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

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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.

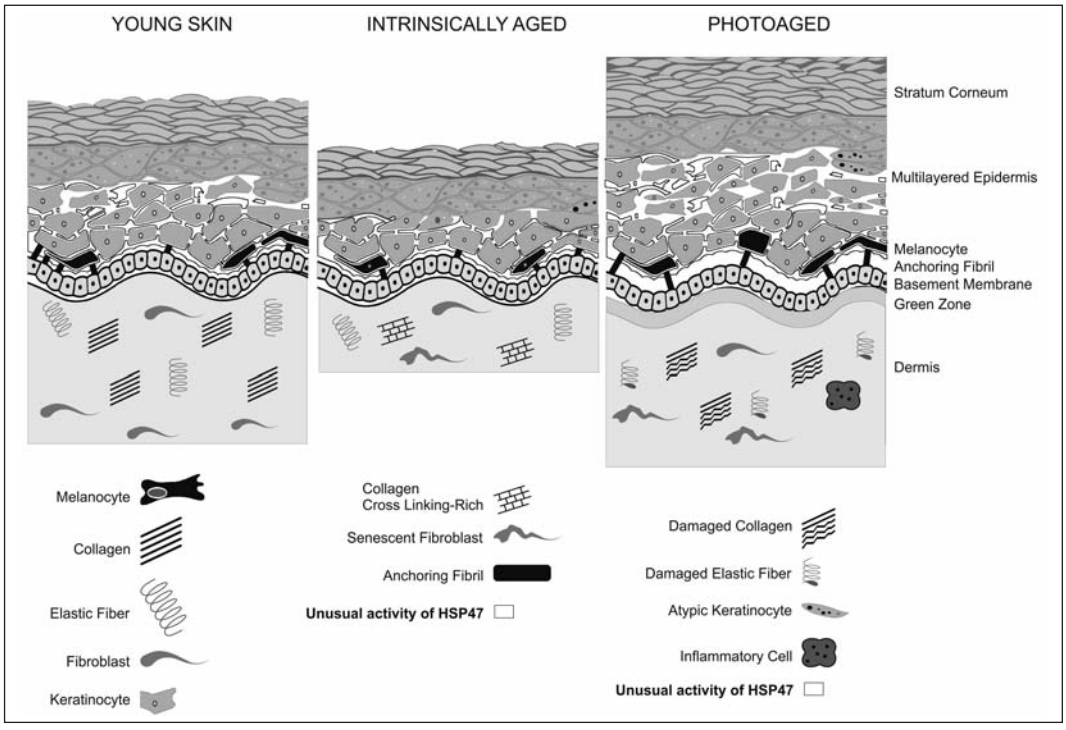


Fig. 1 *Reduced synthesis of HSP47 has been underlined in intrinsically skin: aged and photo-aged.*

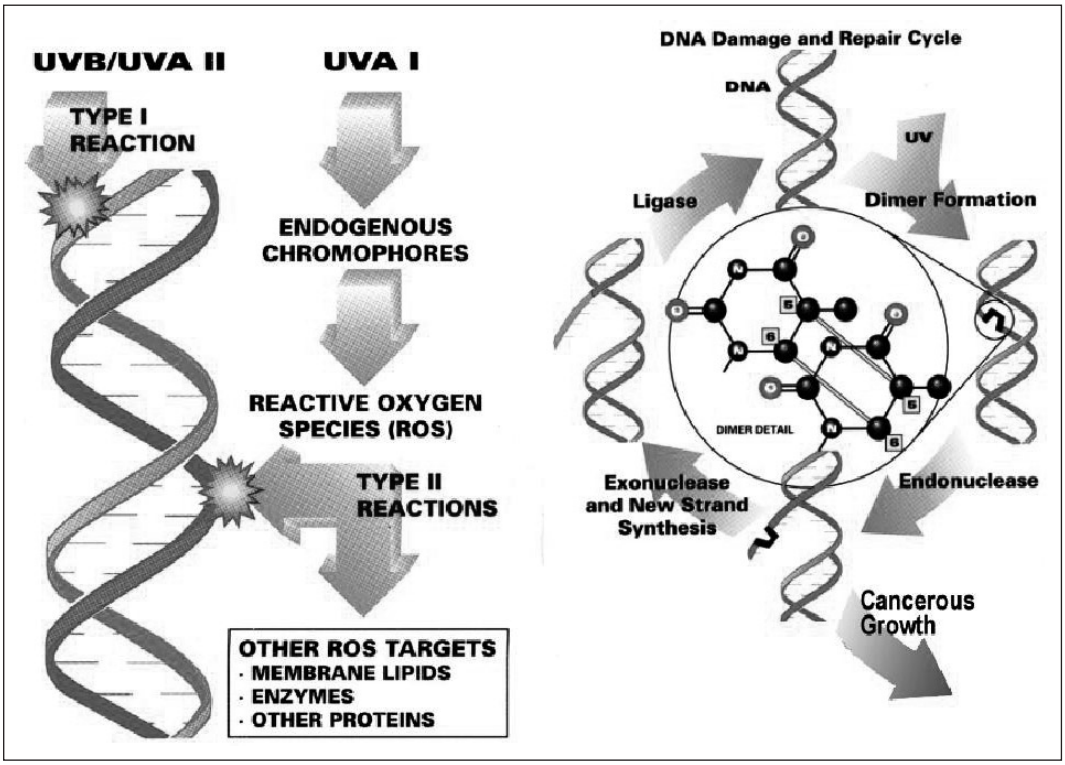


Fig. 2 *Direct and indirect activity of UVB/UVA rays on skin DNA.*

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]:

