be produced more easily and in sufficient quantities to be used therapeutically. Therefore, a cross-disciplinary approach will be necessary to solve the complexity of these different approaches, optimizing the properties of the biomaterials used in this important area.

What the characteristic necessary to produce scaffolds for the tissues regeneration?

Porosity is considered one of the more important parameters when designing and discussing scaffolds for the regeneration of tissue, irrespective of the scaffold material or the tissue type being regenerated. Thus the use of calcium phosphate (CaP), as scaffold for the repair or regeneration of diseased or damaged bone, is based on its chemical similarity to the mineral component of bone, particularly for its specific chemistry and porous architectures obtainable. The high porosity of CaP scaffolds enables invasion with blood vessels, essential for new bone regeneration.

Another parameter in temporary scaffolds is susceptibility to degradation and resorption in a biological environment. While degradation kinetics of several polymeric materials can be adjusted to the requirements of bone regeneration rate, this is rather difficult to achieve for metallic or ceramic materials. Thus the *bioactivity* and activation of specific cellular responses are probably the most important features of new-generation biomaterials for bone tissue regeneration. This is why the so called *bioinstructive* scaffolds, rather than simply osteoconductive scaffolds, have opened up new opportunities in this field.

And two novel promising approaches are based on (1) supramolecular chemistry of surfactants to obtain highly ordered mesoporous bioactive glasses (MBG), and (2) elettrospinning technique to create nanofibrous bioactive glasses. At this purpose, it is to consider that ECM of bone tissue composite system, consisting of both a mineral phase (hydroxyapatite) and an organic phase (collagen and non-collagenous proteins), can be described in terms of hierarchical levels of organization from nanometers, through micrometers and millimeters scale range.

Thus, according to the biomimetic approach, the biomaterial used has to mimic properties and functions of the ECM bone tissue.

About metallic biomaterials, such as stainless steel, cobalt chromium, or titanium used extensively throughout medicine, dentistry, and biotechnology, the production of a new generation of smart biomaterials have been developed, enabling new interesting advantages.

Depositions of polymer layers on titanium and titanium alloy *substrata* have been used to graft biologically active molecules which accelerate the process of bone integration, and protect the surface of the substratum from corrosion. Thus the surface modifications at nanolevel by TiO2 passive oxide may induce positive aspects, modifying the surface topography and especially its porosity. In this way while on one hand bioperformance of the metal results ameliorated, on the other hand some toxicological negative problems seem to appear.

Research in this domain is, therefore, aiming to enhance biocompatibility, ensuring a better bone structure' integration of the implant material. Such changes in the materials behavior from bioinert to bioactive and biointeractive can be achieved via metallic biomaterial processing, for modifying its structure to induce a better cell response.

All these topics are reported and discussed from **Chapters 5** to **12**.

The loss of function of tissues from disease, injury, or aging causes serious health problems as well as tremendous social and economic cost. The substitution of tissues such as bone or cartilage can be performed with allograft materials, introducing a risk of infections, or graft rejection. Thus, while

autologous bone grafts have been considered to eliminate immunological rejection and necessary pathogen transfer, it is affected by potential donor site morbidity and provide a limited volume of possibilities.

Therefore, the need for replacement by many types of metallic implants, including gold, stainless steel, and chromium. However, titanium is today considered the *gold standard* material for repairing damaged bone tissue because of its high biocompatibility. It is well tolerated by the body and has demonstrated strong interactions with adhesion proteins. Moreover, "Ti" can be modified to have the same surface roughness but also a higher degree of wettability necessary to allow cell adhesion and growth, enhance the osteogenic integration without causing chemical harm to surrounding living tissue.

Numerous studies have demonstrated that various tissue engineering approaches can be used to repair damaged myocardium with various cell types, scaffolds, genes, and drug therapies. At this purpose biomaterials have been proven to play an essential role in cardiac tissue regeneration. However, more detailed investigations of suitable degradation rate, specific peptide sequences, porosity, and other chemical and mechanical factors that influence the microenvironment between cells and their adherent substrates are still waiting to be understood, also if matrix metalloproteinases (MMPs) with a specific amino acids sequences have been recognized as potential triggers for smart biomaterial behaviors.

Time-dependent degradation of ECM following the myocardial cell death is, in fact, mainly regulated byMMPs, which are well known to function in the extracellular environment of single cells and degrade both matrix and non-matrix proteins as well. Thus, the activities of MMPs and their inhibitors play central roles in morphogenesis, wound healing, and tissue repair as well as a remodeling response after myocardial infarction.

