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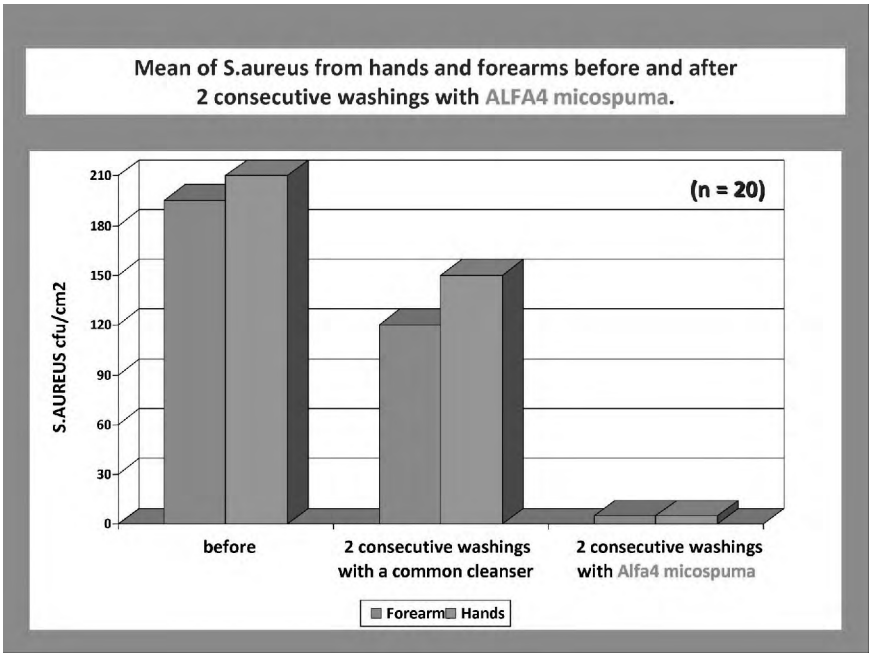

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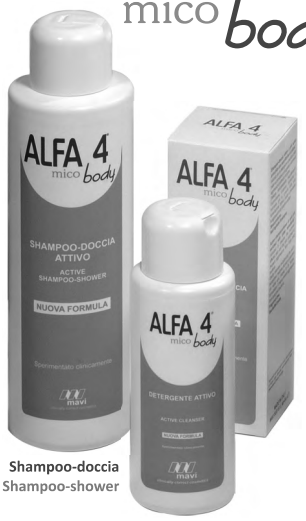

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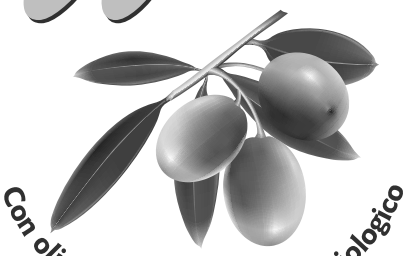

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1

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Trimestrale di Dermatologia Cosmetologica
Quarterly Review of Cosmetic Dermatology

Contents

1 **Message from the Editor in-Chief**

Original Laboratory Studies

3 **Activity of Chitin Nanofibrils Block-Copolymers Entrapping Zn/Al/SA/Allantoin on Seborrheic Dermatitis. A randomized double-blind placebo controlled study**
P. Morganti, G. Fabrizi, M. Palombo, M. Cardillo, A. Cardillo, P. Del Ciotto, F. Carezzi, G. Morganti

General Articles

21 **Biological Application of Chitosans**
U. Cornelli, P. Morganti

31 **Biological Activity of Less Known Minor Components of Extra Virgin Olive Oil**
P. Viola, M. Viola

Book Reviews

41 **Handbook of Immunological Properties of Engineered Nanomaterials**

49 **The Skin Care Ingredient Handbook**

51 **Apply Topically: A Practical Guide to Formulating Topical Applications**

Gentili Lettori,

da 32 anni il Journal of Applied Cosmetology, organo ufficiale dell'International Society of Cosmetic Dermatology (I.S.C.D.), viene stampato su carta ecologica chlorine free" e distribuito via posta a livello internazionale.

Il Comitato Editoriale informa che dal 2015, in linea con gli standard internazionali, la rivista sarà pubblicata solamente in forma digitale online.

La decisione è stata presa per essere vicini alla bioeconomia che prevede minor consumo di energia e di acqua che, come è noto, servono anche per produrre la carta, e limitare i gas inquinanti emessi dai trasporti.

Fiduciosi che questa decisione, oltre ad essere condivisa dai Soci dell'I.S.C.D., sia apprezzata dai numerosi lettori, Vi auguro buona lettura.

L'Editore
Pierfrancesco Morganti

Dear Readers,

for 32 year the Journal of Applied Cosmetology, official organ of the International Society of Cosmetic Dermatology (I.S.C.D.), has been printed on ecological paper "chlorine free", and internationally shipped.

The Editorial Board informs that from 2015, according to the International Environmental Standard, the journal will be published in digital version online only.

This decision has been taken in order to contribute to the development of the Bio-economy, which invites to reduce the consumption of energy and water, in our case used to print on paper, as well as to reduce gas emission also generated by transport.

We believe that, apart from the I.S.C.D. Members, this decision will be appreciated by all the readers.

The Editor-in-Chief
Pierfrancesco Morganti

J. Appl. Cosmetol. ***32,*** *3-19 (January/June 2014)*

Activity of Chitin Nanofibrils Block-Copolymers Entrapping Zn/Al/SA/Allantoin on Seborrheic Dermatitis. A randomized double-blind placebo controlled study

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Received: *April, 2014*

Key words: *Seborrheic Dermatitis; Chitin Nanofibrils; Antioxidant Activity; Salycilic Acid; Zn-Shampoo; Al-Zn complex; Block Polymers; Allantoin;*

Summary

Background: The study was designed to evaluate the topical efficacy and safety of chitin nanofibrils antrapping Zn, Al, salicylic acid (2%) water solution and allantoin combined with the activity of a shampoo based on a Zn based cleansing agent, in the treatment of scalp seborrhoic dermatitis as newly therapy for seborrhoeic dermatitis, preventing sebum-reducing, anti-inflammatory and antimicrobial and antimicotic activity.

Method: A double-blind placebo, 60 patients with scalp seborrhoic dermatitis were treated by a16 week trial. Efficacy was determined *in vivo* by the control of population density of *P. ovale* and S. aureus, dandruff scales, and reduction of surface lipids and free fatty acids/triglycerides ratio.

The anti-inflammatory activity of IL-8, IL-1α and TNF-α and the antioxidant activity on ROS, was verified *in vitro*. Safeness of the treatment was evaluated by questionnaire at each visit.

Results: For all the subject treated by the active solution and Zn shampoo the total symptoms significantly improved at week 4 maintaining the activity.

Moreover, from week and until week 16, S. aureus, *P. ovale*, surface lipids and FFA/triglycerides ratio presented a continuous reduction throughout the study maintaining their normal values during the suspension period also.

Conclusion: SA water solution complexed by chitin nanofibrils with Zn Al ions and allantoin, seems to be an innovative and effective therapeutic option for scalp seborrhoic dermatitis.

Riassunto

Presupposto: La soluzione acquosa di acido salicilico al 2% complessato con nanofibrille di chitina e ioni Zn, Al e allantoina, rappresenta una innovativa terapia per la dermatite seborroica, svolgendo una attività sebo-equilibrante, anti-infiammatoria, antimicrobica ed antimicotica.

Obiettivo: Lo studio è stato impostato per valutare l'efficacia topica e la mancanza di effetti collaterali nel trattamento topico di persone affette da DS, mediante l'uso di una soluzione acquosa di nanofibrille di chitina in grado di legare ioni Zn, Al ed acido salicilico al 2% assieme all'allantoina. L'attività della soluzione è stata coadiuvata dall'uso di uno shampoo formulato con un tensioattivo anionico a base di Zn.

Metodo sperimentale: Attraverso uno studio a doppio cieco condotto per un periodo di 3 mesi di trattamento ed un mese successivo di controllo, sono stati trattati 60 soggetti volontari affetti da dermatite seborroica del cuoio capelluto.

L'efficacia è stata verificata *in vivo* controllando la densità del *P. ovale* e dello S. aureus, il numero di scaglie forforali e la riduzione dei lipidi totale con la variazione del rapporto acidi grassi liberi/trigliceridi.

In vitro è stata controllata l'attività antinfiammatoria del trattamento verificando la secrezione delle citochine 1L-1α, IL-8 e TNF-α, riscontrandone che anche l'attività antiossidante nei confronti dei ROS. E' stata anche verificata la mancanza di effetti collaterali mediante la trascrizione di un questionario.

Risultati: Su tutti i soggetti trattati contemporaneamente con la soluzione attiva (CN-Zn-Al-SA-ALT) e lo shampoo a base di Zn, tutti i sintomi caratterizzanti la DS sono quasi totalmente scomparsi dopo il primo mese di trattamento, continuando il loro percorso di apparente guarigione anche nel mese successivo alla sospensione della terapia.

Inoltre, è stata verificata una drastica riduzione della presenza sia del *P. ovale* che dello *S. aureus*, oltre che delle squame forforali presenti al livello del cuoi capelluto, accompagnate da un riequilibrio dei lipidi di superficie e del rapporto acidi grassi/trigliceridi e delle citochine infiammatorie.

Conclusioni: La soluzione acquosa di acido salicilico al 2% complessato con gli ioni Zn, Al e allantoina, si è rivelata un mezzo innovativo efficace e sicuro per il trattamento della DS.

INTRODUCTION

Seborrheic Dermatitis (SD) is a common chronic, relapsing inflammatory dermatosis and one of the most common cutaneous manifestations of AIDS and AIDS-related complex (1-4). It experiences characteristic pattern, for different age groups while the increase of *Pityrosporum ovale* seems to be the probable causative factor, also if genetic and environmental factors may influence the onset and course of the disease. Many adult patients have an oily complexion, the so-called *seborrheic diathesis,* and most individuals periodically experience fine, dry, and white scalp scaling with minor itching, recognized as dandruff.

The overgrowth of *P. ovale,* that accompanies the scaling and the increased amounts of sebum retained by scales, may play an important secondary role through activation of complement that can become available as the stratum corneum is damaged and serum reaches the scalp surface (5). Moreover, only some 20% of patients with SD are colonized by *S. Aureus, while* in psoriasis the organism is present in low numbers in some 50% of cases. In any way, the distribution of scaling and inflammation is quite diffuse and occur in the seborrheic areas, such as scalp and scalp margins, eyebrows, base of eyelashes, nose-lip folds, external ear canals, posterior auricular fold, and pre-sternal area. Therefore, it seems important determine whether the hair scalp micro flora of SD patients could be able to stimulate IL-1 α production, as well as contribute to stimulate the inflammatory cascade of the cytokines release such as IL-1α, IL-8 and TNF-α from keratinocytes irradiated by UV rays. All these phenomena, allowing generally for an over-production of free fatty acids with a consequent exacerbated inflammatory response, cause a high secretion of cytokines often resulting in appearance of redness, itching and other discomforts. Occasionally the scales on the scalp may be diffuse, thick, and adherent so that differentiation from psoriasis is very difficult to distinguish. At this purpose, patients should be reassured that SD does not cause permanent hair loss. They, in fact, tend to attribute hair loss and their condition to a dry scalp, thus avoiding hair washing. As a consequence, scales accumulation and inflammation may increase. For all these reasons, patients should be encouraged to wash hair every day or every other day by the use of anti-seborrheic and anti-dandruff shampoos, regularly applying specific lotions to the scalp twice daily (6). In any way, it has to be remembered that SD tends to persist and does undergo periods of remission and exacerbation. Thus the frequent washing with Zinc-shampoos, that suppress scales and excess sebum together with the alternatively use of topical steroid or salicylic acid (SA) water solutions, are considered the best SD treatments for the quick resolution of the inflamed areas. Therefore the challenge, in formulating these products, was to dissolve SA into water-based medications without using any organic solvent, as the irritative ethyl alcohol. At this purpose it is to remember that, on one hand the water solubility of SA is very poor (0. 2g/100 ml at 20 °C), while on the other hand its required concentration at level of keratinocytes has to be between 0. 5 and 2% to be effective. The SA mechanism of action is, in fact, the disruption of intercellular adhesions necessary to inhibit the inflammatory cascade associated with SD. It is necessary to remember the difficulty to disrupt these adhesive forces, because of the short distance between both corneocytes and lipid lamellae within the nanoscale and the high resistance of the horny layer' keratin structure providing the covering of these cells, chemically non reactive, hard and waterproof. Therefore, the primary goal in delivering SA and other active ingredients in the right concentration for obtaining the best results is to formulate nanoparticles, which can efficiently pass through the

skin barrier, for being deposited onto the different layers of each corneocyte wall. Thus, Chitin Nanofibril-building blocks, realized by our innovative technology (7-10), seem to represent an innovative delivery system to slowly release the active ingredients over the time. These nanoparticles have the capacity to entrap the active ingredients, remaining stable over time when suspended in appositely formulated emulsions. Once applied on the skin the positively charged nanoparticles, incorporated into the emulsion, easily disrupt the intercellular adhesions, penetrate through the horny layer and anchor themselves at level of corneocytes.

On the other hand, the carrier represented from the Chitin Nanofibril (CN) moiety, will be gradually hydrolyzed and metabolized from the skin cells by the human chitinases, breaking down safe and skin friendly compounds, while release over time the active ingredients in a controlled manner.

AIMS

According to our recent obtained results (6), the aim of this study was to control *in vitro* and *in vivo* on patients affected by SD, the antidandruff and anti-seborrheic activity of a topical treatment based on the contemporary use of a new CN-Zn building block shampoo and the innovative topical lotion formulated with CN-SA-Al-Zn moiety, enriched with allantoin, to increase its anti-inflammatory activity.

Population dynamics and protective role of scalp microbiota

Design Project

Bacteria that normally live in the skin may help protect body from infection and environmental aggressions. As the largest organ of the body the skin represents a major site of interaction with microbes in the environment. Although immune cells protect the skin against harmful organisms, until now the beneficial role of the millions of naturally occurring commensal bacteria, collectively known as skin microbiota, has not been studied. However the density of microorganisms' colonization and the constituent species, found in specific sites on SD skin surface or scalp, reflects a unique cutaneous environment, as reported from different authors (11-14). Several factors affect, in fact, the ecology of microorganisms inhabiting a particular site. While the major SD determinants have been clearly identified (moisture, pH variation, presence of nutrients and inhibitor substances, as triglycerides, free fatty acids and ROS), it has been shown that the interactions between microorganisms should also influence their ability to colonize and proliferate (15,16).

A significant correlation exists between the number of propionibacteria and the total amount of sebaceous lipids delivered to the skin/scalp surface in general and triglycerides and free fatty acids in particular (17-21). Moreover, there is evidence that sebaceous glands may also serve as a source for immunomodulatory mediators as TNF-α (mainly produced by monocytes/macrophages upon stimulation with viruses-bacteria, parasites and tumour cells) (22), as well as interleukin 1-α (IL-α) and IL-8, expressed as cytokines during the inflammatory process (23).

On one hand release of TNF-α, as cytocidal sebocyte-derived agent, seems to represent an unspecific host defence mechanism by which the pilosebaceous unit controls residing and invading pathogenic microorganisms; on the other hand IL-8 should play a hugely important role in cellular osmotic shock. As a consequence cells show denaturation of inter-and intracellular tight junctions with a significant loss in barrier function of the epidermis (24). Cells which are in osmotic shock produce, in fact, the inflammatory mediators TNF-α and especially IL-8, as a

consequence of their stressed state (25, 26). Reduction of secretion of the TNF-α and IL-8 should represent one of the key activities of effective ingredients, dealing with these kind of acute inflammation mediators.

For these considerations it was taken the decision to control the superficial keratinized scales together with the total quantity of surface lipids and the triglycerides/free fatty acids ratio, recovered on the scalp surface of all the SD voluntary patients under study. Moreover both the chemokines IL-1 α, IL-8 and TNF-α, together with the microorganisms S. Aureus and *P. ovale* were also controlled, to verify their eventual implication in inflammatory and immune responses. Finally the antioxidant activity of the topical solution was verified, supposing the oxidative stress may influence the increased keratinocytes' turnover and sebum production in SD.

MATERIALS AND METHODS

MATERIALS

Chitin Nanofibril-Salicilic acid-Alluminium block polymer;
Chitin Nanofibril-Allantoin-Zinc block-polymer and CN-Zn-All-SA-Allantoin-block-polymer (MAVI SUD, Aprilia (Lt), Italy).
Zinc Coceth Sulfate (Zschimmer & Schwarz, Tricerro (Vc), Italy).

Formulations:

Shampoo: Aqua (Water), Chitin (Nano-Fibrils), Zinc Coceth Sulfate, Cocamidopropyl Betaine, Sodium Chloride, PEG-200 Hydrogenated Glyceryl Palmate, Hydrolyzed Wheat Protein, Glucosamine, PEG-7 Glyceryl Cocoate, Phenoxyethanol, Imidazolidinyl Urea, Propylene Glycol, Parfum (Fragrance).
Lotion: Trimethylglycine, Aqua (Water), Glycolic Acid, Salicylic Acid, Zinc PCA, Potassium Alum, Allantoin.

METHODS

Study Design

Patient Enrolment in vivo

This 16-week randomized double-blind placebo controlled study, enrolled 60 volunteer patients (mean age ± 5 year) with stable moderate to severe dandruff covering an area for at least 6 cm^2. Exclusion criteria included patients who had used topical antibiotics, antiseptics, or salicylic acid solutions and corticosteroids in the past 15 days, systemic antibiotics in the past 30 days, and any other topical SD treatments including medicated soaps, cosmetic creams in the past 7 days and systemic corticosteroids in the past 12 weeks.

The study was conducted with the principles of the Declaration of Helsinki revised in Seoul for a period of 90 days plus 30 days of regression period. Each patient provided written informed consent and received a unique identification number. Both patients and investigators were blinded throughout the study as to treatments assigned. Group D received the in study Zn-shampoos and topical lotions sufficient for 12 weeks-treatment. Group C received commercial antidandruff shampoos and a corticosteroid lotion (Hydrocortisone 0. 5%). Group B received commercial antidandruff shampoo and placebo watery topical inactive lotions. Group A received a commercial shampoo and a placebo watery topical inactive lotion (Control group). The control was done every month from an expert dermatologist who verified: (a) quantity of scalp' scales determined by the use of our method previously described (27); (b) quantity of total lipids and triglycerides/free fatty acids ratio taken from 4 different areas of the scalp (frontal, parietals and posterior) were controlled by the 3C System, used from our group for other studies (28, 29); (c) number of *P. ovale* and

Staphylococcus aureus colonies were sampled by rubbing the scalp surface with a swab moistened in 0. 075 M sodium phosphate buffer containing 0. 1% Triton X 100, according to Eady (30). The anti-oxidant property of the in study Solution was controlled ex vivo by measuring the ROS variation in samples of the in study subjects.

Patients were instructed to wash the hair each second day applying some drops of the assigned lotion on the affected scalp area by a soft massage, just before retiring in the evening. Other instructions included that they had to use no other hair treatment during the study.

Clinical evaluations were performed on the first day (baseline) and at 4, 8, 12 weeks (end of treatment). Another control was performed on week 16 (regression period).

Micro-organisms count

Isolates of S. *Aureus* and *P. ovale* were obtained before treatment by swabbing the scalp area of the patients in study. Cultures were harvested during the stationary phase, and intact cells were washed and treated two times in phosphate buffered saline at 4 °C and re-suspended in RMMI 1640 (Gibco). Cell wall debris was sedimented by centrifugation at 12, 000 g and the supernatant containing the soluble cellular fraction was freeze-dried. The freeze-dried fractions were reconstituted in tissue culture medium at 100 ng/ml protein. The obtained values of untreated and treated by different block copolymeric nanoparticles and pirythion olamine cultures are reported in figure 1 and 2 as mean Log_{10} cfu/cm^2 ± 95% CL.