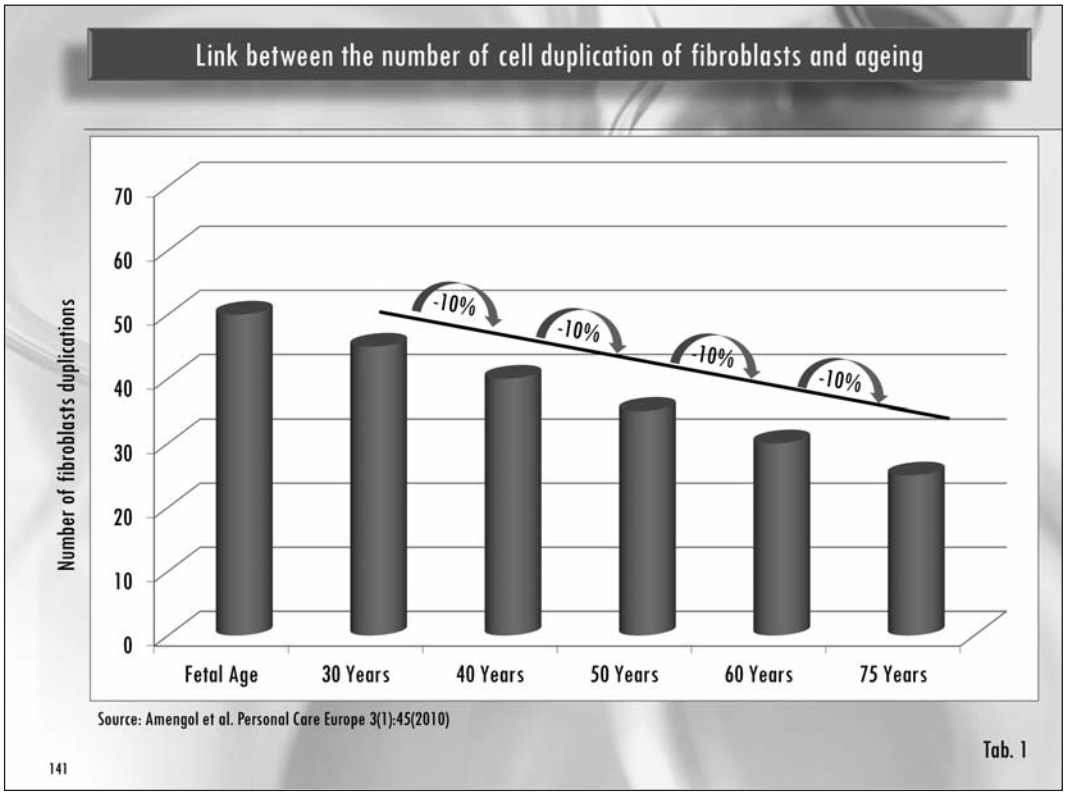
Soluble Collagen, Propylene Glycol, Aqua (Water), Lactic Acid, Glycerin, Pentylene Glycol, Propanediol, Jojoba Wax PEG-120 Esters, Glycine, Arginine, PEG-200 Hydrogenated Castor Oil, Panthenol, Parfum (Fragrance), Sodium Ascorbyl Phosphate.

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.

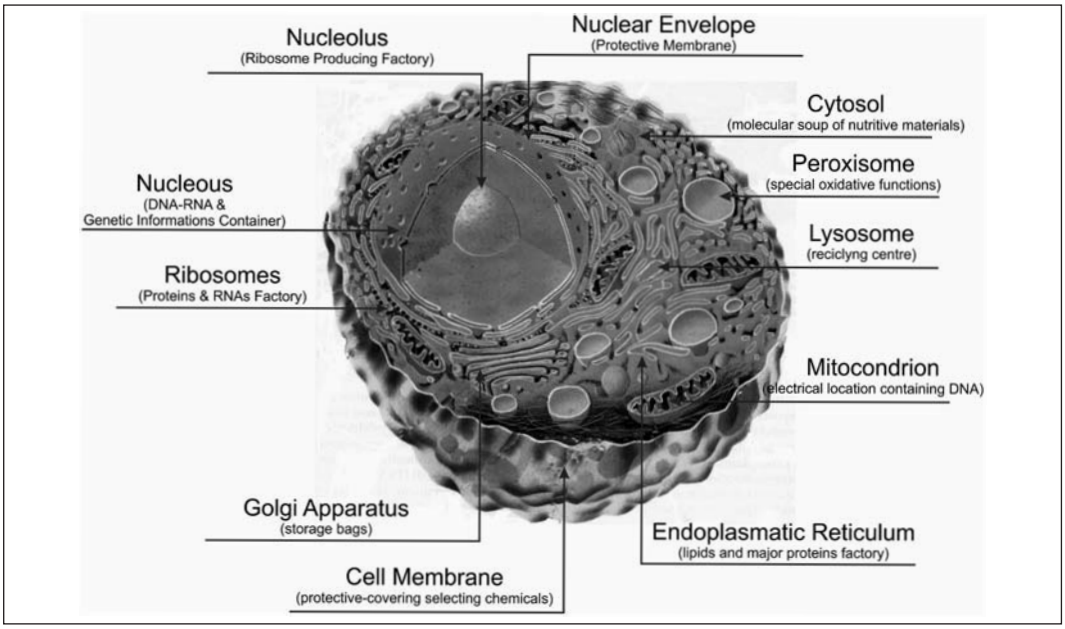


Fig. 3 *View of a cell where HSPs are located at level of endoplasmatic reticulum.*

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).