These the topics reported from **Chapters 13** to **16**, where the interaction between stem cells and biomedical substrates are discussed. Stem cells, found extensively in umbelicus cord and in adult tissue at low frequencies are, in fact, capable of differentiating into specific tissues based on their differentiation potential, location, stimuli, and relocation.

Mesenchymal stem cells (MSCs) present in the adult bone marrow are self-renewing clonal precursors of non-hemopoietic stromal tissue and are capable of differentiating into mesenchymal lineages of bone, cartilage, fat, tendon, muscle, endothelial cells, and marrow stroma. There also exist stem cells and progenitors cells found in adipose tissue and stem cells with more restricted differentiation ability like neural stem cells. Moreover, there are other less well clinically characterized epithelial stem cells in tissue with high homeostasis turnover rates, and regeneration such as the skin which form hair follicles, sebaceous glands, and epidermal cells. There exists, however, a strong clinical need for replacement to conventional transplantation procedures due to the problems of donor availability, cost, and rejection.

Adult stem cells, therefore, are great candidates for such replacement therapy due to their potential for expansion and differentiation into the multiple cell types that constitute an organ.

The three organs most extensively studied are fat, bone, and heart. However, the clinical outcome of using autologous fat is highly unpredictable as lack of revascularization leads to graft resorption. Bone regeneration constitute a greater challenge due to limited availability of healthy donors, donor-site, morbidity, geometry, and cost, as well as heart transplants are limited by donor supply, immunoreaction, and high cost.

In any way, the prime potential of these cells of differentiating into specific cell lineages, is lost as

the cell die in the ischemic, oxidative, and inflammatory wound environment. The cells are lost, in fact, in the presence of low oxygen, low nutrient concentrations, high level of ROS, and pro-death cytokines. Thus, the field needs to develop ways to improve survival during the initial challenge period, as the use of right scaffolds. Therefore, development of bio-compatible polymeric scaffolds that can function as a carrier of both stem cells and proteins, maintaining proteins in their active conformation and restricting the signaling of proteins to the stem and progenitor cell compartment alone for a desired duration of time until tissue repair has been established, will take cell therapy to newer heights.

In conclusion tissue healing is a complex process which involves an orchestrated interaction between many different types of cells including immune, vascular, and stromal cells in addition to the tissue-specific cells. The end result of the healing process depends on a wide range of factors and is not always perfect.

Due to the very attractive properties of MSCs, it is expected that there will be a huge expansion in utilization of these cells for cellularizing biomaterial scaffolds for reconstructive and regenerative purposes. These the topics discussed from **Chapters 17** to **22**, where source, nature and applications of stem cells in tissue engineering are reported.

The final part of the book consisting of **Chapters 23** to **25** is dedicated to the clinical strategies used in regenerative medicine based by stem cell tissue engineering. The goal of such treatment strategies as bioengineered scaffolds and cellular therapies is to act as *smart band-aids*, to replace senescent resident cells and reestablish the anatomical and physiological environment. Scaffolds provide a microenvironment that allows for nutrient diffusion as well as biochemical, physical and cellular stimuli that guides proliferation, differentiation, and migration, while MSCs offer an easy harvest and expansion of a non-immunogenic cell line, guiding the tissue regeneration. Finally a well-designed randomized clinical trials are necessary to scrutinize the true potential of these highly complex therapeutic modalities.

In conclusion a regenerative medicine has made a substantial progress with more technologies ready to help patients, but it needs to have a profitable side to gain the interest of the industrial partner. Accordingly, it is very important that scientists work together with industry from the early stages to understand mutual needs and facilitate the process together with the support of clinical partners, responsible for calling the indications of the medical therapy.

It is necessary to understand in advance all the regulations involved in the category of medical products to facilitate the translational process later on. Technologies together with legal and ethical problems involving the use of biomaterials, represent, in fact, a complex and challenging pathway that requires multidisciplinary interaction between the medical and chemical community.

This book represents a key stone to clarify and understand all the problems connected with the use of stem cells and biomaterials in regenerative medicine, supplying to the readers all the innovations actually *in study* or ready in the market. All the reported information enriched by many interesting and updated references, may be useful for researchers and industrial experts, as well as students who wish to better know and enter into this fascinating and new medical field, understanding also the reasons and necessities for future innovations.