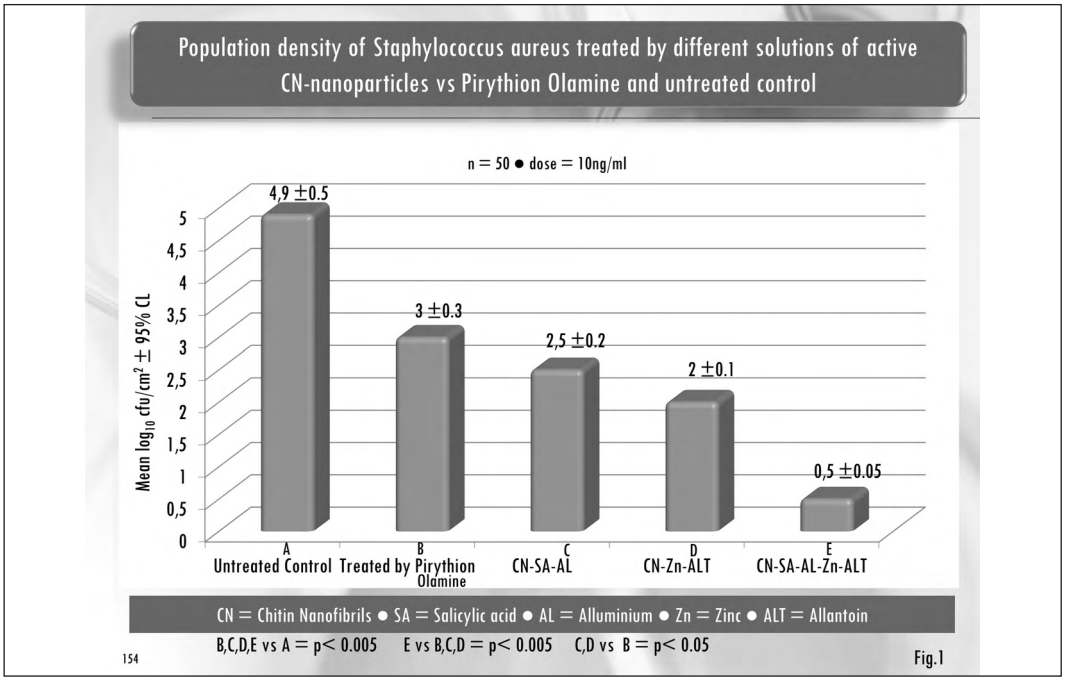

Fig. 1

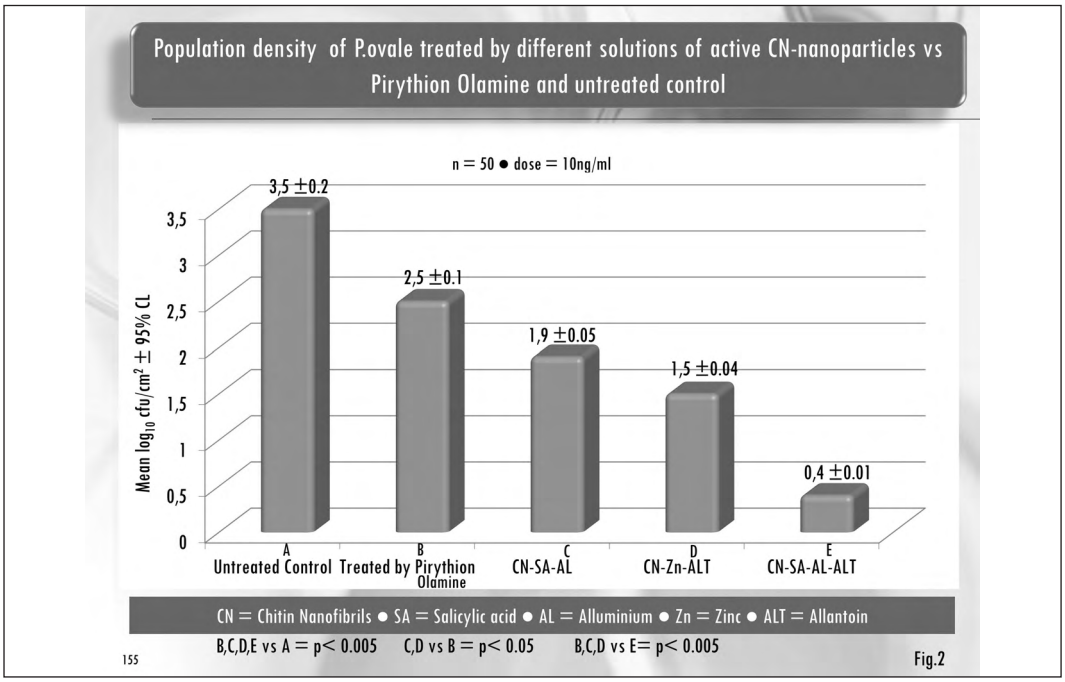

Fig. 2

Keratinocytes cultures co-incubated with microbial fraction

Human keratinocytes, isolated from patients' foreskin and cultured for 3 passages in keratinocyte serum-free medium at 37 °C in 5%(v/v) CO_2 in air using standard procedures (Life Technologies), were seeded in 96-flat-well plates at 1. 5 x10^4 well in keratinocytes serum-free medium. Following overnight incubation at 37 °C in 5% CO_2 in air, the culture medium was aspirated and replaced with 200 microliter of medium plus microbial fraction. Final concentrations of microbial cells were 6x10^5/ well and final protein concentrations of culture supernatants and cellular fractionates were 1μg/ml. Appropriate microbial growth medium controls were included and all tests added with different concentrations of the different nano-particles of were performed in triplicate, while viability of keratinocytes was determined at 0, 24 and 72 h by MTT cleavage assay, previously used from our group (10). The absorbance at 570 nm were determined by a colorimeter (MR 7000 plate reader- Dynatech, UK) (Data not reported).

Anti-inflammatory activity: ex vivo test

IL-1α , IL-8 and TNF-α Determination

The anti inflammatory activity was controlled on keratinocytes' culture supplemented with 5 ng/ml of epidermal growth factor at 37 °C in 5% (v/v) in air using standard procedures.
Keratinocyte supernatants were assayed for IL-1 α by ELISA (Amersham International, UK) and expressed in units of specific activity (pg IL-1 α per optical density at 570 nm). The reduction of UV-induced TNF-α and IL-8 on release kerati-

nocyte cultures was expressed in percent versus untreated controls and on keratinocytes pre-treated with 10ng of the different solutions of our in study nanoparticles, compared with hydrocortisone solution at 0.5% concentration. All samples were controlled by the ELISA luminescence method. To determine the UV-induced production of IL-8 and TNF-α, keratynocites where irradiated with 2 J/cm^2 of UVA and 0. 2 J/cm^2 of UVB.

The results obtained by the keratinocyte cultures treated by different block co-polymeric nanoparticles compared with untreated and hydrocortisone treated cultures, are reported in figures 3-5.

Anti dandruff scales count

The number of dandruff scales was tested by the scrub technique already used from our research group in previous studies (5, 26). This method is based on the use of a hemocytometer to count the number of desquamating scales taken from a 1 cm^2 scalp areas for a given time. Results are reported on Tab I.

Biophysical non-invasive evaluations

Total lipids content and Free fatty acids/triglycerides ratio were controlled by the use of the 3C System (Dermotech, Rome, Italy), according with our previous studies (27, 29).

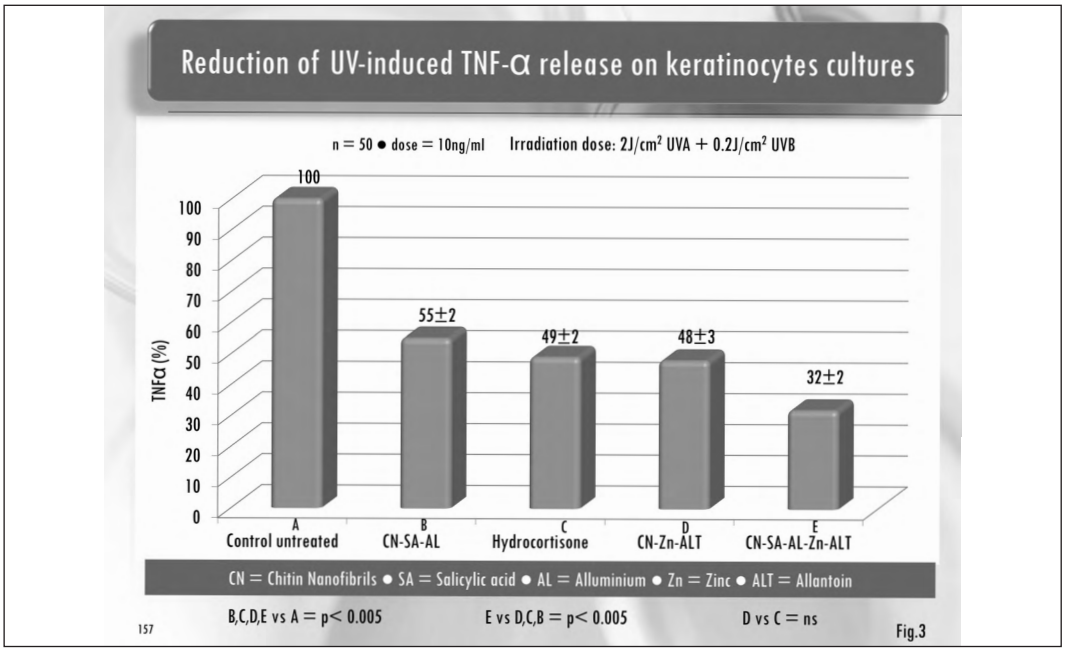

Fig. 3

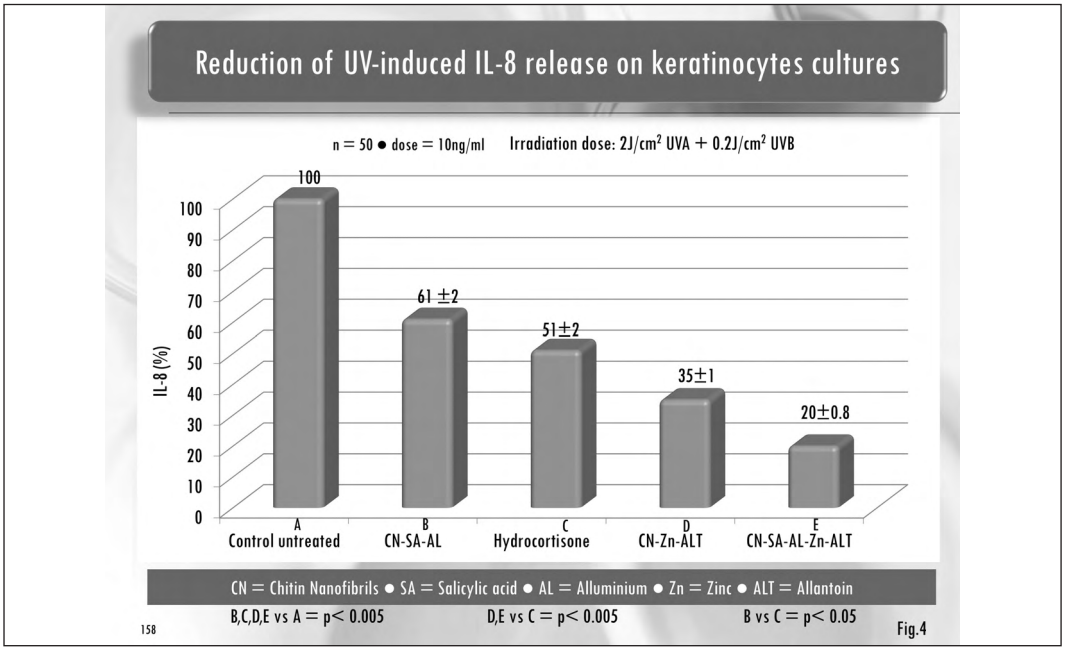

Fig. 4

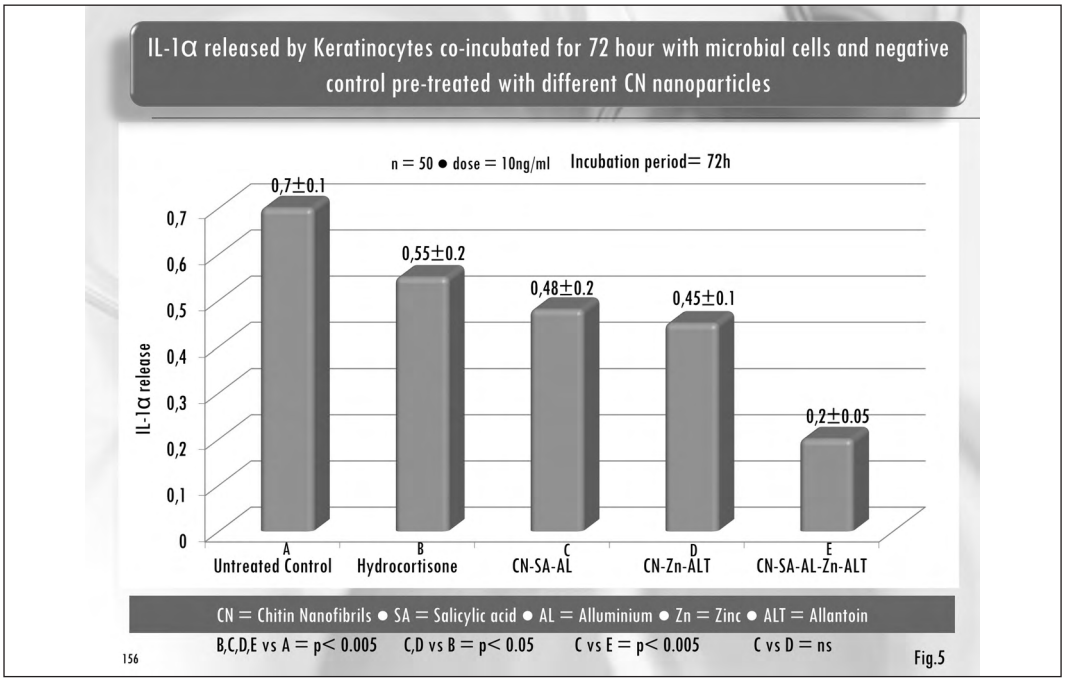

Fig. 5

Dandruff scales on the scalp of subjects affected by seborrheic dermatitis treated by CN-Zn-Al-SA lotion and Zn-shampoo vs traditional and hydrocortisone topical treatment

RH = 50% ● n = 60 ● 12 weeks of treatment with other 4 weeks of regression period

GROUPS	SUBJECT	WEEKS					TREATMENT
		0	4	8	12	16	
D	15	1,458,912 ± 2.87 x 10^5	745,812 ± 3x 10^4	722,456 ± 2x 10^4	711,465 ± 2x 10^4	700,832 ± 3x 10^4	CN-Zn-Al-SA lotion + Zn-shampoo
C	15	1,502,776 ± 1.77x10^4	867,514 ± 5x 10^4	851,892 ± 4x 10^4	794,631 ± 2x 10^3	746,444 ± 2x 10^4	Hydrocortisone Lotion (0.5%) - Comercial antidandruff shampoo
B	15	1,479,471 ± 2x 10^5	1,276,841 ± 4x 10^5	1,111,943 ± 5x 10^3	1,018,632 ± 3x 10^4	1,388,672 ±5x10^4	Watery lotion (placebo) commercial antidandruff shampoo
A	15	1,486,325 ± 3 x 10^5	1,502,113 ± 2x 10^5	1,494,660 ± 4x 10^4	1,498,476 ± 2,57x 10^4	1,587,028 ±5x10^4	Watery lotion (placebo) commercial shampoo

CN = Chitin Nanofibrils ● Zn = Zinc ● Al = Alluminium ● SA = Salicylic Acid

162

Tab. I

Skin surface lipids

Skin lipid level determination was based on photometric measurements of light transmission through skin surface imprints obtained applying to the designed area a frosted plastic foil. It allows adherence of skin lipids in a 1 cm^2 area. The obtained readings, automatically converted in $\mu g/cm^2$, are reported in Fig. 6.

Free fatty acids/triglycerides ratio

Frosted plastic foils were applied on 4 different areas of scalp with gentle pressure by 20 strokes of a gloved finger and carefully removed. Scalp lipids, successively extracted using chloroform/methanol (2:1) for 2 h at room temperature and dried under nitrogen, were separated by silica gel into their individual lipid classes. The isolated free fatty acids and triglycerides were quantified to determine its ratio, reported on Fig. 7.

Anti-oxidant properties Ex vivo test

The antioxidant activity of the in study lotion was evaluated *ex vivo* by measuring the effect on ROS production compared with a Vitamin C solution (10 ng/ml), known for its antioxidant efficacy. The antioxidant activity was tested on neutrophils derived from human blood samples of in study subjects, controlled at day 60th of treatment. The cellular suspension of neutrophils was isolated and incubated in wells with a dose of 10 ng/ml vit C or the active block co-polymeric nanoparticles of the in study solution, at the

same concentration, for 10 min at 37 °C.
The active compounds were composed of mixtures of CN-SA-Al; CN-Zn-Allantoin and CN-Zn-Al-SA-Allantoin block-polymers in water suspension. After incubation, the neutrophils were activated by phorbol myristic acetate (PMA) to stimulate the ROS production measuring soon after the chemiluminescence by a luminometer. This technique enables the detection and quantification of oxidative reaction by the use of lucigenin as fluorescent reagent.
The obtained results, expressed as percentage of the relative chemiluminescence (CL) compared with the 100% control are reported on Fig. 8.

Statistical analysis

Indipendent two-sample t tests were used to compare treatment groups' demographic variable and outcome measures at baseline and each of the follow-up visits, and the change in measure from baseline to final visit. Categorial measures were compared between groups using X2 or Fisher exact test as appropriate. All results achieving two-tailed p value less than 0. 05 were considered statistically significant. Calculations were performed with SAS software, version 9. 1 (SAS Institute Inc, Cary, USA).

Safety Evaluation

The treatment was well tolerated from all the patient who had no side-effects such as erythema, pruritus or pain during or after the treatment of both Zn shampoo and the in-study lotion. No adverse events including desquamation, swelling, crust formation, post inflammatory hyperpigmentation or scarring were observed during the 6 week study.