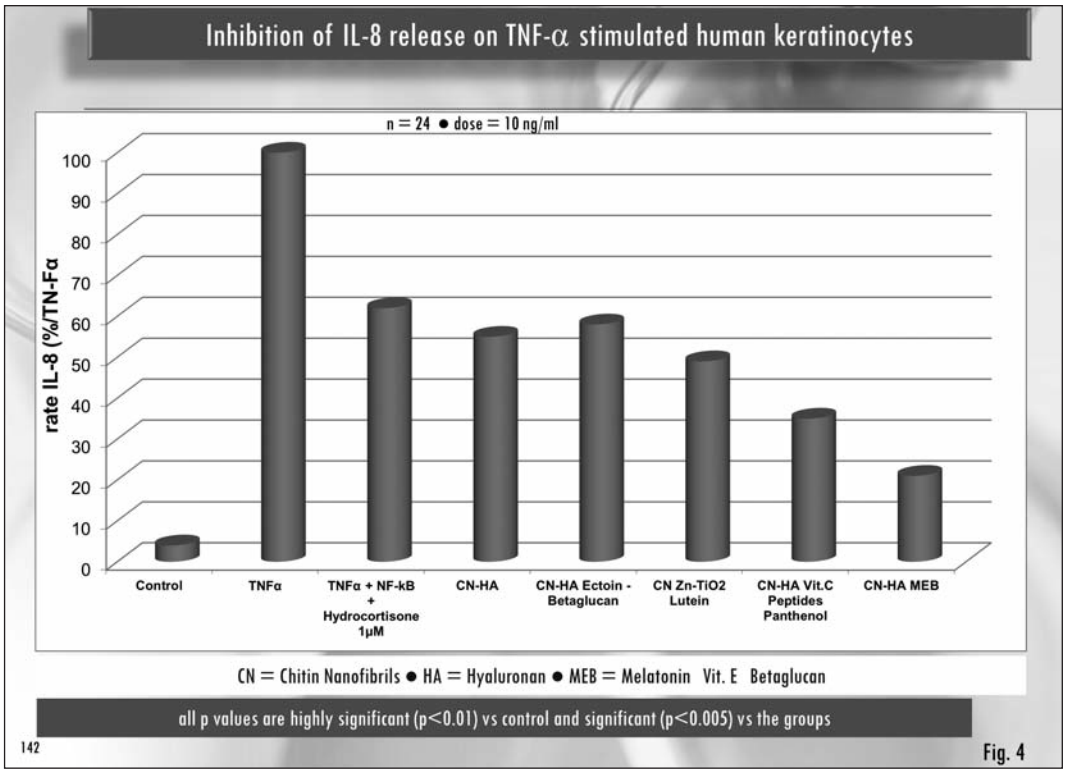


Fig. 4

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).

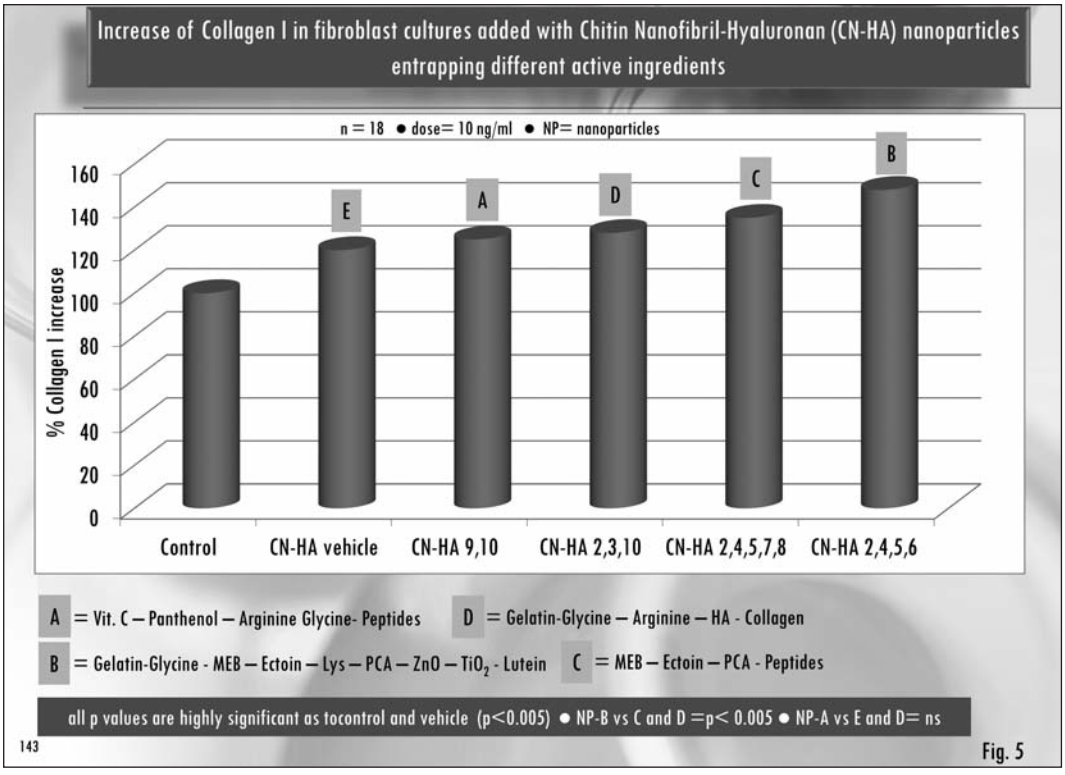


Fig. 5

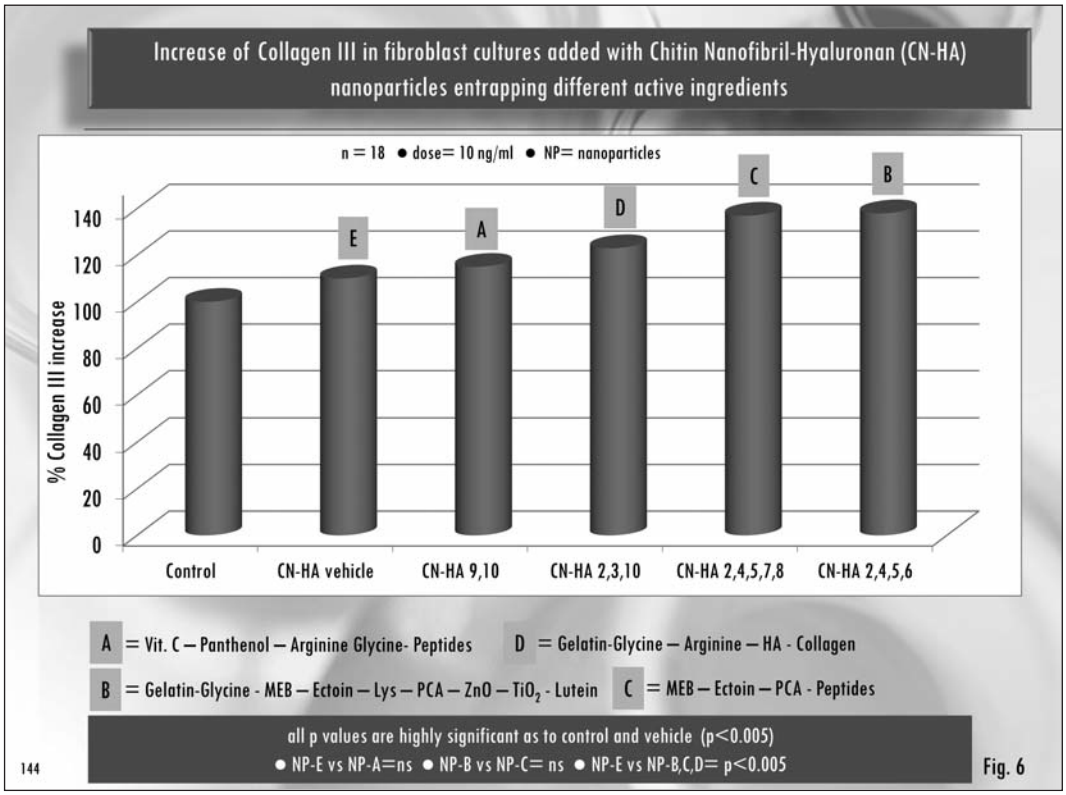


Fig. 6

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).