P. Morganti
Editor-in-Chief

UTILIZZAZIONE DI UN MATERIALE DI SCARTO DI ORIGINE MARINA PER PRODOTTI INNOVATIVI E BIODEGRADABILI PER IL PACKAGING ALIMENTARE

Oggigiorno una importante sorgente per produrre polimeri biodegradabili ed eco-compatibili e'rappresentata da materiali di scarto provenienti dalle lavorazioni di pesci e crostacei.
Di questi scarti rimangono inutilizzate ogni anno 250 miliardi di tonnellate che creano seri problemi di inquinamento sia del mare che del territorio che li ospita. Per incrementare il riciclo di questi scarti industriali è stato organizzato un progetto europeo di ricerca con il relativo Consorzio, denominato **n-CHITOPACK**, per utilizzare le **Nanofibrille di Chitina (CN)** che, provenienti dalla lavorazione di questi scarti, verranno utilizzate per il packaging alimentare.
Il Consorzio Europeo **n-CHITOPACK** coinvolge la PMI MAVI sud (Aprilia) quale produttore della materia prima CN e detentrice dei relativi brevetti internazionali, assieme ad altre PMI quali MICROTEC (Padova) produttrice di packaging flessibili, AROMA SYSTEM (Bologna) produttrice di packaging rigidi, RODAX (Bucarest, Romania) produtrice di macchinari destinati ai packaging alimentari, e BIOZOON (Bremerhaven, Germania) che studierà a fondo il mercato di questi materiali identificandone la domanda.
Le Unità di Ricerca coinvolte necessarie per trasmettere alle PMI le relative tecnologie di produzione e controllo sono: NOFIMA (Tromso, Norvegia), IMC (Praga, Rep. Ceca) e INSTM (Firenze) che metteranno a punto le composizioni ideali dei diversi packaging basati sull'uso di CN funzionalizzato con altri polimeri naturali.
Queste RTD studieranno e caratterizzeranno i relativi packaging per quanto attiene le loro proprietà meccaniche e chimico- fisiche, per valutarne la loro idrofobicità e biodegradabilità con quant'altro richiesto dalle normative EU per i packaging di uso alimentare.
La RTD NOFIMA controllerà le proprietà antimicrobiche e tossicologiche di questi materiali, valutandone la loro vita media e sicurezza nell'uso.
Il Progetto n-CHITOPACK è cordinato da MAVI sud, Italia.
www.n-chitopack.eu

USING BIO-WASTE OF MARINE ORIGIN TO PRODUCE INNOVATIVE BIO-DEGRADABLE FOOD PACKAGING

Today a significant source of potential renewable feedstock for polymers is represented by waste material from the fishing industry, exceeding 250 billion tons/years, and is considered hazardous due to its high perishability and polluting effect, both on land and sea.
To improve the recycling of this marine-waste the EU project **n-CHITOPACK** started to use a sugar-like polymer, the **Chitin Nanofibrils (CN)**, coming from this waste, to produce innovative food packaging processes and products.
The n-CHITOPACK consortium involves SME ingredient supplier of CN and patent holder MAVI sud srl (Aprilia, Italy), and complementary SME's packaging producers: MICROTEC (Padova, Italy) for flexible food packaging, AROMA SYSTEM (Bologna, Italy) for rigid packaging, RODAX IMPEX (Bucharest, Romania) for packaging equipment, and BIOZOON (Bremerhaven, Germany) for marketing analysis and identification of consumer demand.
The RTD Performers IMC (Praha, Czech Republic), NOFIMA (Tromos, Norway) and INSTM (Firenze, Italy)) will perform research on the optimal composition of CN-based packaging, functionalized with other different biologically active polymers in order to meet and characterize its mechanical and chemicalphysical activities together with its hydrophobic and biodegradable requirements for food packaging.
The RTD NOFIMA will analize the products selected in order to evaluate the antimicrobial properties, shelf life extension, and food-safety of their materials.
The Project is coordinated by MAVI sud, Italy.
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Note

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In copertina / Front cover
Block co-polimero formato da Nanofibrille di Chitina (CN) e Acido Ialuronico (HA).
Foto al microscopio elettronico a scansione (SEM). Archivio privato MAVI SUD S.r.l.
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Block co-polymer of Chitin Nanofibrils (CN) and Hyaluronic Acid (HA).
Scanning Electron Microscopy (SEM) micrographs. MAVI SUD S.r.l. Private Database.
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