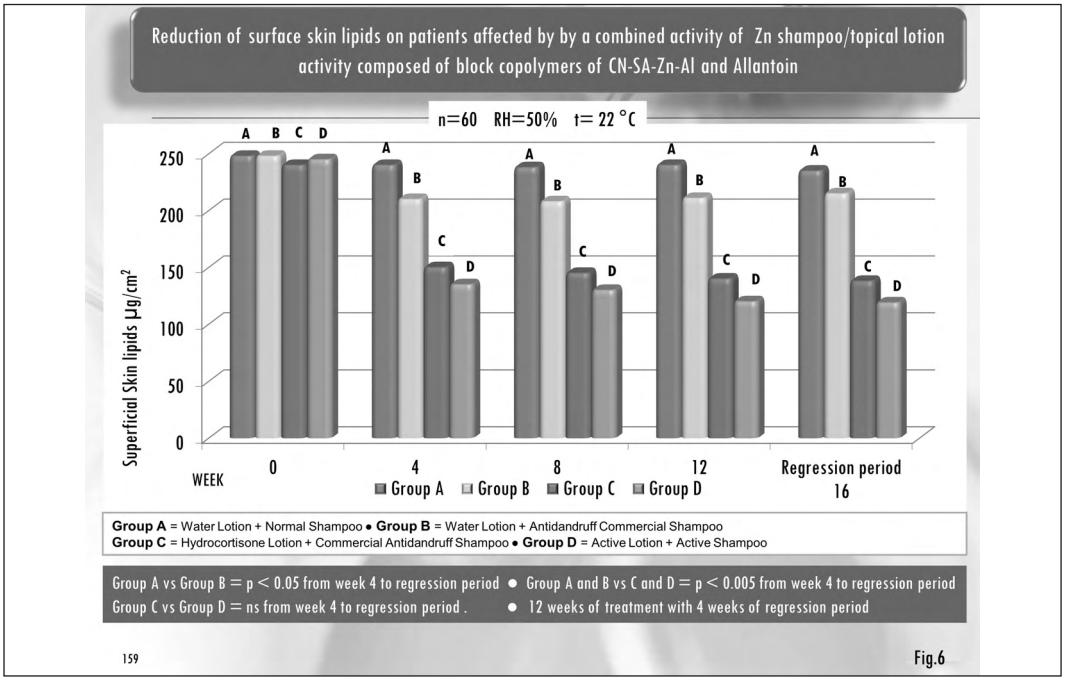

Fig. 6

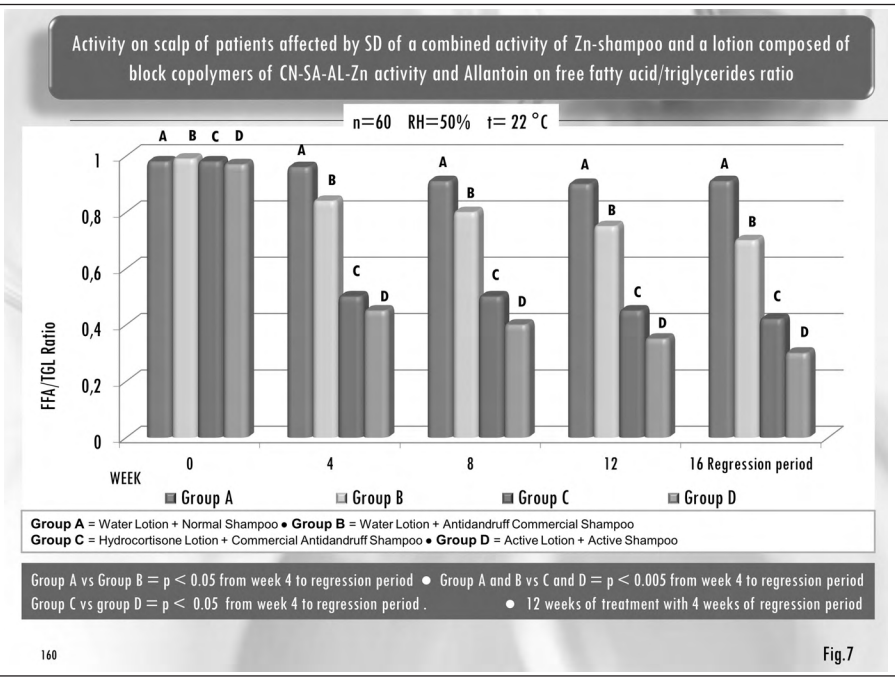

Fig. 7

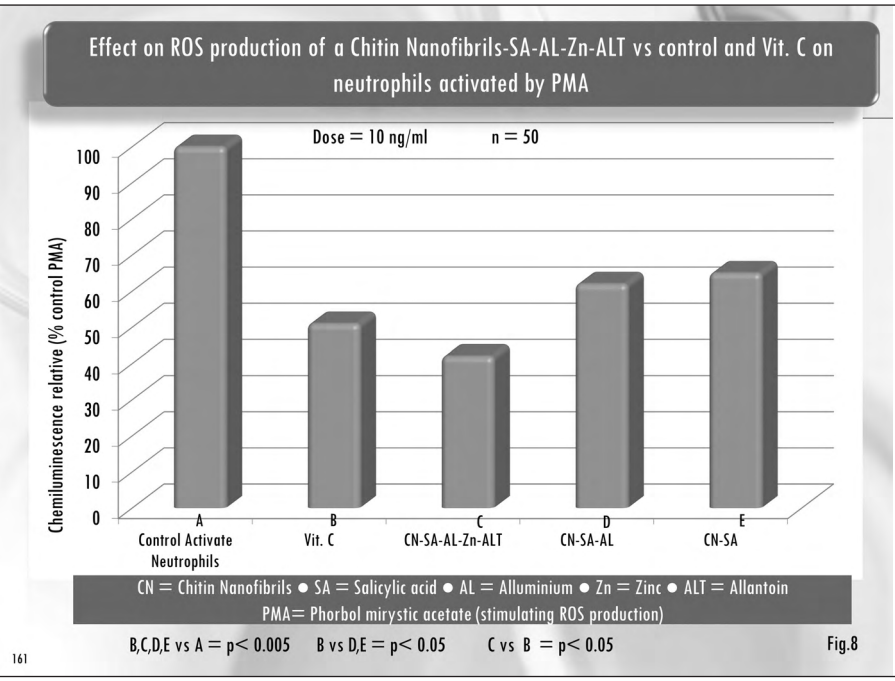

Fig. 8

RESULTS AND DISCUSSION

In the current study we developed a new organic-inorganic formulation based on the use of Chitin Nanofibril block polymers, entrapping 2 % salicylic acid, as non steroidal ant-inflammatory agent, intercalated with Zinc and Al ions together with allantoin as further anti seborrheic/anti-inflammatory agents to be used on patient affected by SD at level of scalp area.

This research was catalyzed by the challenge in obtaining a 2% water solution of salicylic acid, without the use of organic solvents, comparing it to a 0. 5% corticosteroid solution.

The aim was to obtain satisfactory results in decreasing the major SD determinants, such as *P. ovale* and S. aureus, free fatty acids, ROS, and scalp' keratinized scales. The obtained results have been interesting, also because CN-nanoparticles carrier, as previously shown (7, 10), can slowly release the loaded active ingredients in order to maintain concentrations at the desired levels for an extended period of time.

Figures 1 and 2 show that the high density of S. aureus and *P. ovale*, recovered on all the patients affected by SD, decreases on *ex vivo* cultures treated by the use of the different CN-nanoparticles, the more effective being those entrapping all the actives studied (SA-Al-Zn-Allantoin), compared with the untreated control. It is interesting to underline that the activity of CN-SA-Al, CN-Zn-ALT, and CN-SA-Al-Zn-ALT were higher in comparison with a solution containing the same quantity of Pirytion olamine, generally very active especially versus *P. ovale*. Probably the different mixtures of ingredients used have shown a higher effectiveness because of their nanostructured dimensions and the probable synergistic activity they have because of the CN structure, capable to entrap them as a spider net.

The contemporary anti-inflammatory activity of our nanoparticles was confirmed from the obtained results on keratinocytes cultures, verified by the control of TNF-α, IL-8, IL-1α reported on Fig 5. It is known as microbial cells stimulate the production of biochemical signals, as cellular defence to the microbial aggression.

Keratinocytes, incubated for 72 h with microbial cells, have shown in fact an increased release of IL-1α which was drastically reduced when pretreated by the in study nanoparticles. This cascade of anti inflammatory compounds notably increases especially when keratinocytes are under the influence of UV irradiation.

Thus to an increased production of both TNF-α (Fig.3) and IL-8 (Fig.4), obtained from keratinocytes UV-exposed, corresponded an evident decreased release of the same cytokines shown on the cultures pre-treated by the in study nanoparticles, as a further demonstration of the anti-inflammatory activity of these block co-polymers.

It seems interesting to underline that the release of these anti-inflammatory signal-compounds was tangentially lower in comparison to the release obtained by the use of the hydrocortisone solution. This means that the in study composition of nanoparticles has not only an interesting anti microbial/antimicotic and an antidandruff activity (Figg.1,2) (Tab I), but has also revealed an optimum anti inflammatory effectiveness, as shown *in vitro* on figures 4 and 5.

These data are justified from the slow down of ROS, reported on Fig. 8.

All the tested nanoparticles in fact, showing an antioxidant activity, may be considered specific ingredients active in lowering the ROS presence in the interior cell environment. Fig. 8 shows that all the CN-nanoparticles, entrapping the active ingredients used (SA-Al-Zn-ALT), are more active as antioxidant compounds in lowering the endogenous ROS release, than vitamin C. In any way it has been shown from other studies and by different parameters that the CN-nanoparticles synergize the activity of many active ingredients resulting more effective when

entrapped into this natural nanostructure (31-33).

The *in vivo* studies have confirmed all the *in vitro* results obtained by the keratinocytes cultures, regarding also the microbial density of S. aureus and *P. ovale*.

Moreover, by the use of our final formulation based on CN-nanoparticles entrapping SA-Zn-Al-ALT, it was possible to obtain a drastic reduction of superficial lipids recovered on the SD-affected patients during all the treatment period, as shown on Fig. 6, as well as a reduction of the free fatty acid/triglycerides ratio, reported on Fig 7. It is interesting to observe how the levels of free fatty acids, released on the SD-scalp area considered as the main cause of the local irritation, decrease continually and regularly on the patients treated by the in study lotion, also during the 30-days regression period following the treatment end. In conclusion, SA and all the other active ingredients entrapped into the CN-nanoparticles have been elicited significantly at skin level. Thus they had the possibility to improve the antimicrobial and anti-micotic effect of the in study lotion together with its anti-inflammatory activity, as determined by the recovered lower colony-forming units of micro-organisms, accompanied by the corresponding reduction of the surrogate inflammatory indicators IL-1α, IL-8, and TNF-α. Moreover, the *in vitro* results confirmed by the *in vivo* data, have highlighted the effectiveness of the CN-nanoparticles used on SD-affected patients. It seems interesting to underline how the activity and efficacy demonstrated from the super saturated water solution of salicylic acid encapsulated into the chitin nanoparticles seems to possess a higher topical effectiveness than the 0.5% corticosteroid solution used. Probably the effectiveness of SA and the other active ingredients used has due also to the protective and synergistic activity shown by the use of CN carrier. By this new natural carrier it was possible, in fact, to release all the active ingredients at the more effective concentration, at the designed period of time. Thus, CN-nanoparticles seem to be excellent candidates as delivery carrier for cosmetic and drug due not only to their effectiveness, but also to the safety and good biocompatibility and low toxicity they possess, being of natural, origin obtained from by-products and easily hydrolyzed by human and the environment enzymes. In conclusion, according to the last EU guardlines (34) an higher use as active carrier of CN and other natural ingredients, obtained from plant and fishery's biomass, seems to represent the right way for realizing a planty *green economy* (35) that, based on more sustainable industrial processes with a lower consume of energy and water, could safeguard the natural sources and the biodiversity of our planet (36).

This is the goal of our research group.

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Biological Application of Chitosans

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Key words: *Chitosan; Fat binding; Antimicrobial activity; Pharmaceutical technology; Wound healing;*

Summary

Chitosan is a natural cationic polymer used for many medical and non medical applications. It is deriving mainly by the crustacean shells and fungal micelia following deproteinization and is considered as a non toxic and biodegradable polymer. Chitosan carries abundant amino groups with positive charges reacting with negatively charges substances. The most common uses will be presented, focusing on the main characteristics of this fiber.

The activity as food supplement/dietetic/fat binder, its antimicrobial potential, the orthopedic application, the use in the pharmaceutical technology/drug delivery systems, and finally the use as wound and burns healing will be discussed.

Riassunto

Il chitosano è un polimero cationico utilizzato per molteplici applicazioni mediche e non. Viene prodotto industrialmente e principalmente dai residui della lavorazione dei crostacei e, liberato sia dai sali minerali che dalla componente proteica, è considerato un polimero non tossico completamente biodegradabile. Nella struttura del chitosano sono presenti numerosi gruppi amminici caratterizzati dalla presenza di cariche positive utilizzate per complessarlo con altri polimeri a carica negativa.

Nel lavoro verranno posti in evidenza gli usi principali di questo polimero naturale evidenziandone anche le caratteristiche più salienti.

Verranno prese in considerazione le attività del chitosano utilizzato come supplemento alimentare oltre che come potenziale antibatterico e veicolo ideale per applicazioni ortopediche e farmaceutiche quali, ad esempio, il suo uso per la cicatrizzazione di ferite ed ustioni.

INTRODUCTION

Chitosan is a natural nontoxic biopolymer produced by the deacetylation of chitin that after cellulose is the second most abundant polymer found in nature.

Chitin is an insoluble linear polysaccharide composed by (1→4) linked 2-acetamido-2-deoxy-β-d-glucopyranosil units and occurs in three forms with different orientation (α-, β-, and γ-chitin). The α- chitin is the most stable form and is prevalent in crustaceans and insects, whereas the β-chitin is less common and is present in pens of mollusks, and γ-chitin can be found mainly in cocoons and insects.

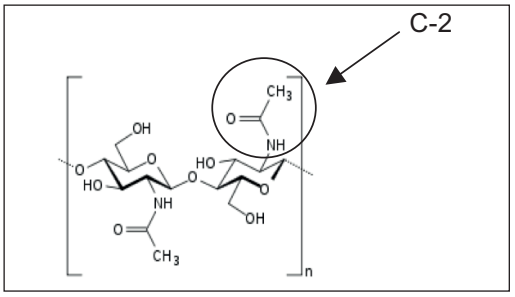

Fig. 1 *Chitin.*

Structure of chitin is slightly related to that of cellulose where the hydroxyl (-OH) group in the C-2 position is replaced by an acetamido [-$NHCOCH_3$] group. His commercial production is usually associated to sea food industries, such as shrimp canning, following the removal of proteins.

Deproteinization is made by hot basic solution (usually sodium or potassium hydroxides 40-45%, 120 C°, for 1-3h) and calcium carbonate. The production of chitin in different part of the world is extremely abundant since it exceeds 10^{10} tons.

The first observation that certain substances (chitin) found in mushrooms did not dissolve in sulfuric acid was reported in 1811 by Henri Braconnot, a French chemist and pharmacist (1).

Later in 1859 Rouget (2) discovered that boiling chitin in potassium hydroxide rendered the material soluble in organic acids, and later in 1894 Hoppe-Seyler named it chitosan (3), despite its structure was defined only around 1950.

Chitosan [poly-(β1/4)2-amino-2-deoxy-D-glucopyranose] consists of a family of highly basic polymers primarily produced by deacetylation of chitin and characterized by different reactive groups: the amino group and hydroxyl groups at C2, C3, and C6 positions.

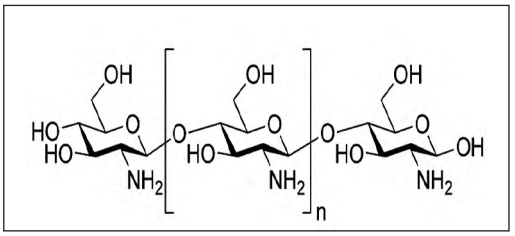

Fig. 1 *Chitosan.*

In essence chitosan cannot be considered as a single entity.

The polymers are usually characterized for deacetylation 40-98 % -usually 75-95 %- considering that those with < 20 % deacetylation are still considered chitin and the generic term of chitosan can be applied when the extent of deacetylation is > 70 %.

Native chitosan is insoluble in water (PK_a about 6.3), alkaline medium and organic solvents, however water soluble salts can be obtained by neutralization with organic acids.

Upon dissolution chitosan forms viscous solutions within the range of 10-100 mPa.s. The molecular weight of raw chitosan preparations is influenced by the deacetylation process, and can be usually within the range of 50 to 2000 kDa. However, oligosaccharides with MW < 5 KDa are also available.

The reactivity, solubility and biodegradation depend basically by the amount of protonated group (NH_2), and by the dimension of the polymer, and all these characteristics can be tailored

for the use following different chemico/physical methods of depolymerization.
Chitosan was introduced in the market in the 1990s and has been the subject of much research regarding its potential use (4).
However, the source of chitin and the technology for chitosan degradation can end up with very variable products such that the GMP for their production and batch/batch variations are one of the most important problems.
The safety profile has been evaluated in rats and results showed no toxicity up to 5 % (approximately 3g/kg/day) for 3 months (5). Some authors was describing apparent toxicity at dietary level of 1 % (approximately 653-720 mg/kg/day) using chitosan oligosaccharides. However, it has been suggested that symptoms were topical (erithema, hair loss, swelling of the snout and forelimb) might be due to the chitosan adhering to the skin and four which are soiled with saliva during grooming (6). A further experience carried out by the same authors at the same dosage level was not detecting these symptoms (7).
This is a confirmation that toxicological data belong to bath production differences.
Due to the general safety and variable characteristics chitosan may have many uses as food supplement/dietetic/fat binder, antimicrobial, orthopedic device, pharmaceutical technology/drug delivery matrix, wound and burns healing bandage.
An overall view of the most common applications of chitosan is reported in (8) and some of the most important will be analyzed here with some detail.

Food Supplement/Dietetic/Fat Binder

One of the most debated aspects of chitosan is the use for body weight and cholesterol reduction.
There are trials showing positive (8-14) as well as uncertain or negative results (15-19). These differences can be due to the type of polymer that in most of the trials is not reported, and also to the presence of organic acids in the formulations that may modify the fat binding capacity of chitosan (20, 21).
The conclusion of an extensive analysis for overweight and obesity (22) was that some evidence that chitosan is more effective than placebo in the short treatment of overweight and obesity despite is unlikely to have clinical significance. In terms of cholesterol, a beneficial effect was limited to total cholesterol (23).
In Europe the activity as hypocholesterolemic product has been approved in 2012 by EMEA provided that the daily dosage will be at least 3 g/day.
However, the action of chitosan in experimental animals on the body weight reduction was found to be more consistent than cholesterol reduction, and the mechanism of action seems to be the fat binding allowing the emulsion fat/chitosan to reach the colon where bacteria use fats as a fuel (24). This mechanisms probably belongs to chitosanases present in some colonic bacteria (8) and can explain why chitosan is not causing steathorrhea. A further important aspect was that the emulsion chitosan/fats is easier with oxidized fats (24) being more polar than other fats. This may indicate that chitosan reduces the propagation of oxidation within the gut behaving as a "scavenger" for oxidized fats.
The association of a chitosan (MW 120-145 kD) with organic acids (named polyglicosamine or L112) was found helpful in the treatment of metabolic syndrome (25). The same type of product was added to the preparation of pasta (in the amount of 2 %) and in a controlled trial (26) subjects were fed daily with 80 g of normal pasta or with pasta added with polyglucosamine (controlled for caloric intake and physical activity) for a period of 2 months. The use of pasta added with

polyglucosamine was significantly reducing body weight whereas cholesterol levels were minimally modified.

Antimicrobial Potential

The antimicrobial potential of chitosan has been described by many authors (8,27-35) and two excellent reviews report all the aspects of the antimicrobial potential (8,34). The activity have been found either alone or with chitosan blended with other natural polymer (starch, gelatin, alginates) and may have many applications in food preservation, dentistry, manufacture of wound-dressing and antimicrobial-finished texstiles. The spectrum of antimicrobial activity includes fungi, yeasts and bacteria. The activity was found more evident in Gram-positive than Gram-Negative bacteria and fungi.

In vitro antibacterial activity was described against several oral pathogens such as *Actinobacillus actinomycetem comitans, Streptococcus mutans, Porphyromonas gengivalis* (30,31).

The antimicrobial activity was more evident in products with high degree of deacetylation, probably due to the higher percentage of protonated amine group (32). The addition of metal ions resulted in almost a complete reduction of the antibacterial activity due to the formation of a complex metal/polymer (33).

The mechanism of antibacterial action is not completely defined. It has been proposed that the interaction between positively charged polymer and negatively charged microbial cell membrane leads to a disruption of microbial membrane, causing a leakage of intracellular constituents (8). The antimicrobial activity was found directly proportional to the degree of deacetylation and inversely affected by pH (at pH 7 the polymer loses the antimicrobial activity). A minimum molecular size (> 10 KDa) is necessary to be effective and at lower concentration cause agglutination, whereas at higher concentration it maintains bacteria in suspension.

However, chitosan's activity is mostly growth-inhibitory and resistant subpopulation might emerge as a result of physiological adaptation (35).

These characteristics stimulate research in the field of postharved diseases in fruits that can be determined by many phytopatogens (e.c. *Botrytis cinerea, Fusariun solani, Penicillum*) that may cause decay of fruit (e.c citrus, stawberry, apple) and chitosan was found active both in preharvest and postharvest periods (36).

Orthopedic application

The antimicrobial potential, the biocompatibility and biodegradability make chitosan useful in tissue engineering application (37). It can be fabricated into a range of architectures, from the nanosphere for drug delivery to the porous scaffold that can support bones and cartilage structures. Scaffolds made from chitosan can substitute autologous bone grafts and an extensive literature is available on the matter (38).

It has been shown to stimulate mineral deposition by osteoblasts (39) and the cationic nature allows for the interaction with proteoglycans, activating the repairing process. The biocompatibility, the slow biodegradation and the antibacterial activity make chitosan ideal for bone and cartilage regeneration (40).

Pharmaceutical technology/drug delivery systems

Two main aspects can be analyzed concerning the activity as matrix for control delivery systems, and the adjuvant/binder activity for vaccines.

Sustained and controlled delivery can be achieved using chitosan based formulations, alone or

in combination with other components (e.c. alginate, lecitin) to form beads, microparticles and nanoparticles, coated tabs, capsules, gels.
The chitosan gel bead received attention for controlled release preparation because of the interaction of its positively charged molecules with the anionic counterion that may allow the formation of gelled spheres (41).
Application have been tested with some drug such as diclofenac (42), thoephylline (43), curcumine (44) to achieve colon specific drug delivery. In the pH range of the colon (5.5-6.0) chitosan can get soluble and releases the drug from the matrix. Protein and peptides also that have to reach the colon to be absorbed could take advantage from the chitosan coating.
Chitosan nanoparticles-based topical delivery systems also have been extensively studied.
The encapsulation of retinol in chitosan to be used for acne and anti-wrinkle treatment can minimize irritation and toxicity (45). Encapsulation of aciclovir into chitosan TPP (tripolyphosphate) enhances drug penetration into the skin (46). Chitosan-lecitin loaded with quercetin allows the flavonoid to reach the epidermis (47).
In addition to topical application into the skin, chitosan-based nanoparticles were capable of delivering drugs into the nasal mucosa as was shown for insulin (48) and ondasentron (49).
Because of its mucoadesive property, chitosan is a promising support for intranasal vaccination (50-51).
Traditionally vaccines are parenterally administered, and the use of needles can cause problems of compliance for needle fobia or contamination, and the administration need trained personnel also. Furthermore, the parenteral administration is characterized by short half-life that can limit the efficacy of vaccination. The chitosan matrix prolongs the retention time at the mucosal areas (52) and reduces the degradation of vaccine by proteases.
The intranasal vaccination can be a solution for these limitations. In this case chitosan may behave also ad adjuvant since it may enhance both umoral and cell mediated immune response (53).