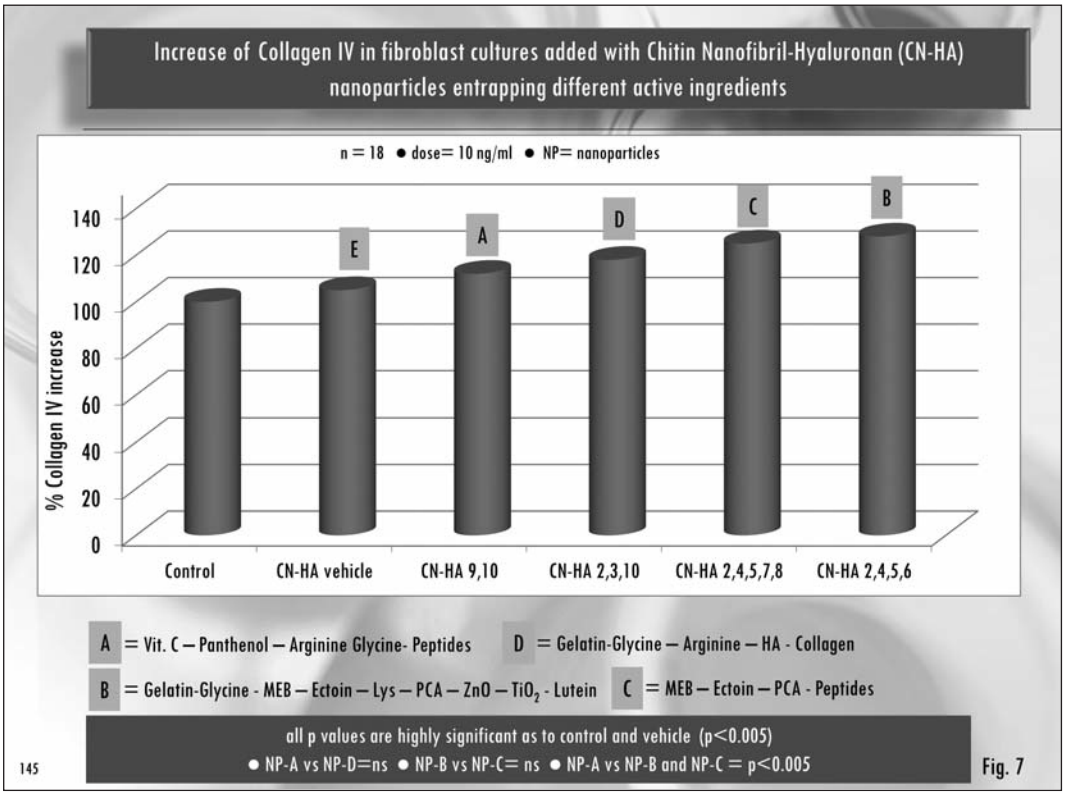


Fig. 7

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.

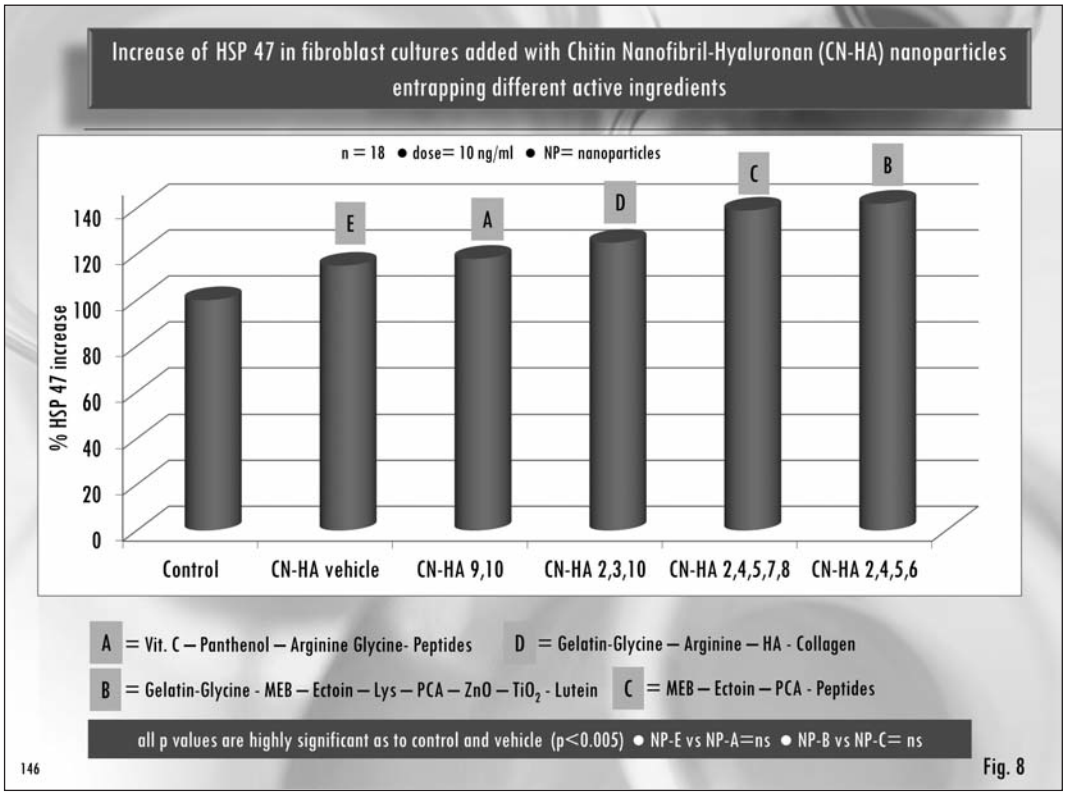


Fig. 8

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.