Wound and burns healing

One of the most prominent commercial application of chitosan is the use as hemostatic and wound healing.
The hemostatic mechanism is independent of the coagulation cascade. It appears that the interaction with the cell membrane of erythocytes allows clot formation in the absence of coagulation factors because chitosan matrix tends to attract circulating plasma proteins and platelet adhesion allowing the thrombus formation (54).
The chitosan bandages were approved by FDA and are used for emostasis in the emergency (55) and military setting. In addition it maintains a sterile wound exudate and promotes granulation (56). It can be also a matrix to deliver antibiotics in a view that compromised wound sites contain avascular zone that can prevent the delivery of systemic antibiotics to the infected tissue (57).
Extensive reviews on these issues are available (57,58).

Allergy

The derivation of most chitosans from crustaceans can be a problem for allergic subjects. According to some authors (59), chitosans of all grades once purified should not considered as "crustacean derivatives" because the isolation procedures have removed proteins (in particular the allergenic tropomiosin), fats and other contaminants. This can be true, provided that producers will release the product in terms of GMP. To minimize the production cost and use every kind of chitin most of the chitosan sold around the world is produced without GMP.

CONCLUSIONS

Chitosan is an example of a biological polymer that allows a variety of applications.
The biocompatibility, the physical stability and processability are the ground for these proteiform characteristics. However, due to the variability of MW and charge density that are characterizing different products with specific activities, we may not consider this polymer as a unique entity, either as single compound or in association with other components.
Very frequently the source of the product and the chemico/physical properties are not reported, and this represents an extremely important limitation. The lack of specifications generates confusion and make hard to compare data on the activity. The only shield against "trivial products" is the reliability of the producers, particularly in case of the treatment of overweight/obesity and cholesterol reduction, but also in case of all the other applications.
The GMP for chitosan production will be the only way to solve these problems.

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Biological Activity of Less Known Minor Components of Extra Virgin Olive Oil

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Summary

Extra virgin olive oil is composed of 70-80% oleic acid (18:1 ω-9), 6-8% linoleic acid (18:2 ω-6), 0.5-2% α-linolenic (18:3 ω-3), 8-12% palmitic acid (16:0), 0.5-2.5% palmitoleic acid (16:1 ω-9), and 2-4% stearic acid (18:0). Also present, on an average of 2%, are some minor components made up of α-tocopherol, carotenoids, polyphenols, phospholipids, phytosterols, triterpenic hydrocarbons (squalene), chlorophyll and aromatic composts.

Saturated fatty acid content is modest, polyunsaturates are present in sufficient quantity, with an optimum ω-6/ω-3 ratio (medially 8:1), while monounsaturates predominate, this being a positive health factor. It's balanced acidic makeup and the presence of these minor components of high biologic activity (principally the antioxidant properties of α-tocopherol, polyphenols and carotenoids), makes extra virgin olive oil extremely protective for health.

Among the minor components, besides those with antioxidant properties, there are lesser known ones with biologic value that should not be underestimated. These are the phytosterols and squalene, which though present only in modest amounts act synergistically with the other components, increasing their protective action on health.

Riassunto

L'olio di oliva extravergine è composto per il 70-80% da acido oleico (18:1 ω-9), per il 6-8% da acido linoleico (18:2 ω-6), per lo 0,5-2% da acido α-linolenico (18:3 ω-3), per l'8-12% da acido palmitico (16:0), per lo 0,5-2,5% da acido palmitoleico (16:1 ω-9) e per il 2-4% da acido stearico (18:0). Sono presenti inoltre, in una percentuale media del 2%, alcuni componenti minori costituiti da α-

tocoferolo, carotenoidi, polifenoli, fosfolipidi, fitosteroli, idrocarburi triterpenici (squalene), clorofilla e composti aromatici. Il contenuto in acidi grassi saturi è modesto, i polinsaturi sono presenti in quantità adeguate, con un rapporto ω-6/ω-3 ottimale (mediamente 8:1), mentre prevalgono i monoinsaturi, la cui ricchezza costituisce un fatto positivo per la salute dell'organismo. La sua composizione acidica equilibrata e la presenza di componenti minori dotati di marcate attività biologiche (tra cui principalmente l'attività antiossidante svolta dall'α-tocoferolo, dai polifenoli e dai carotenoidi), conferiscono all'olio extravergine d'oliva un notevole valore protettivo per l'organismo.

Tra i componenti minori, accanto a quelli citati dotati di attività antiossidante, non va sottovalutata la presenza ed il valore biologico di alcuni componenti minori meno conosciuti, quali i fitosteroli e lo squalene, che, anche se presenti in modesta quantità, agiscono potenziandosi sinergicamente con gli altri componenti, contribuendo ad incrementare la loro attività protettiva nei riguardi della salute.

INTRODUCTION

The European Union regulation n.2568/91 defines "virgin" to be oil obtained from mechanical pressing of olives just harvested. More precisely "extra virgin" defines oils whose acidity (expressed as oleic acid) is inferior to 0.8%, and "virgin" if acidity is less than 0.2%. The simple term "olive oil" refers to a refined oil (refinement is necessary because of high acidity e/o impurities present), to which 5-10% virgin oil is added, and with resulting acidity inferior to 1%. The term "olive sansa oil" refers to oil produced from the residue that remains after pressing the olives, named "sansa", that is refined and mixed with a certain quantity of virgin oil and has an acidity inferior to 1%.

Olive oil contains a high percentage of monounsaturated oleic acid (18:1 ω-9), a modest amount of saturated fatty acids and an adequate amount of polyunsaturated fatty acids with a balanced ratio between linoleic acid (18:2 ω-6) and α-linolenic acid (18: ω-3) that averages 8:1 (tab. 1).

Extra virgin olive oil, besides a balanced amount of fatty acids, has a notable content of minor components (1.5-2%) of high biologic value, like Vitamin E, polyphenols, carotenoids, phospholipid, phytosterols, squalene, chlorophyll and numerous aromatic composts.

The simple term "olive oil" designates an acidic composition analogous to "virgin oil", but its content of minor components is notably reduced. It's understandable that "olive oil" and "olive sansa and olive oil" possess an inferior biologic value compared to extra virgin olive oil.

In some nations outside the European Union, they sell an oil defined "light olive oil" usually composed of refined olive oil with a small quantity of virgin oil and an unspecified amount of seed oil (sunflower oil, soy oil, and rapeseed oil). Obviously this oil has low biologic value.

The European commission establishes precise rules to verify the quality and purity of its products. The European Union's Competent Authorities exert rigorous controls which are continuously updated. The entire production of olive oil is highly controlled under the *European Regulation n. 1019/20002 that prohibits the sale of olive oil* that is not officially authorized.

DIETARY LIPIDS

Dietary lipids are essentially triglycerides, constituted by a glycerol molecule bonded to three molecules of fatty acids which can be of different types (saturated, monounsaturated, polyunsaturated ω-6 and polyunsaturated ω-3). Besides triglycerides there are some minor components, as liposoluble vitamins, sterols, carotenoids, polyphenols, phospholipids, aromatic composts and triterpene hydrocarbons.

TABLE I

Percentages of fatty acids contained in olive oil.

Palmitic Acid	(16:0)	7.5 -20
Palmitoleic Acid	(16:1 ω-7)	0.3-5.5
Stearic Acid	(18:0)	0.5 – 5.0
Oleic Acid	(18:1 ω-9)	55.0 – 83.0
Linoleic Acid	(18:2 ω-6)	3.5 – 21.0
α-linolenic Acid	(18:3 ω-3)	0.3 – 1.5

Maximum limits fixed by the International Oleic Council - Madrid

The composition in fatty acids and the presence of the minor components vary quite a bit according to the particular fat under study (terrestrial animal fats, marine animal fats, olive oil, seed oils, tropical plant oils) modifying the palatability and especially the biologic value.
Relating the health virtues of extra virgin olive oil, besides those of it's balanced content of fatty acids (high in monounsaturates and an optimum polyunsaturated ratio ω-6/ω-3), one should not forget those minor components of high biologic activity that offer protection for skin, aging and chronic degenerative diseases (tumors and atherosclerosis),not to mention its pleasing gastronomic qualities.
Especially acclaimed are those minor components (α-tocopherol, carotenoids and above all polyphenols) that intervene against peroxidative risk caused by oxygen free radicals, to whose damaging effects we are constantly exposed. However, it should not be forgotten that in this oil there are other components (phytosterols and squalene) which, though present in small quantities, offer interesting protective biologic activity.

Sterols Present in Dietary Lipids

Among the non saponifiable fraction of fats, sterols are biologically important, especially cholesterol in animal and phytosterols in plant foods. Cholesterol is present in many animal foods as eggs, butter, cheese, cream, and most meats. Plants contain no cholesterol but phytosterols which represent important structural and functional components of plant membranes and which have a similar structure to cholesterol. Phytosterols (and phytostanols, their saturated counterparts) are present in virgin olive oil in a percentage of 125-160mg., represented by β-sitosterol (prevently), by stigmasterol, by campisterol, by fucosterol and by Δ-5-avenasterol, all possessing interesting protective properties.
The only difference between cholesterol and β-sitosterol is in the length of the lateral chain (C-17) which in β-sitosterol consists of 10 and not 8 carbon atoms and in C-24 there is an ethylic group.

Antiatherogenic activity of phytosterols

As well known, high cholesterol levels in humans play a relevant role in cardiovascular disease and thus its reduction is highly recommended through dietary corrections, such as less intake of fats of terrestrial animal origin, which besides their high cholesterol content, intervene by inhibiting LDL membrane receptors (due to saturated fatty acids that block LDL entry into the cell).Thus to avoid high cholesterolemia, the use of vegetable oils are recommended, not only for their polyunsaturated acid content, but also for their phytosterol content which reduces intestinal absorption of cholesterol.
Vegetable sterols are structurally similar to cholesterol, and its this chemical affinity that allows vegetables to compete with animal sterols at the intestinal level limiting their absorption, by impeding their esterification speed in the intestinal mucosae (1) determining consequently a reduction of their plasma level (2).
Cholesterol present in plasma is however only in part of dietary origin (c.25%), in fact total cholesterol is mainly of endogenous origin, bio-synthesized by the body (c.75%) particularly by the liver because the organism tends to maintain plasma levels constant, since cholesterol is indispensable for the biologic membrane structure and for the synthesis of steroid hormones (sexual and cortico-adrenal). In fact mammals have a bio-feedback mechanism that compensates a reduced exogenous intake with an increase of endogenous production, and vice-versa. This equilibrium can however malfunction with a pathologic increase of plasma levels, due to an excessive intake and/or for modifications of

LDL cellular receptors (acquired or congenital). In any case this pathologic increase must be fought through a correct diet, and, if necessary, also with proper medication.

As for plasma cholesterol levels, the exogenous aspect of nutrition is correlated not only to cholesterol and high saturated fat content in foods, but also to intestinal absorption which can be inhibited by vegetable sterol competition. In fact scientific clinical and experimental studies (3, 4) have shown how phytosterol consumption leads to an 8-13% reduction of plasma LDL cholesterol levels with a consequent reduction of 25% for vascular risk.

TABLE II

Minor components present in extravirgin olive oil.

Tocopherols
Carotenoids
Polyphenols
Phytosterols
Triterpenic Hydrocarbon
Chlorophyll
Aromatic Composts

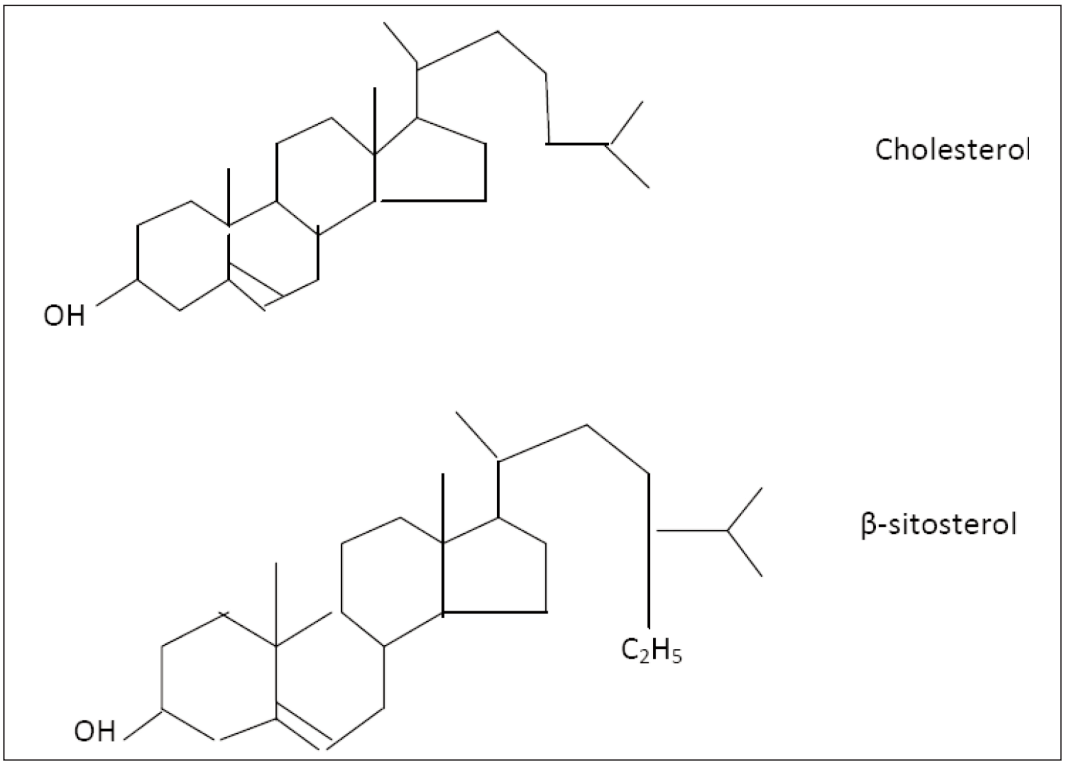

Fig. 1 *Chitosan.*

MEANS OF ACTION

The mechanism by which phytosterols lower cholesterol levels is not however totally clarified, though highly accredited research confirms the competitive interference at the intestinal level. However it has been seen, especially regarding β-sitosterol, that there is a capacity to increase bile salt excretion (5) and to separate cholesterol from the micelle that form with bile salts, thus increasing its elimination in the feces (6). Therefore consumption of phytosterols should be regarded as a positive factor that limits intestinal cholesterol absorption, reducing its plasma levels, even though the bio-feedback mechanism does not seem to entirely compensate its reduced absorption (7, 8).

Vegetable foods with the highest phytosterol content are vegetable oils, followed by nuts and dried fruits, cereals and legumes, but they are present even in olives (green or black), broccoli and cauliflower. The richest vegetable oils are wheat germ oil and corn oil, but also olive oil has content, even if moderate.

Given cholesterol lowering phytosterol properties, high doses (up to 4gm. daily) have been recommended, even by enriching normal foods like yogurt, or dietetic products. However recent data indicate that even lesser quantities such as 1gm daily, which is the amount usually consumed in the Italian diet (where extra virgin olive oil is associated with the above mentioned foods), can determine significant beneficial effects (9). These effects can be further improved by increasing the dose to 1, 6 gm daily, which is considered the optimal dose. Ulterior increases do not seem to augment the beneficial effects (10), and as we shall see, can pose problems (11).

On the whole, the phytosterol content of virgin olive oil, even if not that high, has a concomitant positive effect in the prevention of cardiovascular disease by enhancing the beneficial action shown by the other minor components as well as by the oil's balanced acidic composition.

Other properties

Phytosterols, besides their vascular protective action by lowering cholesterol plasma levels, are also active against tumor growth. Though this action is only based on *in vitro* and animal studies, there seems to be promising results for phytosterol anti-neoplastic effects especially as regards the prostate.

Fucosterol and Δ-5-avenasterol have shown *in vitro* anti oxidant activity, due to their resistance to rectification and to high temperatures. Thus protecting olive oil from thermo oxidation during frying, from the point when such protection is no longer available from tocopherols and polyphenols which during frying gradually undergo destruction from the continued high temperatures. (12).

Phytosterols have also been shown to have cosmetic properties, seen in β-sitosterol (the major phytosterol found in olive oil) which inhibits testosterone transformation into diidrotestosterone, thus regulating sebum and reducing oily skin (13).

Virgin olive oil contains an average of 125-160 mg to100 ml of phytosterols, not very much, but as said before, their presence reinforces the protective effects given by the other components of olive oil. However it must be repeated that phytosterols are found in other vegetable foods regularly consumed (legumes, cereals, olives, broccoli, cauliflower and nuts), especially in the Mediterranean Diet in which extra virgin olive oil is of primary importance, and, along with these foods, creates a diet that has no need to add special dietetic supplements.

The amount recommended by experts is 1,5-2gm per day, higher doses do not seem to offer better results (13), in fact, when taken in dietary products enriched pharmacologically, there is risk

of phytosterol peroxidation (11). It should be noted that phytosterols are inactive if taken in pure form and must therefore be made soluble to have biologic action. It is therefore important that their intake be through natural products, active within their natural dietary composition.

In conclusion, the phytosterols present in extra virgin olive oil, even if not in high quantity, offer a direct, moderate therapeutic action, but also reinforce the other constituents of the Mediterranean Diet. This diet is normo-caloric, has a balanced lipid composition, and is rich in vegetables with the presence of minor components having protective biologic action. A diet, which when consumed over time, offers an effective preventive action against chronic, degenerative diseases and the aging process (14).

Triterpenic Hydrocarbons (Squalene)

Squalene (term derived from "squalus" the Latin term for shark, whose liver is particularly rich in the substance) is another less known component of olive oil. It is present in virgin oil (400-450mg/liter) and in lesser amounts unidentified oil. Compared to other dietary vegetable oils, virgin olive oil contains on an average 18 times more squalene.

Squalene is an isoprenoid structurally similar to β-carotene that is found ubiquitously in the body (15, 16, and 17). In plasma and tissues it is present in minimal amounts, being concentrated mostly in the stratum corneum of the skin. It is one of the most important lipids of the skin to which it provides protection. It constitutes 12% of the total lipids of sebum and acts as a biologic sun filter, in particular of oxygen singlets (16). This contributes to reinforce the structural balance of the lipidic film necessary for the barrier function, as well as offering hydration, anti-aging and antitumor activity (18).

In the body's internal tissues squalene is present in very small amounts. Its action in this location is still object of study and discussion, but it seems to have mainly antioxidant and antitumor properties. However squalene is noted for its stimulation of immunity, in fact some pharmaceutical companies put it in their vaccine formulas (19).

Biochemically squalene is a step in the synthesis of cholesterol. The body however does not have a tendency to transform squalene taken orally into cholesterol, unless its intake is massive, equal to 1000 milligrams a day. Taken in lesser amounts, as can be had by taking 50 grams of olive HMg-CoA-reductase, an enzyme that intervenes in the endogenous synthesis of cholesterol (20). At that dose it also has notable anticancerous activity, so much so that some studies have proposed that olive oil's anticancer properties are mainly tied to its squalene content (17, 20, and 21).

TABLE VI

Sebum skin composition.

Free fatty acids	6.3 – 56.0
Squalene	6.5 – 18.0
Other hydrocarbons	0.5 – 10.0
Wax	12.3 – 25.0
Try-glicerides	5.5 – 37.5
Mono and di-glicerides	3.1 – 13.5
Unidentified minor components	5.0 – 12.0

As regards to this, some authors have stated that the well documented fact that southern Italy, Spain and Greece have markedly lower incidences of breast cancer as compared to northern Europe and North America, can be attributed to the oral intake of olive oil for its high monounsaturated fatty acid content, its balanced ratio omega-6/omega-3 and the presence of polyphenols, in particular to lignans. Without excluding or underestimating the action of these components, these authors (21, 22) support the hypothesis that the protective effect could be due also, if not mainly, to squalene present in olive oil.

This hypothesis, based on epidemiologic elements, seems supported also be experimental studies conducted on animals, which demonstrate an anticancer effect also on other organs, as the colon, and in particular on the prostate where (together with β -sitosterol) squalene exercises a protective effect even as regards benign hyperplasia.

It must be added however that squalene molecule has 6 unsaturated bonds, and is therefore susceptible to peroxidative phenomena with formation of mono-hydro-peroxide that could favor the appearance of seborroic dermatitis. To exert the described protective action against oxygen singlets, squalene must be associated simultaneously with the presence of antioxidants such as α-tocopherol, polyphenols and carotenoids, as those contained in extra virgin olive oil.

CONCLUSIONS

In closing this presentation of the lesser known components of extra virgin olive oil, it seems reasonable to specify that phytosterols and squalene, even if present in small amounts, act positively towards increasing its protective action. This seems important for the physiopathology of metabolism in that it has been demonstrated that the protective agents (in particular antioxidants) act synergetically. The phytosterols and squalene together with tocopherol, polyphenols and carotenoids, contribute to increase the biologic protective activity explicated by extra virgin olive oil, which, we reiterate, constitutes a fundamental element of the Mediterranean Diet.

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Handbook of Immunological Properties of Engineered Nanomaterials

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The immuno system functions to defend the host from pathogenic microorganisms, such as bacteria, viruses, fungi, and other invaders. This defensive function is performed by leukocytes and many other specializes cells distributed in different organs, specially in the lymphoid and haemopoietic systems. However, the different cells interact each with other giving an immunologic reply coordinated and direct to eliminate the pathogenic microorganism or to minimize the produced damages. The immune system is highly versatile to recognize and destroy foreign elements, building a multistage response against them. It includes a large variety of cells and many types of soluble elements. Non-specific immune cells, such as macrophages, granulocytes, dendritic, or natural killer cells, together with specific cells (T helper, T regulatory, B lymphocytes, etc) are the most important cellular components. The cooperation between non-specific cells with specific ones (B and T lymphocytes) and the secretion of soluble factors are crucial for a good adaptive immune response. Antigenicity is a subtype of the immunogenicity response characterized by the formation of an antibody specific to the given type of foreign substance (i.e. Nanoparticles and therapeutic proteins), called antigen. Thus, although the main purpose of the immune system is the recognition of pathogens, it is possible to develop immune responses against non-pathogens or foreign elements. This the reason why some people develop quite strong immune responses to certain components. These responses are called *allergy* or *hypersensitivity* reactions, which include exaggerated humoral or cell-mediated responses to non-pathogen components. However, despite many years of intensive studies, it is still not known why a particular person develops an allergy and others do not.

This book, organized by **21 Chapters**, tries to give a reply to this enquire, reporting and discussing the new studies published on the engineered nanomaterials. In recent years, in fact, a large variety of nanostructures have been designed and produced by many research groups and companies. These nanocompounds (NCs), deliberately engineered, biodegradable or non-biodegradable, can enter the organism inadvertently or accidentally by unsuspected routes and, for their characteristics, can have potential undesirable adverse effects.

In this contest, one important issue to be carefully addressed is the potential capacity of NCs to activate the immune system. Nanostructures and Nanoparticles can interact with biological systems, inducing a response to these foreign elements in different ways, including the induction of phagocytosis and activation or inhibition of immune cells. In fact, both immunostimulation and immu-

nosuppression can be beneficial or detrimental depending on the intended use of the engineered nanomaterials, that can also respond by triggering hypersensitivity or allergy reactions. In any way, reviews of available data suggest that engineered nanomaterials are intrinsically no more immunotoxic than traditional drugs currently in use. Additionally, the incorporation of conventional ingredients into nanotechnology-based pharmaceutical or cosmetic platforms helps to decrease immuno toxicity of traditional formulations. However, it is important to underline that each nanoparticle is unique, and a positive experience with one formulation does not guarantee similar success with another. The bio distribution of an active ingredient due to the nanocarrier used is, in fact, an important point to think when evaluating nanoformulations. For this reason is necessary organize why through pharmacokinetic studies assessing the distribution of both the active ingredient(s) and nanocarrier(s) are needed to identify potential changes in the active *ingredient 's* bio-distribution and new sites of undesirable toxicity caused by the change.

Chapter 2 describes the physical and chemical properties, composition, identification, quality, and stability of nanomaterials, giving a comprehensive overview of their critical points prior to their use in immunological tests. The routes of exposure of these materials and the ensuing biological effects may be significantly different because of their size dimension and surface chemistry, specifically engineered for a better bio distribution, localization, and elimination, or to be recognized by the immune system.

Size, in fact, is one of the most critical parameter as it influences the nanoparticle 'accumulation, as well as its surface charge that plays an important role in its biocompatibility and bio-distribution. It is to remember that nanotechnology is "the understanding and control of matter at dimensions between approximately 1 and 100 nanometers" and, at this molecular dimension, the matter possesses completely different properties.

Thus, nanotechnologies could provide new approaches for delivering small molecules, proteins, nucleic acids and drugs, at the optimal dose in a controlled release to specific tissues, cells, and even cellular organelles.

Nanoparticles (NPs) could also re-integrate drugs that previously failed in clinical trials, and displace certain classes of drugs by achieving suitable pharmacokinetic and toxicological properties. This chapter has been focused on understanding the use of instrumentation necessary to define the physical and chemical properties, composition, identification, quality, purity, and stability of the nanomaterials. Characterization in batch mode and through separation have been discussed also, and guidelines and issues associated with size by dynamic light scattering and zeta potential measurements, have been provided. A comprehensive overview of critical points related to nanomaterials engineering, characterization, sterilization and role in their activities and toxicological processing, are reported in **chapters 3, 4,** and **5**.

Nanotherapeutics are complex systems with multiple components, each of which could be susceptible to the damaging effects of the sterilization procedure in a different way. Thus, the necessity to use a suitable battery of tests for understanding the impact of sterilization procedure on the short-term effects and the stability of the product during its shelf-life. The observations by many studies suggest, in fact, that several different tests may need to be employed to identify the most appropriate sterilization method for a specific nanoparticle' formulation. Sterile filtration, for example, appears to have the least impact on formulations in cases where it can be used, while gamma irradiation and ethylene oxide seem to cause the most extensive degradation, which include the forma-

tion of cytotoxic degradants. In addition, the sterilization procedure can modify the size distribution and morphology of the particles, altering, for example, the amount of the ingredient bounding and release, as well as the amount and the type of impurities and the final toxicity of the designed formulation. Hence, "due to the negative consequences of sterilization on Nanoparticles, it is necessary to perform all characterizations after the sterilization procedure, and establish a stability program suitable for the detection of chemical degradants during the shelf-life of the product ". This the conclusions of the **chapter 3**.

Pyrogenicity assessment is another important component of preclinical characterization of engineered nanomaterials. It often coincides with the testing of the material's sterility. Any material, including engineered nanoparticles, may be contained with endotoxin during preparation, unless it is produced under aseptic conditions and with sterile, pyrogenated reagents. In addition, due to the nanoparticles large surface-to-volume ratios, nanomaterial formulations are thought to be at particular risk of endotoxin contamination. High concentrations of endotoxin can induce immuno stimulatory reactions and result in the unfair disqualification of a given formulation from further development. There is, therefore, a need to develope and harmonize standard approaches and algorithms for selecting appropriate methods for a given nanoformulation. **Chapter 4** provides practical suggestions for overcoming these challenges.

Surface characteristics of nanomaterials are well recognized to contribute to their unique properties and applications. These size-dependent properties facilitate rapid, ubiquitous surface adsorption and contamination from many sources: storage containers, nanosynthesis components, by-products, and stabilizers, pollutants, etc. In addition, surface adsorption may alter otherwise benign or innocuous adsorbed materials rendering certain antigenic or inflammatory responses on a nanomaterial surface. These specific surface properties and their modifications over time are, therefore, critical to elucidate the nanomaterial presentation to various immune and inflammatory conditions. Currently detectable cell-based toxicities and immune responses can only be correlated indirectly to Nanomaterials, requiring sensitive and versatile analytical methods. Methods to detect adsorbate classes, understand cell-material interactions, and the ways in which nanomaterials present chemistry to host inflammatory mediators, are reported and discussed in **chapter 5**.

Whenever NPs come into contact with biological fluids, a layer of proteins (*protein corona*) may adsorb onto their surfaces and, utilizing the endocytosis machinery to intrude cells, interact with the biological systems. **Chapter 6** describes the nature and formation of a corona under physiological conditions and the efficiency of its effect on cellular uptake and bio distribution of NPs. All the aspects of protein adsorption to NP surface are also reported and reviewed together with the significant role of this corona for contemporary nanomedical applications.

Understanding the formation and persistence of the protein corona is a formidable task which is of paramount importance not only for the elucidation, interpretation, and assessment of biological effects of unintended exposure to NPs, but also for their intended use in nanomedicine. Protein adsorption onto NP surfaces may, in fact, involve conformational changes of the proteins, leading to a loss of biological function. In any way, the use of NPs in targeted delivery applications in nanomedicine needs a reliable foundation on the molecular scale. Thus the necessary to better understand the real impact of physical and chemical NP characteristics on protein adsorption before using systematically the protein corona.

Engineering and naturally occurring nanomaterials may come in contact with blood through diffe-

rent pathways, either directly or indirectly. Understanding the conditions in which red blood cells (DBCs) and nanomaterials come in contact with each other and their mechanisms of interaction, is critical for safety and the exploitation of the real potential these nanoparticles have in cellular therapies and cosmetic/drug delivery. Thus, special attention must be given to the interactions between NPs and proteins together with the potential changes which may affect their physicochemical properties and toxicity. Moreover, more comparative studies have to be performed *in vivo* using the same NPs at different concentrations. Naturally, nanoparticle interactions with red blood cells are largely determined by their physicochemical properties, which may differ among different classes of engineered nanomaterials. For example hemolysis, caused by silica Nanoparticles, was recently attributed to surface charge, as well as the shape was also named as primary parameter in determining hematocompatibility of gold colloids. Thus, nanotechnology has been shown to benefit the formulation of hydrophobic macromolecules with reduced hemolytic activity by improving solubility and reducing zeta potential through the preparation of polymer-based nanosuspensions. **Chapter 7** provides a comprehensive overview of the current literature on this topic, suggesting solutions to overcome the mechanisms of interactions between DBCs and NPs.

The vascular endothelium(EC) forms the inner lining of the vasculature and is an essential system in multicellular organisms because it delivers nutrients and oxygen, removes CO2, and raw products, facilitating the inter-organ communication. On the other hand, endothelial dysfunctions may lead to life-threatening vascular disorders, including organ damage and/or thrombotic and bleeding pathologies. The oxidative stress, pro-inflammatory activation, induction of apoptosis and/ or inhibition of proliferation, migration, and tube formation are, in fact, the most common adverse effects of nanomaterials on EC. As many engineered nanomaterials, designed for biomedical applications, may reach the vasculature, it is necessary to evaluate them on cultured endothelial cells. This is the topic of **chapter 8** where numerous microscopic methods are reported to study and/or serve the morphological and structural changes employed to control the EC and nanomaterial uptake.

In biomedical applications, in fact, different types of nanomaterials are under development for use in diagnostic biosensors, drug delivery nanosystems, imaging nanoprobes for intravascular use, and other devices that come into contact with blood and vasculature. This is the reason why the interaction of biomaterials with the endothelium has been a focus of extensive research. EC plays a key role in maintaining the anti thrombotic and fibrinolytic potential of the vascular wall, also, participating in the regulation of vascular tone and microvascular perfusion, and controlling micro vascular permeability. Furthermore, healthy ECs have antioxidant and anti-inflammatory properties, eliciting the defense process at the tissue level. In this chapter, on one hand the physiology of endothelial cells are reported together with the possible adverse effects of nanomaterials on ECs; on the other hand the methods that are used for the *in vitro* evaluation of the effects of nanomaterials on ECs are briefly reported and discussed.

The effects of nanomaterials on the plasma coagulation system is the topic of **chapter 9**, while the effects on platelets (PLT) are reported in **chapter 10**. The plasma coagulation system (PCS) consists of plasma proteins and other factors that, together with platelets and vascular endothelial cells, maintain hemocoagulation balance, preventing blood clotting under physiological conditions. Platelets, small anucleated cells, result fundamental in the formation of the primary hemostatic plug, at the site of vascular injury. Thus, PLTs, together with the PCS and vascular endothelial cells, maintain the hemocoagulation balance. Under physiological conditions, these systems prevent blood clot-

ting, while in cases of vascular injury, they facilitate hemostatic to prevent blood loss. As the majority of the engineered nanomaterials that come in contact with the blood are being designed for biomedical applications, they may reach the circulation as result of occupational, environmental, or other routes of exposure. Also the effects of engineered nanomaterials on PLT and PCS are reported and discussed on these two chapters, together with the review of the actual methodologies used to control their activity and effectiveness. In any way, nanoparticle interactions with platelets seem to depend on the nanoparticle composition, size, and charge. Of interest is the data suggesting that nanoparticles activate platelets through uncanonical pathways, e.g., those involving matrix metalloproteinases. Thus, *Complement activation* is the topic of **chapter 11**. This system, made up of a biochemical cascade of about 35 soluble and cell surface proteins, has the function to *complement* cells of the innate and adaptive immunity in protecting the host from invading pathogens. It plays many protective functions including opsonization, chemo taxis, and cell lysis.

Complement proteins are also important for supporting the adaptive immunity through the activation of dendritic cells, T cells, and B cells. To date, the leading causes of death in the industrialized countries include cancer, cardiovascular, neurodegenerative diseases, and diabetes. While new treatments are urgently needed to treat these diseases, nanotechnology may potentially provides new ingredients and nanocarriers that can deliver drugs/cosmetics in a specific and controlled manner, minimizing side effects. But the success of bioengineered nanocarriers for drug/cosmetic delivery purpose requires a deep understanding of their interaction with the complement system. This chapter assesses the most important parameters and procedures adopted *in vitro* and *in vivo* to influence the activation of the complement system, evaluating also the most recent engineering approaches necessary to prevent or reduce the complement activation.

Phagocytosis denotes the uptake of large particles and occurs by close opposition between a segment of plasma membrane and the particle' s surface, excluding most of the surrounding fluid. The enzymes, mainly responsible for the digestion of the ingested material, are the lysosomal acid hydrolases. With regard to the mechanism of immune phagocytosis, it is noteworthy that the initial interaction of immune ligands on a particle's surface with receptors on the membrane of phagocytic leukocytes does not trigger the ingestion of the particle. It merely initiates a process that requires the continuous apposition of receptors and ligands until the particle is fully enclosed within a phagocyte vacuole. Nanoparticle(NP) uptake by the mononuclear phagocytic system (MPS) and the resulting clinical manifestation is the topic of **chapter 12**. It is to underline that many NP agents attach to traditional small molecules and deliver them in a novel way. For this reason, it has been observed a therapeutically greater exposure and efficacy in the body.

The opsonins circulating and tissue phagocytes, in fact, represent a very complex network and the interaction with NPs often depends on the biological atmosphere, as well as on the physicochemical properties of the NP components, that play an important role in their recognition by macrophages. Therefore, size and charge result as very important parameters in determining their interaction with macrophages and the blood. Clearance NPs with smaller sizes (<100) have shown advantageous being removed more slowly from blood, as well as a larger surface area seems to increase the density of recognizable sites on the surface of liposomes. Nevertheless, the data suggested that phagocytoses may depend not only on the particles' charge, but can also be influenced by their surface coating. This is why polymer confirmation has been proven to be important for the protection of NP surface from immune recognition. However, an increase in the particle size regardless of the type of

coating was shown to either shift particle bio distribution from hepatic (for smaller particles) to splenic uptake (for larger particles), or can even completely eliminate protection. Moreover, while most of the NP components are biodegradable, some are not and lead to bioaccumulation in tissues and cytotoxicity.

Thus, *in vitro* and *in vivo* clinical studies are reported and discussed by the use of different NPs, but new researches are needed to create an ideal NP capable to balance efficacy and toxicity in the same time. The success of biomedical application of NPs, in fact, depends on their interactions with the different components of the immune system.

Chapter 14 reviews the observations made during the last decade in the area of nanoparticle effects on bone marrow cells, such as nanoparticle biodistribution in the host including the bone marrow, nanoparticle-assisted imaging of the bone marrow, immunomodulatory effects, toxicity to bone marrow cells, and the use of nanoparticles for the radioprotection of the bone marrow.

For their interesting characteristics NPs have found wide range of applications for imaging of the bone, but also to help in cancer treatment, to distinguish infection from inflammation, and for delivering of numerous drugs to this organ. In terms of their toxicity to the bone marrow, much more work has to be done as nanomaterials have only relatively recently entered the consumer market and healthcare.

In any way, nanoparticles used today seem to be non-toxic, while the metal and metal oxide-based ones, such as TiO2 and ZnO, do present some toxicity concerns. As previously reported, surface charge and chemistry of NPs play a significant role in determining the type of immune response generated, since the chemical nature of the particles strongly influences phagocytosis. For example, positively charged cationic particles, in general, have greater affinity towards the cell surface than negatively charged or neutral particles, probably due to the strong ionic attraction between the positively charged particles and the negatively charged cell surface. Furthermore, cationic nanoparticles have the ability to escape endolysosomal degradation compared to anionic nanoparticles of the same size. Thus, the beneficial ionic attraction between the positively charged nanoparticles and the negatively charged surface of the cells is likely to represent a strong stimulus that initiates binding and subsequent internalization. The shape of the NP also effects it's cellular uptake and distribution. Spherical particles have been shown to be taken up by cells more efficiently than rod-shaped ones. On the other hand non-spherical nanoparticles have been shown to have higher accumulation and retention rates in cancer tissues *in vivo*, compared to shoe ricks nanoparticles. Therefore, NPs has to be engineered, as much more possible selective for the intended targets, by choosing the appropriate ligand. For example, chitin nanofibrils and chitosan have emerged as useful drug delivery matrices because of their polycationic nature, biodegradability, biocompatibility, mucoadhesiveness, and ease with their physicochemical modification. All these topics are reported and reviewed on the **chapter 15**, where the Nanoparticles 1-1000nm in diameter, to be used as vaccine carriers and adjuvants, are discussed for their effects on the immune system, including drainage to the lymph nodes and uptake by the antigen-presenting cells. One of the most successful nano-tools that have emerged in recent years are, however, nano-drug delivery devices. These nano-sized carriers used to load therapeutic agents, such as vaccines and enzymes, aid in overcoming the pharmacological and toxicological hurdles through site-specific and controlled drug delivery. The large surface area of these nano-compounds which include polymeric nanoparticles, micelles, dendrimers, liposomes, solid lipid nanoparticles, proteins, carbohydrates and inorganic ingredients, allows enhanced bioactivity

and efficient surface modification, facilitating their solubility, targeting, circulation, and stability. Moreover, their biocompatibility serves the purpose of conferring protection to the body from the toxic action of the drug payloads on normal tissues, while facilitating the therapeutic action on diseased cells.

The essential characteristics of the more used different polymeric nanocarriers together with their biocompatibility and biodegradability are reported and discussed in **chapter 16**, while the allergenicity potential and the antigenicity of all the nanostructures are reported in **chapters 17** and **18** respectively.

As previously reported, nanostructures can interact with the immune system, inducing a response on these foreign elements in different ways, including the induction of phagocytosis, activation or inhibition of the immune cells, also triggering hypersensitivity or allergy reactions. Although the main purpose of the immune system is the recognition of pathogens, it is possible to develop immune responses against non-pathogens or foreign elements, as these nanostructures. These responses are called *allergy* or *hypersensitivity* to non-pathogen components.

On the other hand, *Antigenicity* has to be considered a subtype of the immunogenicity response, characterized by the formation of an antibody specific to the given type of foreign substance, called an antigen. Due to their small size, nanoparticles are not antigenic themselves, but some of them may act as happens and become antigenic when conjugated to a protein carrier. Conversely, engineered nanomaterials not conjugated to a protein, and those specifically designed to carry therapeutic proteins, were shown to be non-antigenic and, on the contrary, to help the antigenicity of therapeutic proteins attached to them.

Growing day by day the interest to assess the correlation between *in vitro* and *in vivo* studies of the nanoparticle' immunotoxicity, these topics are extensively discussed on **chapters 19** and **20**, where comprehensive protocols are reported. In any way, due to significant and organizational differences between the human system and those of animal species used for testing, traditional toxicology studies conducted in rodents and non-rodents are often not predictive, especially of moderate and functional effects.

Furthermore, there are ethical concerns regarding animal use and a worldwide 3R efforts, where 3R stands for the *replacement* of animals in research with non-animal alternatives, the reduction in the number of animals used in experiments, and the *refinement* of scientific procedures and animal husbandry with the aim of minimizing animal suffering. Thus, the high throughput nature and lower costs of *in vitro* tests on cellular cultures, makes them a very attractive alternative. However, according to the author of the book, four are the main challenges for the *in vitro* testing of nanoparticle immunotoxicity: (1) the selection of an appropriate model, (2) the selection of an appropriate endpoint, (3) the selection of appropriate positive and negative control, (4) nanoparticle interference with *in vitro* assays.