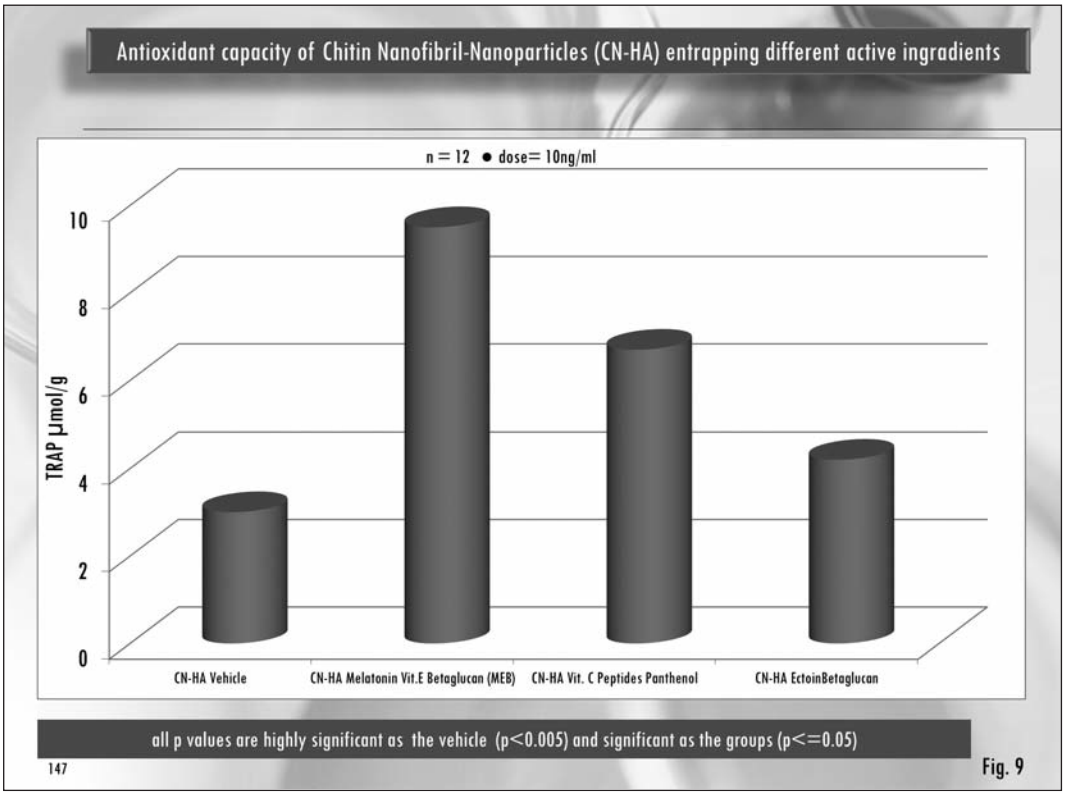


Fig. 9

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).

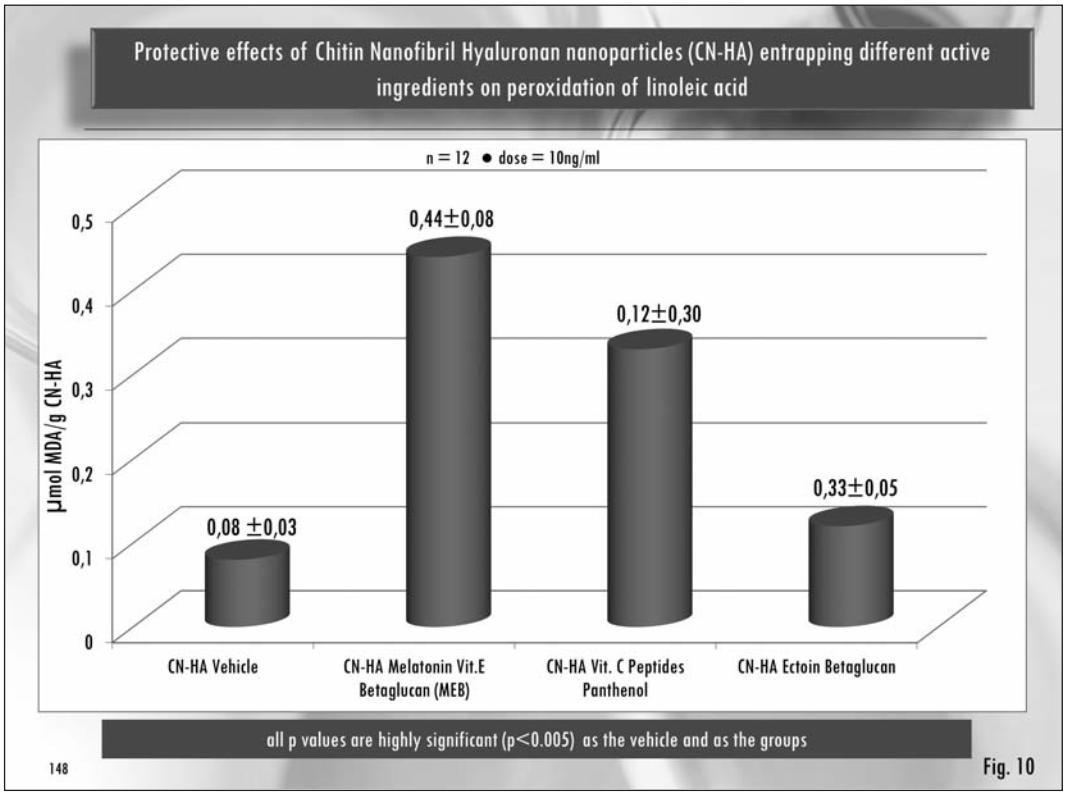


Fig. 10

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).