Chapter 21 has been dedicated to the regulatory considerations by the US FDA. Actually these novel nanostructures are subject to the same toxicological rules and regulation concerning drug development that apply to small molecules and therapeutic proteins.

This interesting book, regarding the immunological properties of the engineered nanomaterials, written from expert scientists and in a very clear manner, provides an overview of all the today problems reported on the fascinating topic of the nanostructures used for medical applications. Nanomedicine provides, in fact, an opportunity for improved drug development because clinical phases often cast

light on the side effects, limiting the drug's therapeutic dose.
It is possible to develop a nanocarrier that will accumulate in certain tissues owing to its physico-chemical properties, as well as select a therapeutic load based on the distribution properties of the nanocarriers. The interesting data and objectives reported in all the chapters, can improve and inspire research ideas and topics to expert clinicians, as well as to material and mechanical engineers, biomedical engineers, molecular cell biologists, physiologists and students also of both the chemical and medical community.

Pierfrancesco Morganti
Editor-in-Chief

The Skin Care Ingredient Handbook

by Linda Walker

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According to the author "Creating a cosmetic product is like creating a beautiful cake.
The base of the cake may have *functionality* ingredients like moisturizing fillings and stabilizing fondant layers, as well as emulsifying ingredients like eggs to bind the flour to oils and liquids. Star ingredients like chocolate, marzipan, or fruits must be carefully added to the cake to preserve form and flavour, just like key *actives* in a skin care product.
"To obtain a stable and effective product is the challenge of cosmetic formulator". Of course the cosmetic chemist must expertise a greater level of preparation for developing an effective formulation which requires the establishment of an increased number of project requirements, goals, and criteria for success. It is imperative, in fact, that the final new product's description has to include details of the desired physicochemical attributes, aesthetic properties, functional benefits, cost parameters to respect the consumer expectations. Moreover the packaging accuracy and reproducibility, has to be the results of a well designed component formula of a multifunctional product, to allow it to be conveniently dispensed.
Finally, the functional evaluation of any formulation has to be considered as a continuum, starting with instrumental laboratory evaluations and ending with consumer/market testing.
Results of consumer research must be used, in fact, to actually help shape and optimize the product, not just to elicit approval or rejection of decisions already made. Thus, on one hand the laboratory phase has based on the greater control of environmental conditions, substrate uniformity, applications parameters, test methods, etc, providing the greatest amount of accuracy and reproducibility. On the other hand, consumer testing provides less accuracy and reproducibility, but gives results that are more representative of real life product usage than those gained from instrumental laboratory investigation. In any way any protocol, adopted for the formulation, requires the storage of samples under stress conditions (45 °C, refrigerator and freezer) with carefully documented, periodic observations as well as analytical, microbiological, and functional assays. Remembering that millions of end users with common yet compromised skin conditions (atopic Dermatitis, eczema, psoriasis, etc) will also routinely use the cosmetic products, clinical trials have to be designed to support each specific marketing claims, such as *Clinically Correct Cosmetics*. To day clinical techniques are, in fact, available that will demonstrate how substances as water and petrolatum, once thought to be inactive, alter the structure and function of skin. Thus many scientists agree that there is a need for a term, such as *Cosmeceutical or Clinically Correct Cosmetic*, to describe products that moved beyond the simplistic cosmetic definition for practical purpose, but are more effective than a cosmetic formula-

tion, being not drugs or Medical devices. These so-called Cosmeceuticals or 3C Cosmetics have both cosmetic and therapeutic properties.

This book has been developed by **Part I:** *Fundamentals* organized by **4 Chapters**, focused respectively on *Cellular Functions and Skin Aging; Ingredient Selection; Skin Care Trends,* and *Definition of Ingredients* and *Technologies*.

Moreover it has been enriched by **Part II** reporting a *Glossary of Ingredients* and **Part III** where on *Appendix A* are discussed the FDA Labelling Regulations, and on *Appendix B* The Definition of terms, and on *Appendix C* the INCI Listing of Ingredients, are reported respectively.

Being written for beauticians and for young cosmetic chemists the area dedicated to the skin is well written and easily understandable, reporting the fundamental components of the skin interested and involved in the cosmetic activity. Half of the book has been dedicated to the main ingredients used for formulating the cosmetic products, the fundamental characteristics of which have been reported together with their commune use in skin care. In conclusion The Skin Care Ingredient Handbook may be useful for students in Cosmetic Chemistry who wish to enter in the fascinating technical *art* of formulating innovative Cosmetics, but may be of help for Dermatologists who like to have general ideas on the techniques and ingredients used to formulate and control the skin care products.

P. Morganti
Editor-in-Chief

APPLY TOPICALLY: A Practical Guide to Formulating Topical Applications

By Nava Dayan

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The main objective of Pharmaceuticals and Skin care Development is to create effective products based on the state-of-the-art active ingredients with improved patient compliance and usability. Thus, the vehicle/carrier used to delivery topical active ingredients through the skin layers and mucous membrane, can considerably influence the performance of the *actives'* effectiveness. The carrier can have direct effects on both skin and mucous membrane barrier, as well as it can enhance or retard the delivery of the active agent(s) to the target site of action. In addition it can affect the skin and mucous membranes appearance and the relative sensory properties of the final formulation, often influencing the patient compliance.

Skin and mucous membranes (MB) are, in fact, the first line of the human body' defence and, acting as a biological barrier, offer thermal insulation, prevent water loss, and protect the internal organs from the external environment, foreign toxic substances, and pathogens.

Skin, generally less than 2 mm thick, is composed of several layers, the outermost of which the *stratum corneum* (SC) (10-30um thick) represents the primary barrier. On one hand, SC is composed of corneocytes filled with keratin filaments enclosed by an envelop of cross-linked proteins, and surrounded by a semi continuous matrix of lipids, which create a strong biological barrier. On the other MBs, covered by adhesive gel-like mucus and composed of a densely woven network of natural mucin polymers interspersed with a variety of glycoproteins, create another effective barrier to diffusion across mucosal surfaces. Both the Skin lipids -organized in lamellar fashion and produced/secreted during the maturation of the keratinocytes and mucus, constantly secreted and turned over, represent strong cellular and molecular barriers to penetration of any kind of compounds. Therefore the necessity to overcome these barriers by the use of right vehicles which, able to disturb the lipid structure within the stratum corneum and/or the mucus molecular organization within MB can increase the permeability of the active ingredients through these structures.

The goal of this book, consisting of **VII Sections** and **22 Chapters** is to describe and discuss the key elements necessary to formulate stable and effective cosmetics by different steps.

In **Section I, Chapters 1 to 5**, *The Preliminary Considerations and Selection of Raw Materials* are reported and discussed. Thus the necessity to design and organize a detailed description of the cosmetic to be produced.

First of all concept, goals, and objectives of the project should be stated, reporting the benefits to the

consumer that will be delivered by the product. At the same time, distribution area, potential market, and the related budget have to be determined, describing the desired technologies to use, and defining the desired site of applications and claims. Finally the regulatory rules and the eventual patent restrictions have to be controlled. Once these questions have been considered, a product design flow will begin to form, following the logical tenets of initial formulation and product scale-up.

Emulsions, responsible for delivery the active ingredients into the skin, are the most common forms of vehicles for skin care products. Active ingredients are a mixture of compounds that provide the product with its consistency and effective activity. Recently, the use of nano-emulsions has become a popular approach for formulating more effective and elegant cosmetics. In any way, the emulsions should contain the right concentration of *actives* and bio-based raw materials possibly obtained from renewable resources, having stability features, programmed release kinetics and possibly clinical efficacy, being also skin-friendly, environmentally-friendly and aesthetically pleasing. New innovative cosmetics and services could be brought to the market, for example, by promoting further exploration of marine biodiversity and strengthening marine biotechnology. The unexploited potential of the sea is, in fact, even bigger since more than 90% of marine biodiversity remains unexplored, offering a huge potential for discovery of new species and applications derived from biotechnologies, which is foreseen to generate a 10% annual growth.

A major use in the cosmetic field of raw materials from plant biomass and fishery's by-products should represent a new strategy for an innovative economic growth and development, socially and environmentally sustainable. Transitioning to this *inclusive green economy* is increasingly recognized as an alternative that can deliver low-carbon and climate-resilient development, significantly improving resource efficiency, healthy and more resilient ecosystems, and greater economic opportunities and social justice for disadvantaged groups also. How to formulate skin care products with the right ingredients is the topic reported in section I.

Formulation, Processing and Production Techniques is the topic of **Section II, Chapters 6 to 9**.

Emulsions are complex and versatile systems, which allow the skin care chemist to combine otherwise immiscible ingredients into effective skin care products. This gives the advantage of developing custom-made commercially desirable formulations, designed for various skin types or addressing many skin disorders or conditions. The success of a product depends not only on its effectiveness, but often on its sensorial attributes, in which case the consumer is the best candidate to judge. Thus sensorial and textural properties of the emulsions play a pivotal role in a formulation's acceptance as a final product by the consumer. The Texture Profile Analysis, reported and described in chapter 6 is a promising and novel technique that can help the cosmetic chemist to better verify the final designed formulation. But this formulation has to be stable and effective also, for example, for its moisturizing or healing properties. Moreover it has to be formulated by the use of the right vehicle capable to deliver the active agent(s) to the target site of action in the right time. Finally an effective scale-up to pilot plant manufacturing should occur within good manufacturing practices framework. Scale-up success means designing and implementing an efficient, cost-effective process resulting in a product which consistently meets a comprehensive and appropriate set of pre-determined quality attributes. Working within a structured system of good recordkeeping, documentation, and good manufacturing practices is, in fact, a necessary foundation to successful scale-up. These topics are reported and discussed in chapters 7 to 9.

Section III, Chapters 10 to 13, is dedicated to *Testing and Measurements Methods*.

Carefully constructed and statistically designed experiments, facilitate the analysis of collected data in a logical and expeditious fashion. The cosmetic chemist works with a plethora of cosmetic raw materials which have to be carefully selected. Thus, to obtain a well-designed formulation it is necessary to organize sets of experimental batches to fit an experimental model that can make predictions within the range of parameters established. The rationale is to achieve an optimized product that will be the most cost-effective, while still offering the best overall performance and customer satisfaction. Furthermore, a well-planned statistical approach will provide in-depth knowledge and greater understanding of the product with a minimal amount of experimentation necessary to accelerate the speed-to-market and achieve cost-saving. Naturally a number of prototype-formulations have to be developed and evaluated before the approval of the final ones to be launched. Thus various rheological measurements have to be done to characterize the product, such as flow profiles, crepe/recovery, yield value measurements, viscosity recovery strain sweep and frequency sweep and so on, made by different instruments.

In conclusion a general guide to design and develop a target profile for topical formulations are reported and discussed by these four chapters.

Sensory and Elegancy is the topic of **Section IV, Chapters 14 and 15**. The key to gaining a profitable repeat business in the skin care market is to offer products that should be effective, having also an emotional appeal through their aesthetic appearance and performance. The cosmetic product, in fact, can make a person look better, providing him/her with a sense of wellbeing and helping to achieve success in a modern society that values physical attractiveness. The *what is beautiful is good* stereotype is, in fact, extended to the older also, so that unattractive elderly individuals are perceived significantly less favourably. Today, attractiveness is equated to youthfulness. Thus, the consumer is not only interested in what a product can do but also in the promise it holds. The expectation is, therefore, that cosmetics have to stir the emotions while performing on a high technical level, possessing both rational and emotional aspects. As a consequence the primary determining variable for engineering the tactile aspects of a topically applied emulsion is the emulsifier choice. Formulation chemist can help marketing professional, communicating aesthetics needs and desires by offering a wider array of emulsion options up front in the development process from which to choose. In any way, almost every ingredient in a personal care product plays a key role and has a practical function: emulsifier, preservative, emollient, and so on. However, fragrance seems to be the most important product attribute at the point of sale, influencing soon the consumer evaluation, as emotionally potent component of most personal care products. By using the information and the processes reported and discussed on these two chapters, more a aesthetically pleasing topical formulations that resonate with a target audience can be designed and scaled up to produce successfully cosmetics and short their development cost.

In any way, formulation scale-up may present unexpected complications, depending on the complexity of the formulation, compounding procedure design, and the availability of manufacturing equipment. Moreover, a well prepared product needs to be stable for at least three years. Thus, the necessity of the *stability testing* necessary to evaluate a product's ability to maintain its original aesthetic, physical and chemical characteristics designed under controlled conditions to accelerate the aging process. Such testing can provide and indicate many of the problems that may occur in formulations over time. Therefore, stability testing can guide the chemist during product development to ensure that it will remain safe to use, continuing to be aesthetically acceptable to the consumer for

use over time. The length of time a product remains fit and acceptable for use, is termed its *shelf life*. It should be sufficient to provide adequate time for manufacture and distribution, the expected time duration in retail, and the probable length of time the product will be used by the consumer, all under the environmental conditions anticipated in each segment.

The changes affecting a cosmetic product can be both chemical and physical and are often affected by environmental conditions, such as temperature, humidity, and sunlight exposure, as well as physical stresses such as what experienced in transport. Naturally the topical product' stability testing will depend up on the type of product and its stage of development. At this purpose, stability considerations with respect to the concepts of absolute and relative stability, the stage of product development, and the product/package interactions with both theoric and pragmatic applications of accelerated conditions are discussed in **Section V, Chapters 16 to 18**, *Stability and Preservation* reports all the key factors that have to be considered in the evaluation of topical product stability.

Microbial contamination of topically applied formulations, in fact, can not only affect the cosmetic stability, but can be a consumer safety concern. At this purpose, it is to underline that also if the skin care products are typically not designed to be sterile, they need to adhere to appropriate regulatory criteria for safety with set limits. Most often, the presence of low levels of microorganisms is to be expected as long as they are inhibited from proliferation and are non-pathogenic. A product that adheres to such local regulatory qualifications is considered clean and safe. Preservation is, therefore, an integral part of the success, but it is still only a part of the requirements of a successfully cosmetic product. This is the reason why an adequate preservation of topically applied formulations is required to protect the consumer and the product during its normal intended use and shelf life. Thus, an essential aspect of stability of a formulation is establishing its microbiological safety, which determined through performing a preservative efficacy test, measures the capacity of a formulation's preservative system against microbial contamination.

In conclusion, formulation science is the art of combining a variety of ingredients creating a single coherent physical form while maintaining balance among all ingredients to maintain stability and effectiveness of the final product designed. *Color Cosmetics*, reported in **Section VI, Chapters 19 and 20**, is a particular cosmetic technology and formulation design that require specialized equipments, knowledge and expertise. Thus, for example, as lip product sales continue to grow worldwide, marketers and formulation chemists have to come up day by day with innovative ideas and claims to attract consumers. Moreover, while formulating lip care products is not an easy task, the market of this color cosmetic is highly competitive, with new trend emerging year after year. At this purpose, new ingredients and technology from skin care are now being introduced to provide moisturizing and antiaging benefits for colored lipsticks also.

On the other hand, nail lacquers are other color cosmetics characterized from a high market increase. Fingernails and toenails are, in fact, important anatomical structures that, regarding as an individual's *calling card*, require periodic maintenance for good overall hygiene and health. Thus, nail cosmetics are used to make fingernails and toenails look and feeling well groomed, attractive, and protected. These two chapters report the current developments of all the colours, the polymers and *green raw* materials, used to day for formulating innovative color Cosmetics sensitive to global environmental, safety, and regulatory issues.

The book ends with **Section VII, Chapters 21 and 22**, totally dedicated to *Sunscreens*.

The increasing awareness about the damaging effects of sunlight has led to a significant demand for

more protection from sunscreens and to an enlargement of the concept of sun protection toward global photoprotection. Thus, a topical and a nutritional activity to protect the skin against damages from sunlight are increasingly advocated to the General Public.

The use of UV filters in skin care and cosmetic products together with the oral intake of specialized and protective diet supplements represent, therefore, a key benefit that these products can provide consumers. The predominant physical forms of sunscreens currently on the market are emulsion-based lotions and alcohol-based continuous sprays. Solid sticks and oily formulations are also available. In any way, formulating effective sunscreens requires a number of considerations beginning with the selection and combination of approved UV filters for desired SPF and UVA protection.

In vitro and *in silico* methods can be very useful during development to estimate the right balance of filters within the FDA, Japan, Australia, or EU monographs. Being the lists of sunscreens different among these countries, harmonization of ingredients and efficacy testing methods is on going as well, to provide the end-consumer with adequate protection and clear label information. In any way, a limited menu of UV filters for incorporation into sunscreens is available for the formulating chemist, depending on regulatory requirements in an individual country or jurisdiction.

With the demand for higher SPF's, the trend has been to use more individual and a wider variety of agents in new products. But recent research in sunscreens efficacy has emphasized the need for products protecting skin and mucous membranes against the full UV spectrum with a limited number of available agents. However, while sunscreen efficacy depends on vehicle formulation also, solvents and emollients of the formulation can have a profound effect on the strength of UV absorbance by the active ingredients and at which wavelengths they absorb. Moreover, film formers and emulsifiers, which determine the uniformity and thickness of the film on the skin surface, may determine SPF level, durability, and water resistance of the final formulation also.

These are some of the problems and the challenges the formulator has in developing new and effective formulations. A detailed discussion of incorporating UV filters into various vehicles to achieve defined goals for efficacy and aesthetics is reported on these two chapters.

This interesting book completed with an updated references list and enriched with a glossary of terms, offers the formulator a simple and practical approach to design effective skin care products for topical use, helping him/her to select the right ingredients according with and respecting the today international rules. All the aspects of a modern cosmetic formulation from the selection of raw materials, to the scale-up and pilot production process completed by stability tests and the necessary marketing supports are reported.

Written with the support of well known experts, *Apply Topically* may be useful not only for students in chemistry who wish to know and understand philosophy and technologies necessary for formulating cosmetic products, but may be of useful support for cosmetic chemists in their daily work and for all people of the chemical and medical community who want to have a better technological specific knowledge on the fascinating field of Cosmetic Dermatology

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2

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Quarterly Review of Cosmetic Dermatology

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Contents

Original Laboratory Studies

57 **Skin Lightening Efficacy of New Formulations Enhanced by Chitin Nanoparticles Delivery System. Note I.**
P. Morganti, P. Del Ciotto, F. Carezzi and Yip Jui Yeo

General Articles

73 **Selected Synthetic and Natural Actives as Inhibitors in Androgenic Alopecia**
K. Pytkowska, M. Kubacka, J. Arct

85 **Cosmetic Regulation of Sebaceous Gland Activity**
S. Skalec, B. Winiarska, J. Arct, K. Pytkowska

Book Reviews

99 **Memories of a Cosmetically Disturbed Mind**

102 **Health Challenged Skin: The Estheticians' Desk Reference**

Announcements

V **11th International Congress of Cosmetic Dermatology**

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Skin Lightening Efficacy of New Formulations Enhanced by Chitin Nanoparticles Delivery System. Note I.

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Summary

This preliminary study reports the whitening efficacy obtained *in vivo* by the contemporary topical use of 3 cosmetic emulsions enriched with the designed nanoparticles and applied for a period of 6 months on the skin of 40 voluntary subjects, female and male, affected by hyperpigmentation in different skin areas.

Before the skin treatment, different Chitin Nanofibril-Hyaluronan (CN-HA) block copolymeric nanoparticles, entrapping known whitening agents, were controlled *in vitro* to verify their activity on the melanogenetic process. It was controlled *in vitro* their antioxidant and anti-inflammatory activity together with the possibility to interfere on melanin synthesis and melanosome transfer.

From the obtained results it has been shown that the combined activity of different whitening agents entrapped into natural nanoparticles as carrier, gives as result an interesting depigmenting effectiveness without any side effect.

A new study is in progress to confirm these data.

Riassunto

Mediante questo studio preliminare si pone in evidenza l'attività depigmentante ottenuta *in vivo* con l'uso contemporaneo per 6 mesi di 3 emulsioni cosmetiche arricchite con nanoparticelle appositamente formulate, applicate sulla cute affetta da aree iperpigmentate di 40 soggetti volontari di ambo i sessi.

Prima del trattamento *in vivo*, è stata verificata *in vitro* l'efficacia di block copolimeriche nanoparticelle di nanofibrille di chitina-acido ialuronico (CN-HA) incapsulate con diversi ingredienti depigmentanti inseriti nelle 3 emulsioni utilizzate. Di queste nanoparticelle è stata verificata l'attività antiossidante ed antiinfiammatoria assieme alla capacità di ridurre la sintesi della melanina ed il suo trasferimento ai cheratinociti attraverso i melanosomi.

L'attività combinata di diversi ingredienti incapsulati nelle nanoparticelle ed inseriti in adatti veicoli ha posto in evidenza sia l'interessante efficacia depigmentante delle 3 emulsioni cosmetiche che la loro innocuità d'uso.

Durante i 6 mesi di trattamento non è stato evidenziato alcun effetto collaterale.

Un nuovo studio dermatologico è in corso per confermare i risultati ottenuti.

INTRODUCTION

Epidermal melanin unit, composed of a melanocyte and associated clusters of keratinocytes, is responsible for the colour variation of human skin by the presence of *melanin* in melanosome.
Melanin, as bio polymer formed by the action of the enzyme tyrosinase on tyrosine and levodopa, has the principal function to provide the skin protection against the ultraviolet (UV) irradiation. Tyrosinase is, therefore, the key enzyme involved in the synthesis of melanin and, as a rate-limiting enzyme, catalyzes different oxidative processes, leading to the synthesis of DOPA (dihydroxyphenylalanine), dopaquinone for further producing eumelanin and pheomelanin (1-3).
Finally, melanosomes are the melanin carrier that, synthesized in melanocytes, are transferred to keratinocytes and transported to the epidermal surface by means of the dendritic process (Fig. 1).

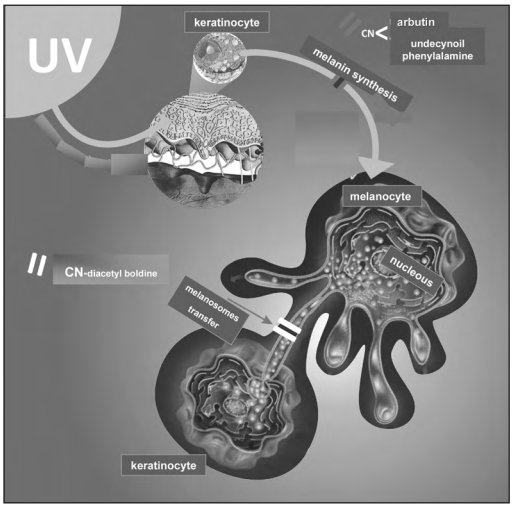

Fig. 1

However while the exposure of melanocytes to UV radiation is of more critical importance, environmental factors (temperature, hormones, chemical reactions) play also an important role in the synthesis of melanin. In conclusion the visible skin colour arises essentially upon the visual impact of the melanin units plus the effects of UV light, hormones, and genetic predisposition.
When function of this epidermal unit is altered, many disorders can take place, giving rise to hyperpigmentation phenomena, such as freckles, ephelides, age spots or melasma. Possible mechanisms of hyperpigmentation, promoted by sun exposure through enzymatic reactions include: (a) increased production of normal melanosome, (b) possibly increased melanosome size, (c) increased transfer of melanosomes to keratinocytes, (d) increased survival of keratinocytes, and (d) increased production of Reactive Oxygen Species (ROS) and pro-inflammatory cytokines.
In any way, type and amount of melanin synthesized by the melanocyte, and the distribution pattern in the surrounding of this cell determines the actual colour of the skin both in physiological and pathological conditions.
Preventive methods such as sun avoidance or control, and sunscreen use, attempt to reduce the UV contribution to the hyperpigmentation appearance. Whitening or bleaching agents are implemented for the elimination of dark spots ameliorating the skin lightening tone.

MARKET OF WHITENING PRODUCTS

For the Asian culture *bright* and *white* skin is considered at the base of the female beauty, and consequently all the products active in eliminating or slowing down the uneven pigmentation, such as age spots, post inflammatory hyperpigmentation, or melasma, represent the first product to buy together with antiaging cosmetics. As a consequence, the global market for skin whitening and/or brightening products in Asia Pacific has today a year turnover of US$ 13 billions with a expected increase of 13% per year

until 2020 (Euromonitor Data 2013). However, all the brightening agents are used by European, Hispanic and Americans also, while in EU and USA these products are principally used to reduce age spots, freckles and acne scars.
In China and in all the Asia Pacific area they are used not only for slowing down hyperpigmentation, but also to lightening the skin colour daily. However, being hyperpigmentation an often cause of psychosocial distress worldwide (4), multiple interventions are available to whitening the skin, such as chemical peels, lasers, physical methods and naturally whitening cosmetics.
The latter category continue to represent the mainstay of approach for either lighten skin or treat abnormal hyperpigmentation, such as melasma or age spots because of their simplicity of using and economical convenience (5-8).

Whitening agents

To address problems presented by skin hyperpigmentation, whitening agents are commonly used to formulate cosmetic products including, arbutin, kojic acid, ascorbic acid and its derivatives, together with many other different compounds isolated from plant such as liquorice, flavonoids and polyphenols. In any way it is to underline that consumers are looking for whitening cosmetics active not only in the long term, but having also a fast and immediate effect, when applied on the skin.
To obtain such result it is necessary to use botanical or chemical agents really effective embedded into the right vehicle with a delivery capacity scientifically proved. It is to remember, in fact, the four R's Skin delivery rules: to deliver the Right chemical to the Right site in the skin at the Right concentration for the corRect period of time (9).
For all these reasons our purpose has been to design a new vehicle having the fundamental characteristics to delivery the whitening ingredients selected at level of melanocyte and melanosome.

AIMS

At this purpose, the aim of the study was to design whitening cosmetic nanoemulsions by the use of known active ingredients entrapped by the block co-polymeric nanoparticles CN-HA, as active carrier. This innovative and natural vehicle has been obtained by the gelation method and the use of the electronegative Chitin Nanofibrils (CN) together with the electronegative Hyaluronan (HA), according to our previous experiences (10-12).
The formulations have been designed to act at different levels: (a) to inhibit the melanin synthesis, (b) to slow down the transfer and distribution of melanosomes to keratinocytes, (c) to absorb part of UVA/UVB rays, (f) to reduce the darkening process, (d) to neutralize ROS in excess, (e) to control the inflammatory process, to trigger the melanin synthesis.

STUDY DESIGN

Due to the observation that chronic UV exposure and hyperpigmented skin present an alteration at different levels of the melanogenetic process together with an unusual synthesis of epidermal free fatty acids and triglycerides, and a slow down of skin hydration, which play important roles in epidermal barrier homeostasis (13-15), these parameters were controlled by *in vitro* and *in vivo* studies. Moreover, it was controlled the fibroblast and keratinocyte activity, which seem to be up-regulated and associated with an increased production of reactive oxygen species (ROS) and skin pro-inflammatory cytokines (16).

Selection of actives and vehicle

After the selection of the active ingredients to be

used as whitening agents, they were bonded to CN-HA nanoparticles and encapsulated by the use of our technology, previously described (17). Successively the activity of the different block copolymeric nanoparticles was verified *in vitro* on keratinocytes cultures and emulsified with the nanoemulsion designed to control *in vivo* the whitening effectiveness of the final formulation.

The nanostructured vehicle

The nanostructured vehicle, organized as a nanoemulsion, was prepared by phase inversion temperature and high-pressure homogenisation method, according to our previous study (17). It is characterized by a dispersion of nano scale droplets (~10-500nm) fundamental to obtain a controlled delivery of the block copolymeric nanoparticles entrapping the selected whitening ingredients and to improve their bioavailability and bioefficacy (18).

Similar to liposomes, these nanoparticles support the skin penetration of the active ingredients entrapped into the block polymeric nanoparticles, increasing their concentration in the skin and allowing their effective transport at level of the different skin layers (19). Moreover, depending to the electrical charges covering their surface they can penetrate through the skin layers (when positively charges) or remain on the surface of the *Stratum a Corneum* (SC) (when negatively charges) (Fig. 2) (20).

Block polymeric nanoparticles entrapping whitening and UV protective ingredients

6 different CN-HA block polymeric nanoparticles were prepared entrapping different active ingredients such as: methoxydibenzoyl methane + titanium dioxide + silica + alumina as UV filters; ascorbyl phosphate + *Phyllantus emblica*, ascorbyl tetraisopalmitate and ascorbyl phosphate as antioxidants and anti-inflammatory ingredients; compounds inhibiting the melanin synthesis at level of tyrosinase as undecylenoyl phenylalanine and arbutin; and compound slowing down transfer and distribution of melanin to keratinocytes as diacetyl boldine.

Nanoparticles entrapping the sun screening agents were negatively charges, while all the other were positively charged.

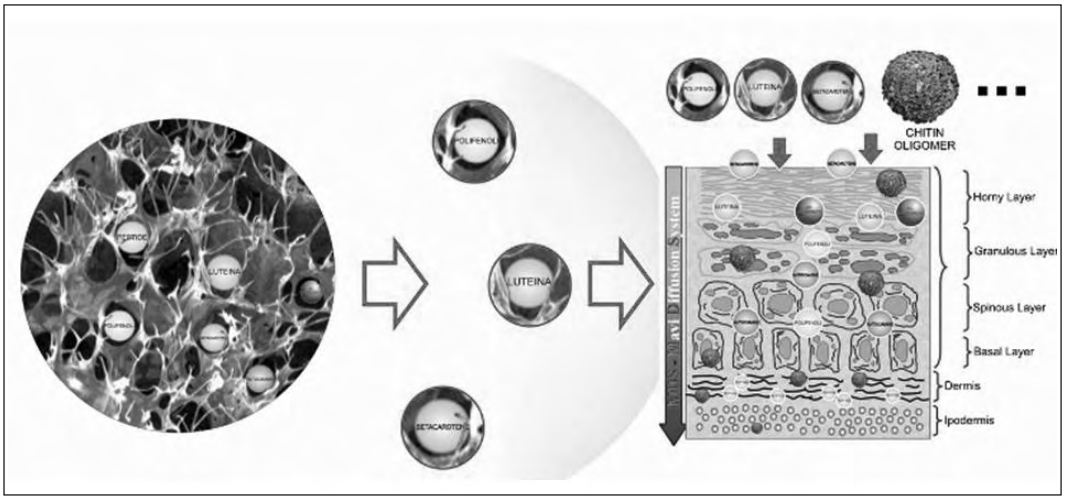

Fig. 2 *Skin penetrability of nanoparticles depends on their size, superficial charge and type and/or polymer used.*

MATERIALS

All the block copolymeric nanoparticles such as: Chitin-Hyaluronan nanoparticles (CN-HA vehicle); CN-HA- diacetyl boldine; CN-HA-arbutine and undecylenoyl phenylalanine;
CN-HA-methoxydibenzoylmethane-titanium-dioxide-silica-alumina; CN-HA-Sodium ascorbyl phosphate and *Phyllantus emblica*; CN-HA-ascorbyl tetraisopalmitate and CN-HA-ascorbyl phosphate were supplied by MAVI sud, Aprilia (LT) Italy;
Sodium ascorbyl phosphate was supplied by Res Pharma (MI);
Diacetyl boldine (lumiskin) was supplied by Sederma (France);
Undecylenoyl phenylalanine (sepiwhite MSH) was supplied by Seppic (MI);
Methoxydibenzoylmethane was supplied by Merck (Germany).

Formulations designed

Formulation A[1]: Aqua (Water), Prunus Dulcis (Sweet Almond Oil), Dimethicone, Sodium Ascorbyl Phosphate, Olea Europaea (Olive Oil), Glycerin, Cyclotetrasiloxano, Ascorbiltetraisopalmitate, Lecithin, Palmitic Acid, Methoxydibenzoylmethane, C12-16 Alcohols, Phyllantus emblica Fruit Extract, Chitin (Nano-Fibrils), Glyceryl Stearate, PEG-100 Stearate, Cyclopentasiloxano, Phenoxyethanol, Xanthan Gum, Tocopheryl Acetate, Bisabolol, Imidazolidinyl Urea, Cetyl Alcohol, Titanium Dioxide, PEG-8, Silica, Alumina, Potassium Azelaoyl Diglicinate, PEG-75 Stearate, Methylparaben, Ceteth-20, Tocopherol, Steareth-20, Propylparaben, Disodium EDTA, Ascorbyl Palmitate, Citric Acid, Ascorbic Acid.
Formulation B[2]: Aqua (Water), Prunus Dulcis (Almond Oil), Dimethicone, Olea Europaea (Olive Oil), Caprylic/Capric Triglyceride, Glycerin, Lecithin, Palmitic Acid, C12-16 Alcohols, Butyl Methoxydibenzoylmethane, Glyceryl Stearate, PEG-100 Stearate, Xanthan Gum, Phenoxyethanol, Benzyl PCA, Cetyl Alcohol, Titanium Dioxide, Silica, Alumina, PEG-75 Stearate, Ceteth-20, Sodium Ascorbyl Phosphate, Steareth-20, Sodium Hyaluronate, Chitin (Nano-Fibrils), Diacetyl Boldine.
Formulation C[3]: Aqua (Water), Prunus Dulcis (Almond Oil), Dimethicone, Olea Europaea (Olive Oil), Glycerin, Undecylenoyl Phenylalanine, Lecithin, Palmitic Acid, C12-16 Alcohols, Arbutin, Glyceryl Stearate, PEG-100 Stearate, Xanthan Gum, Phenoxyethanol, Benzyl PCA, Cetyl Alcohol, PEG-75 Stearate, Ceteth-20, Sodium Hyaluronate, Steareth-20, Sodium Ascorbyl Phosphate, Chitin (Nano-Fibrils).

In vitro studies

Melanin and tyrosinase inhibitory activity

Melanin and tyrosinase activity was measured on B16 murine melanoma cells in DMEM cultures supplemented with 10% calf serum. B16 melanocytes were seeded in culture medium incubated at 37 °C with CO2 at 5%.
After three days, the cell culture medium was exchanged to medium containing 10ng of kojic acid (reference substances), control (without ingredient), CN-HA nanoparticles (vehicle), CN-HA entrapping respectively diacetyl boldine, undecylenoyl phenylalanine+arbutine, ascorbyl phosphate + *Phyllanthus emblica*, and ascorbyl tetraisopalmitate (active) at the same concentrations.
After 3 days of incubation at 37 °C with CO2 at 5%, the cells were washed and divided in two

[1] *Trade name: Acromos forte, Mavi, Italy.*
[2] *Trade name: TS Spotless Day, Mavi, Italy.*
[3] *Trade name: TS Spotless Night, Mavi, Italy.*

groups. In one group the cell number was determined by counting in a haemocytometer chamber, in the other the DL-DOPA was added, and incubation was continued for other 2 hours at the same conditions.
On the first group melanin content was measured, while on the second group the tyrosinase activity was controlled all both by the Bradford's method, measuring the optical density at 475 nm (21).
The obtained results, assayed as DOPA oxidase activity and determined in triplicate is reported on figures 3 and 4

Dendrite length

According to Krishnanoorthy et al. (22), treated and untreated melanocytes were examined by microscopy to determine the cellular dendrites' mean length. Results are shown on Tab I.

Antioxidant and antiinflammatory activity

Melanin synthesis is an oxygen-dependent process that acts as a potential Source of ROS Inside pigment-forming cells, CN-HA-ascorbyl phosphate, and ascorbyl phosphate + *Phyllanthus emblica* not only protect cell from external oxidative injury for its antioxidant and anti-inflammatory properties, but also inhibits the melanin synthesis, contributing to the whitening activity of the formulations.

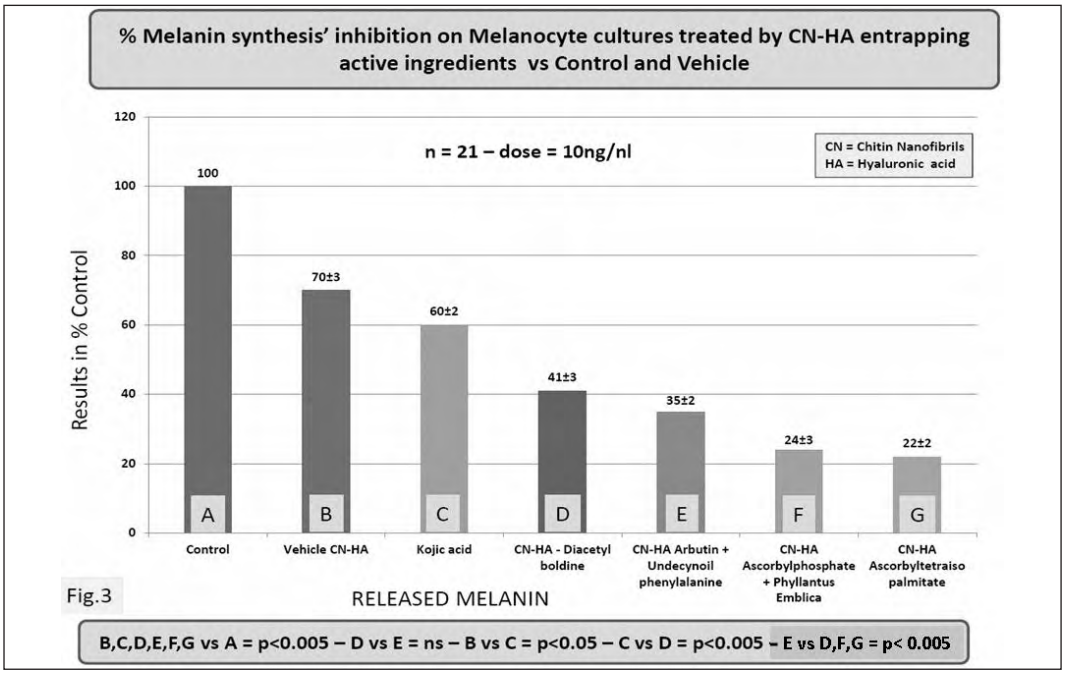

Fig. 3

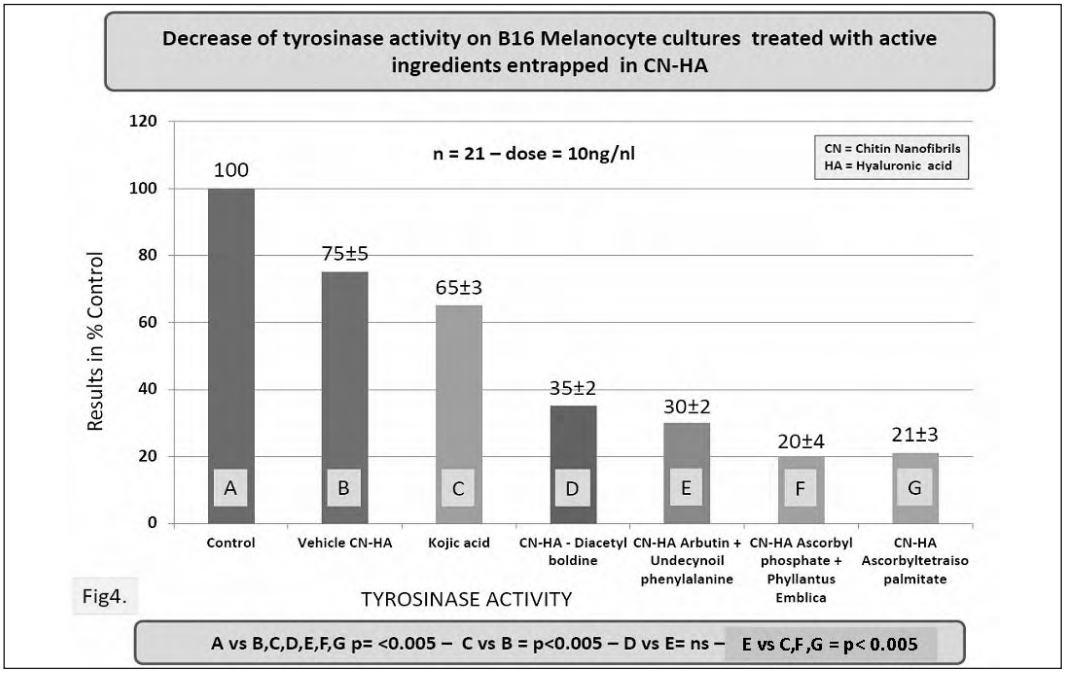

Fig4.

Fig. 4

Dendrite mean lenght (DL)

INGREDIENTS CN-HA entrapped	DL / MICRON
Control untreated	100±5
Vehicle treated (CN-HA)	81±3
Kojic acid (reference active)	95±4
Diacetyl boldine (CN-HA entrapped)	51±3
Arbutin + Undecynol phenyl alamine (CN-HA entrapped)	65±4
Ascorbylphosphate + Phyllantus emblica (CN-HA entrapped)	59±5
Ascorbyltetraisopalmitate (CN-HA entrapped)	53±4

Tab 1

Derma inflammation, induced by accumulation of UV radiation, seems to be associated to the increased melanogenesis of hyperpigmented skin (16, 23).