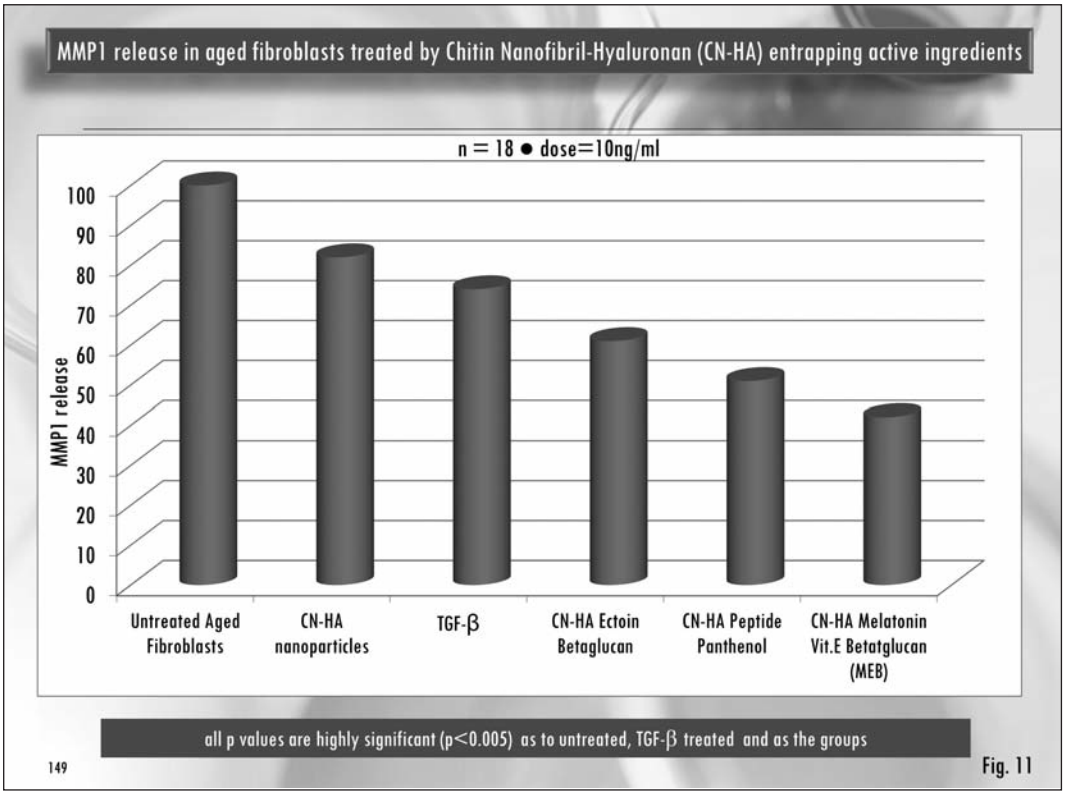


Fig. II

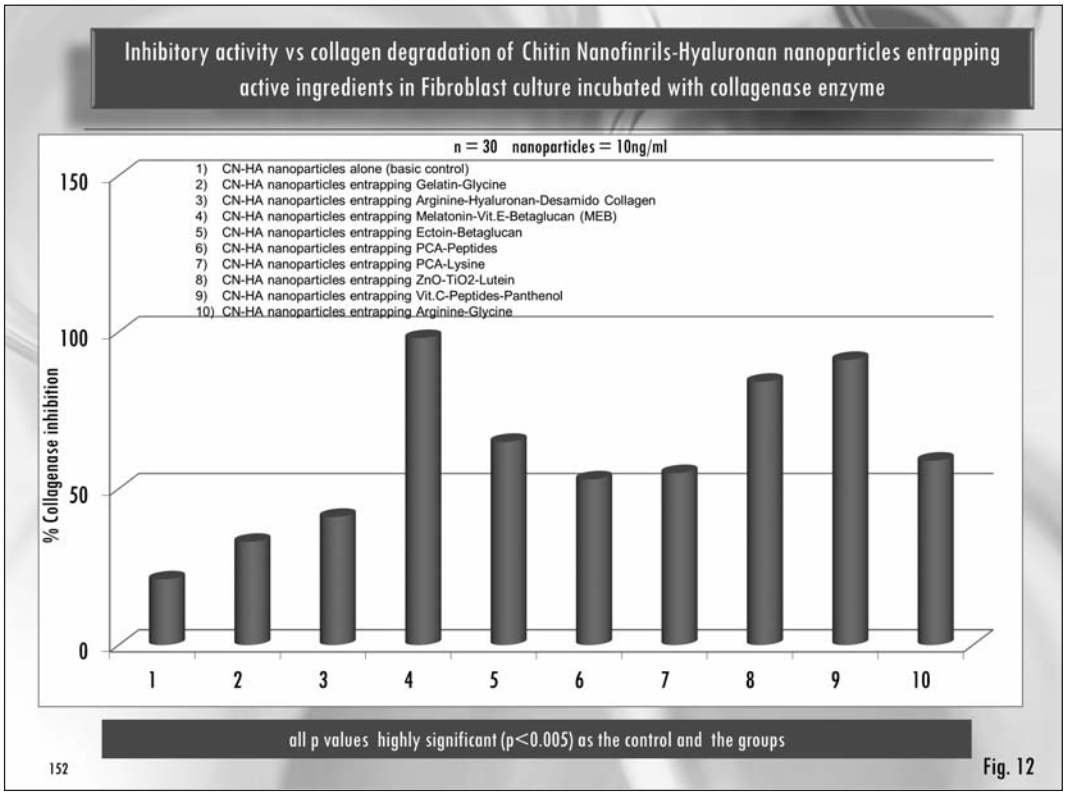


Fig. 12

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.

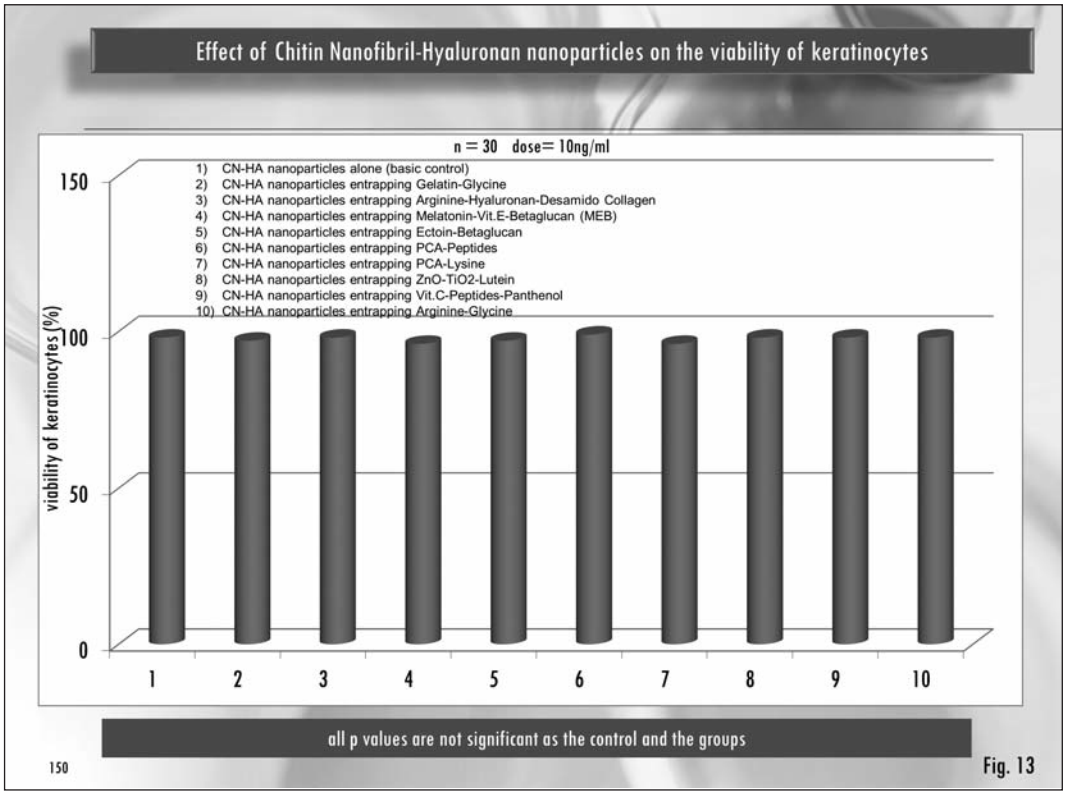

Fig. 13

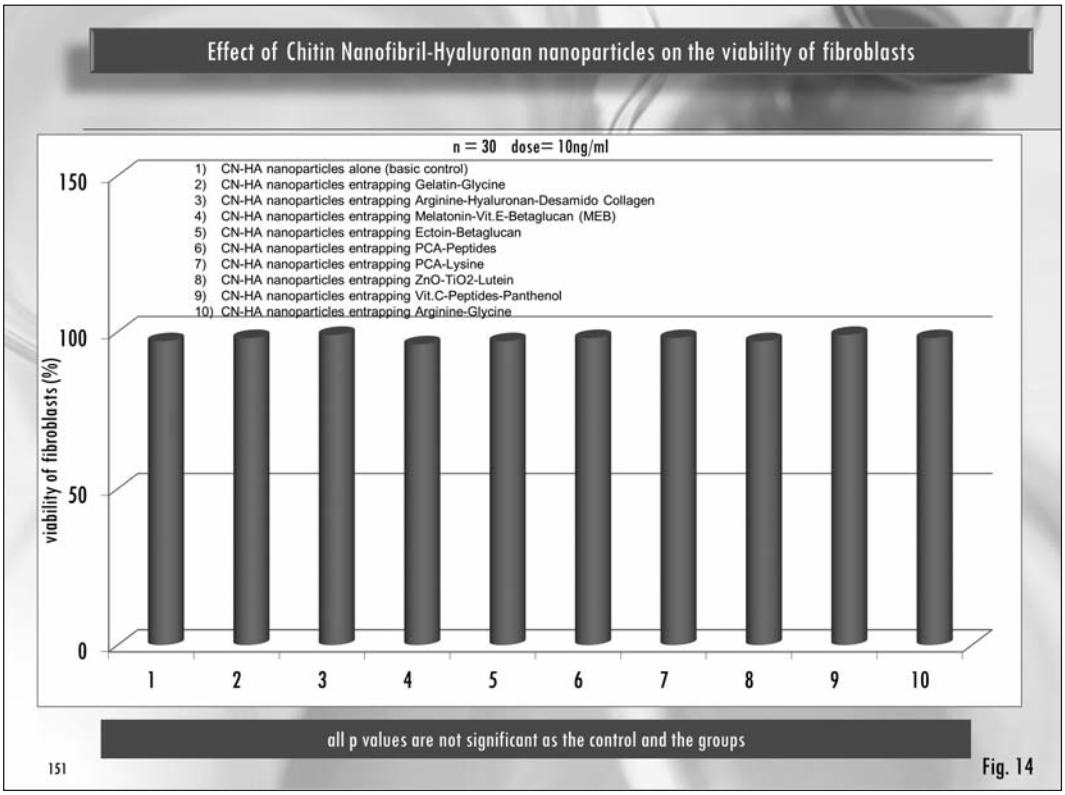

Fig. 14

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.

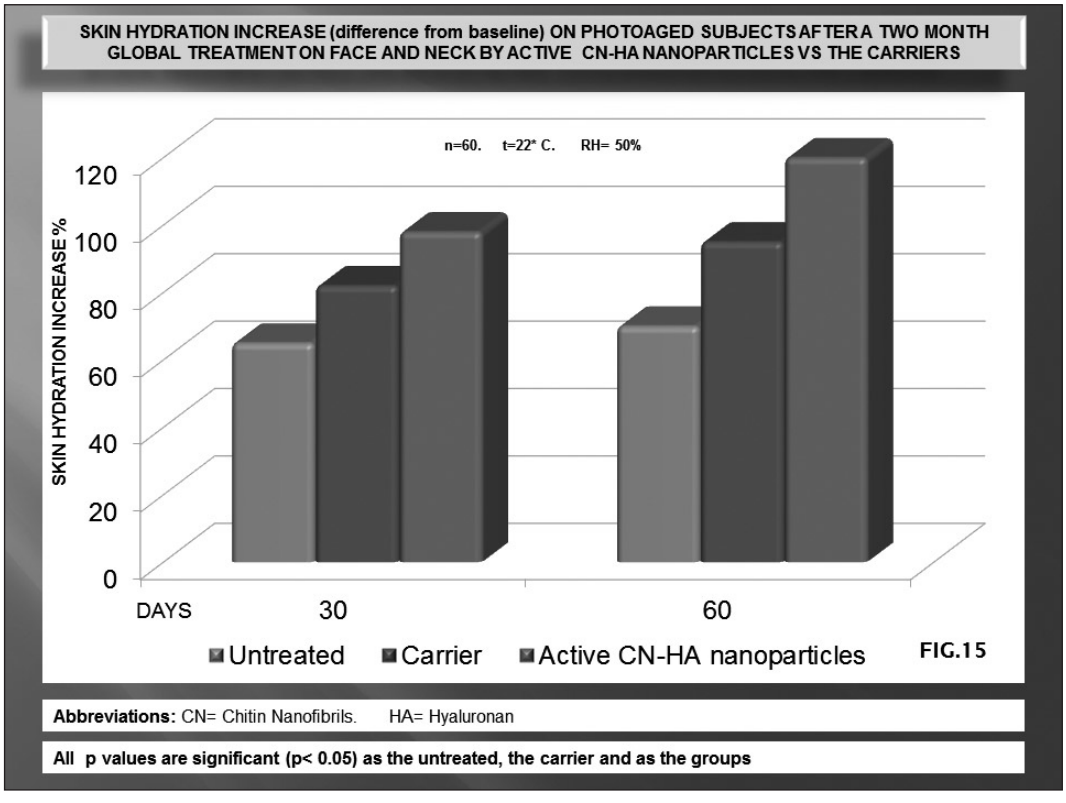

Fig. 15

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.

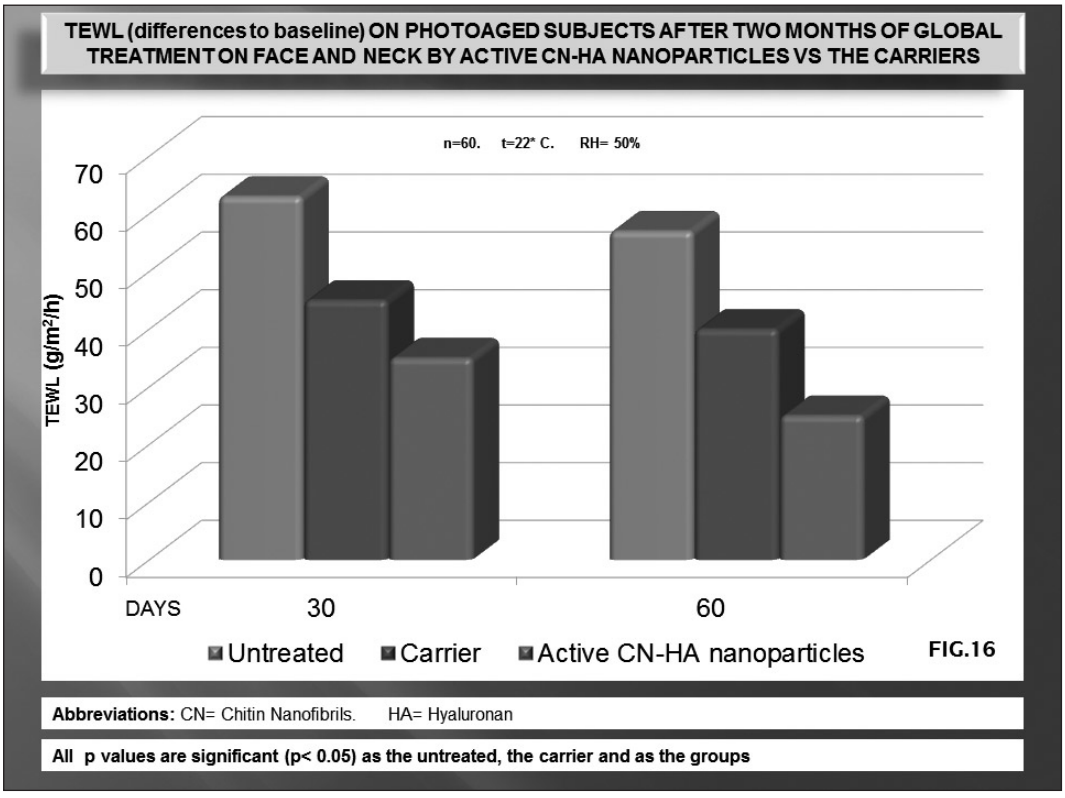

Fig. 16

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).

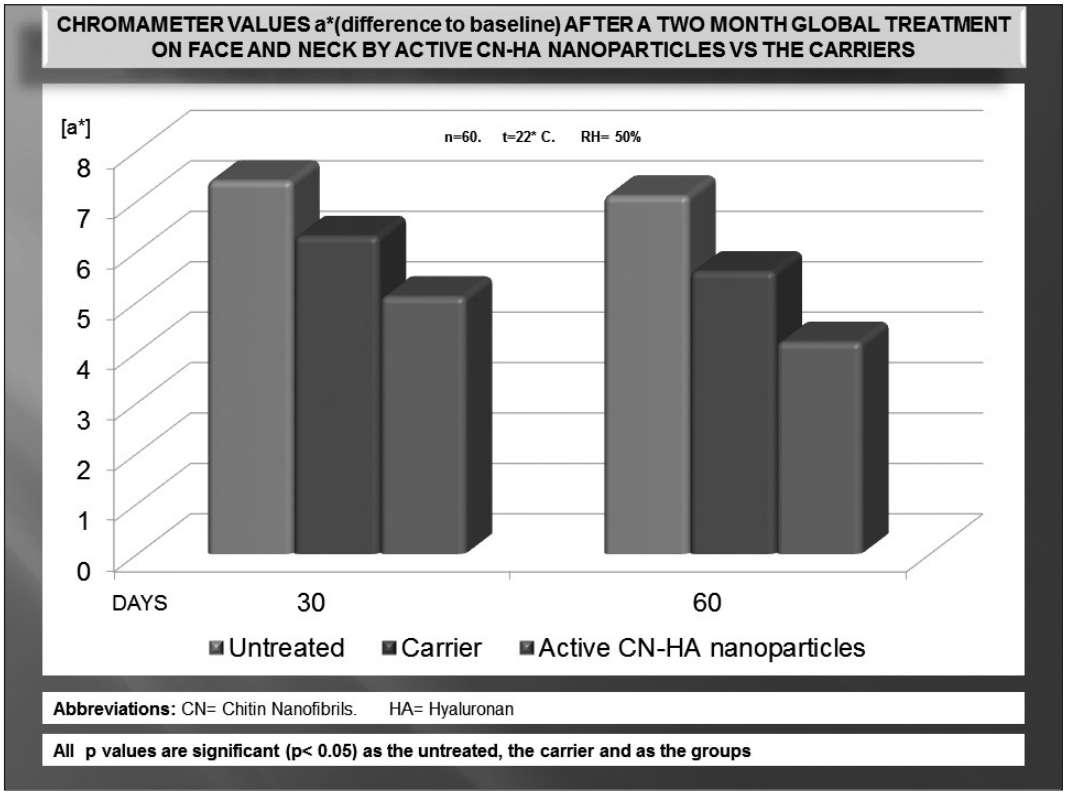

Fig. 17

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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