The *antioxidant activity* was controlled on the peroxidation of linoleic acid with formation of malondialdehyde as final product by the method previously described by our group and briefly reported (24).

The pathological peroxidation of the linoleic acid in the cell membrane produces, in fact, a plethora of primary peroxided compounds that lead to many chronic diseases, causing phenomena of skin hyperpigmentation also (25).

Thus, linoleic acid was dissolved in 1 ml of methanol, dried under nitrogen and redissolved in 2 ml of phosphate buffer. The samples added with the CN-HA-ascorbyl phosphate + *Phyllantus emblica* and ascorbyl phosphate nanoparticles at the dose of 10ng/ml respectively, were oxidated to malondialdehyde (MDA) by the use of 10ul of AMVN for 15 min at 37 °C. MDA, detected by a fluorimetric methodology, was compared to the untreated control. The obtained results in triplicate are reported on figure 5.

The *antiinflammatory activity* was verified controlling the release of IL-8 on TNF-a stimulated keratinocytes, according to the methodology, briefly reported and previously described by our group (24).

The CN-HA-ascorbyl phosphate + *Phyllantus emblica,* CN-HA-ascorbyl phosphate, and CN-HA-ascorbyl tetraisopalmitate nanoparticles were introduced in fresh culture of keratinocytes at the dose of 10ng/ml with TNF-alpha at 100 ng/ml. As control were used an untreated sample and a positive sample with hydrocortisone at the dose of 1 uM. After 24 hours of incubation at 37 °C and 5% of CO2, the quantity of IL-8 was evaluate D by ELISA on the superstant culture. The obtained results in triplicate are shown on figure 6.

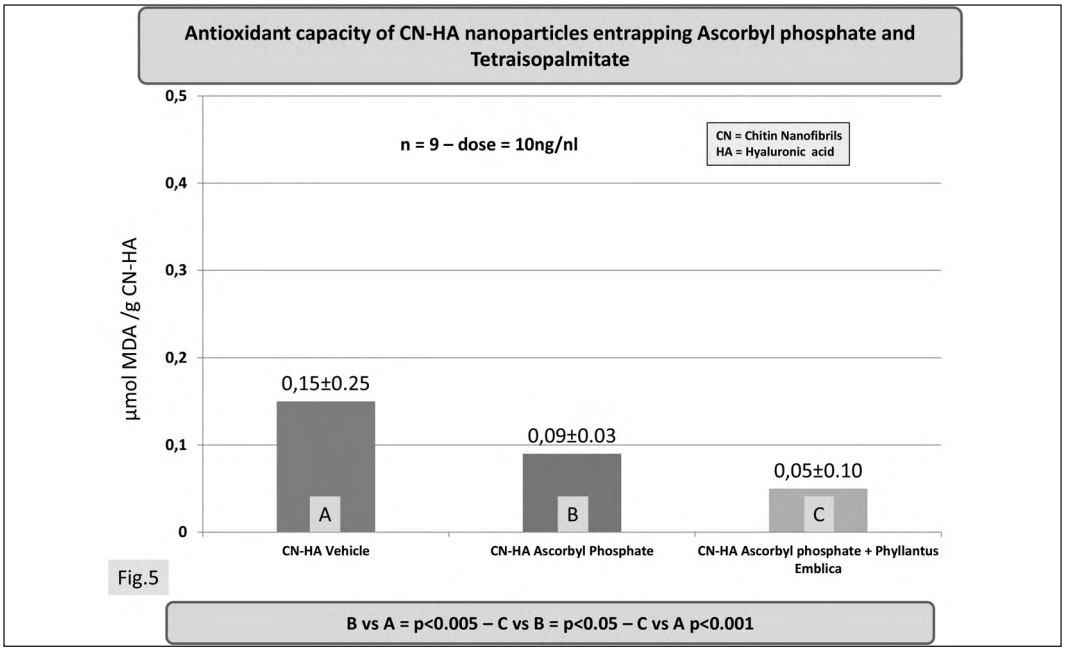

Fig. 5

Study Criteria in vivo

As preliminary study, 40 voluntary subjects (male and female, of mean age 50±4), affected by hyperpigmented phenomena in different skin areas, were treated in the Beauty Academy Impress Centre in Melaka. All the subjects were treated into the Beauty Center, for a period of 6 months, by applying the in study whitening emulsions on all the surface of the face, after a previous application of cleansing and moisturizing lotions, according to the following design: the treatment into the Beauty Centre, organized by this frequency: 4 times/month for the 1st month; 3 times/month for month 2 and 3; 2 times/month for month 4 and 5; 1 time/month for month 6 was controlled monthly by an expert pharmacist.

During all the in study period the subjects have applied at home the whitening cream A[4] on spot area, morning and evening, and the whitening cream B[5] and C[6] on the entire skin surface, morning and evening respectively.

Naturally the application of the whitening emulsions was preceded by cleansing[7] and moisturizing[8] treatments of the entire face area. In addition, during the day they applied a Sun block emulsion[9] on face, arms, and legs before going outside their abitative location.

Examples of the obtained results are reported on figures 7 and 8 at the end of the first period' treatment (6 months).

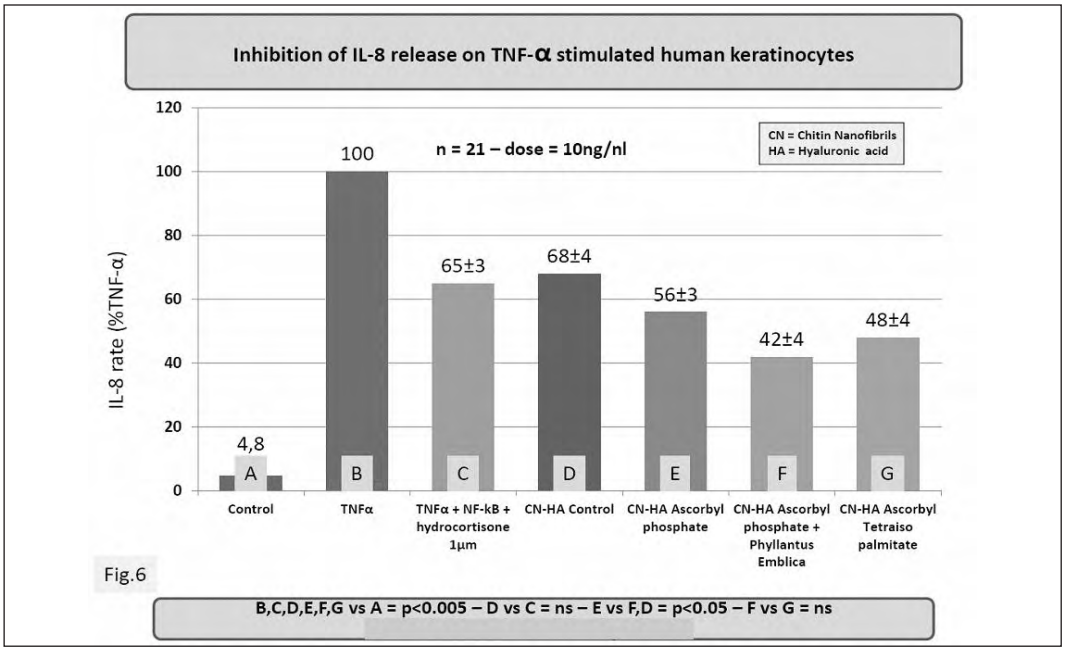

Fig. 6

[4] *Trade name: Acromos forte, Mavi, Italy.*
[5] *Trade name: TS Spotless Day, Mavi, Italy.*
[6] *Trade name: TS Spotless Night, Mavi, Italy.*
[7] *Trade names: TS Gentle Whitening milk, followed by TS Skin Toner lotion. Mavi, Italy.*
[8] *Trade names: TS Face Firming Serum, Idroskin C and Mavioil cream. Mavi, Italy.*
[9] *Trade name: MAVISAN 50+ cream, Mavi, Italy.*

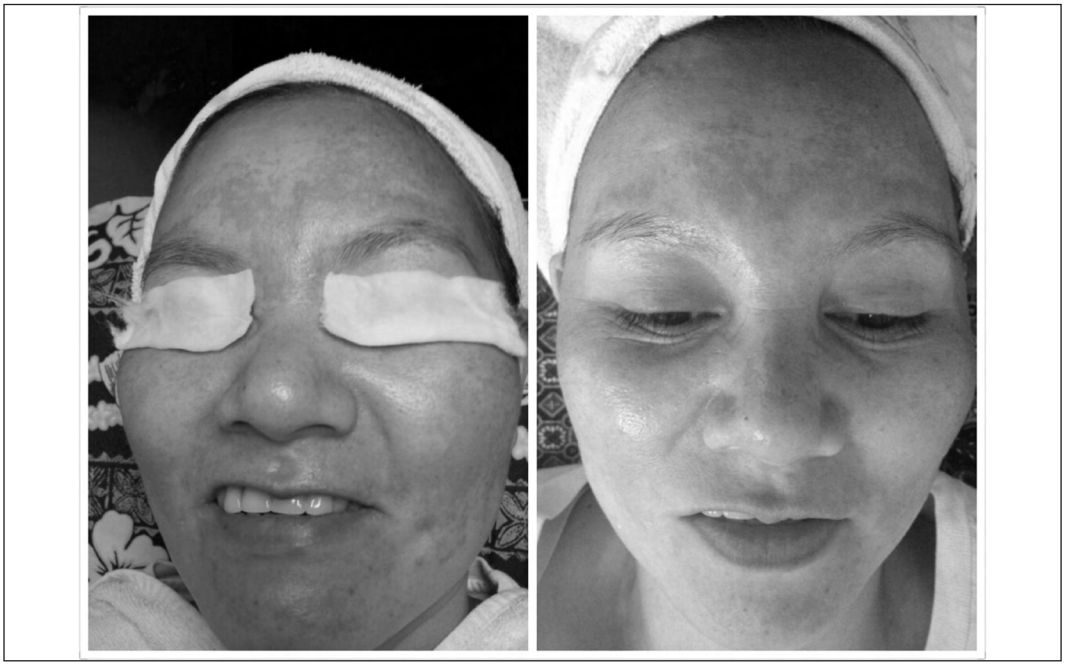

Fig. 7 *Before (left) and After (right) the treatment.*

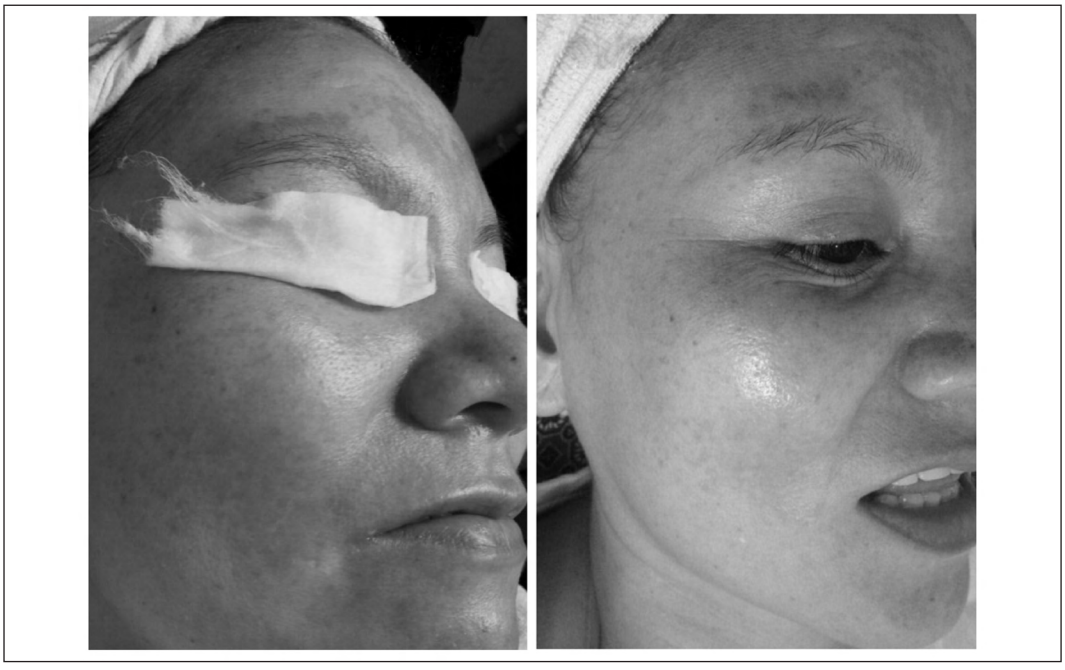

Fig. 8 *Before (left) and After (right) the treatment.*

Statistical analysis

The results are expressed as mean ±SD from at least three independent experiments. Statistical evaluations were performed with GraphPad Prism4 (Graph Pad Software Inc, San Diego, California. USA).
All statistical evaluations were conducted as two-tailed analysis at a minimum of a 95% confidence interval ($p<0.05$) using a repeated measures ANOVA and a Tukey post-test to determine statistically significant differences in the results.

RESULTS AND DISCUSSION

Also if the skin depigmentation of most common signs of photoaging and melasma remain a challenge for both dermatologists and beauticians, the combination of different whitening agents used by different formulations with a unique global treatment has shown to provide an interesting whitening effect.
The *in vitro* obtained results are probably due to the different mechanisms of action the active ingredients have shown. Helped in their effectiveness by the use of the innovative carrier composed by two natural polymers, Chitin Nanofibrils and Hyaluronic acid combined to form block copolymeric nanoparticles positively or negatively charged on their surface, the active ingredients selected have shown to act at different levels of the melanogenetic process.
The probable synergistic activity among the different CN-HA nanoparticles entrapping respectively diacetyl boldine, arbutine/undecynoil phenylalanine, ascorbyl phosphate + *Phyllantus emblica* or ascorbyltetraisopalmitate to form the cosmetic formulations, gave the possibility to decrease the melanogenesis process probably acting both at level of maturation and transfer of melanosomes, as reported on figure 3, and through a decrease of melanin synthesis slowing down the tyrosinase activity, as reported on figure 4.
As evident from the results, the different particles show different effectiveness on melanin synthesis and tyrosinase activity always higher compared to the kojik acid effectiveness. It is interesting to underline that the real effectiveness of the selected ingredients has been confirmed by the different dendrites length which, reducing the melanin transfer to keratinocytes, significantly influence the skin pigmentation, as shown on table I.
On the other hand, the antioxidant capacity of CN-HA nanoparticles entrapping ascorbyl phosphate + *Phyllantus emblica*, ascorbyl phosphate and ascorbyltetraisopalmitate respectively, have shown the intensive activity these nanoparticles have to decrease the excessive production of ROS, also modulating the release of the cytokine IL-8, necessary to reduce the inflammatory process, often present at level of hyperpigmented skin, as shown in figures 5 and 6.
These *in vitro* results have been confirmed by the preliminary *in vivo* study, where large hyperpigmented skin areas have been sensibly reduced, as clearly shown on the subject reported on figures 7 and 8. No side effects to any subject have been found during the 6 month treatment.
The same whitening formulations to control their possible side effects for long period of time were evaluated by another study in progress at one Dermatological Department, where TEWL, skin hydration, skin pH, erythema, oedema, and skin blood flow have been measured, according to Fluhr et all methodology (26).
The first obtained data seem to confirm these preliminary results (Unpublished Data. Work in progress).

CONCLUSION

Further studies are in progress to better elucidate the possible mechanism of action and the best

dosage treatment periods for these new formulations, based on the innovative use of the CN-HA block copolymeric nanoparticles as carrier for whitening agents, as well as to investigate the true role they may have to modulate the different manifestations inducing the skin hyperpigmented phenomena.

However, the possibility to block the chain reactions in different cellular points of the malanogenesis pathway by the use of the bionanotechnology seems the best way for trying to solve the difficult problem of an altered pigmentation without having any side effects.

This is one of the challenges of MAVI Nanoscience Center.

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Selected Synthetic and Natural Actives as Inhibitors in Androgenic Alopecia

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Key words: *Androgenic alopecia; Finasteride; Minoxidil; Zizyphus jujuba; Fallopia multiflora; Asarum europaeum;*

Summary

Androgenic alopecia is a common condition that affects Caucasian males in the age range between 20 and 40. A paper discusses briefly pathogenesis of androgenic hair loss in men and the factors affecting its development.

The main part of the paper describes selected actives inhibiting androgenic alopecia and their mechanism of action, recalling the results of their effectiveness testing in vivo. It discusses the widely used active substances such as finasteride and minoxidil and a range of plant materials used in traditional medicine for potential use in practice. Unfortunately, not for all interesting plants are available results of human efficacy studies.

Riassunto

La alopecia androgenetica è una comune affezione che colpisce la razza caucasica soprattutto di sesso maschile e di età compresa tra 20 e 40 anni. Il lavoro descrive brevemente la patogenesi e lo sviluppo di questa patologia nell'uomo. La parte principale dello studio riporta i più importanti ingredienti attivi utilizzati per inibire lo sviluppo della alopecia androgenetica, descrivendone anche i meccanismi d'azione ed i risultati di efficacia condotti *in vivo*.

Tra gli attivi utilizzati viene discussa l'attività svolta dalla finasteride e dal minoxidil, oltre che da diversi estratti vegetali utilizzati dalla medicina tradizionale.

Sfortunatamente per molte piante medicinali non sono reperibili i risultati ottenuti *in vivo*.

INTRODUCTION

Androgenic alopecia is a common condition that affects males in the age range between 20 and 40, constituting a serious psychological problem. According to research findings, patients suffering from extensive androgenic hair loss have a worse quality of life. Psychic distress is comparable to that experienced in the course of such serious conditions as, e.g. psoriasis. For this reason, it is crucial to cooperate with a dermatologist who will help a patient to comprehend the factors underlying the development of this condition and will help to select an appropriate treatment. The therapy of androgenic alopecia is time-consuming and requires a lot of patience as well as regularity in taking medicines.

PATHOGENESIS OF ANDROGENIC HAIR LOSS IN MEN AND THE FACTORS AFFECTING ITS DEVELOPMENT

The etiology of androgenic alopecia is usually connected with the level of androgens, genetic susceptibility and age. The condition is inherited in an autosomal dominant or multigenic way. Multigenic inheritance is related to the occurrence of early balding in the family, a significant prevalence of the condition in the family and heterogeneity of its clinical manifestation. Premature androgenic hair loss is probably conditioned by an autosomal gene.

Probability for a male to be affected is dependent on the number of relatives suffering from androgenic hair loss of the 1st and 2nd degree. When balding of this type affects the patient's sister or mother, the prognosis is considerably worse (1). In individuals who have genetic predispositions, the androgen level may be correct and does not have to be a factor in the development of the condition. Over 50% males after 40 are affected by the hair loss which is indirectly rooted in androgen activity. In 1942 Hamilton discovered that androgenic hair loss is not present in eunuchs. It appeared when they were given testosterone. In 1974 a hereditary anomaly was discovered, a male pseudohermaphroditism[1].

It was a very important discovery in the context of searching a medicine for androgenic alopecia. Children born with pseudohermaphroditism possessed female phenotypic traits, external sex organs were not fully differentiated, yet until puberty, when male traits appeared, the children were treated as girls. As adults, they possessed thin hair, yet with no clinical balding symptoms, no acne, or the prostate did not enlarge.

Research showed that those individuals had a deficiency of the enzyme, type II 5-alpha-reductase, which affected the correct conversion of testosterone to DHT (2). Androgenic alopecia is not present in individuals who have a geneticall-ly conditioned deficiency of the type II 5-alpha-reductase. Since the fontanelle and forehead areas are formed from the neural crest and are characterized by a higher 5-alpha-reductase activity, a more profound hair loss occurs in these areas, whereas the occipital part develops from the mesoderm, and there the hair remains. This is connected with a different metabolism of testosterone by the hair follicle. Testosterone is transformed into 5-alpha-dihydrotestosterone (DHT) and only then does it function in target tissues [2,1].

5-alpha-dihydrotestosterone has a higher affinity for androgenic receptors, forming more stable complexes. The number of 5-alpha-dihydrotestosterone and testosterone receptors is dependent on the individual's age and head area. In the forehead they are more densely located than they are in the occipital part. The conversion of testosterone into DHT is controlled by two isoenzymes, 5-alpha-reductase type I and type II. In hair follicles 5-alpha-reductase type I is present only in sebaceous glands, whereas 5-alpha-reductase type II is present in the sebaceous duct, outer

root sheath and inner root sheath[1].

Selected actives inhibiting androgenic alopecia and their mechanism of action

Finasteride

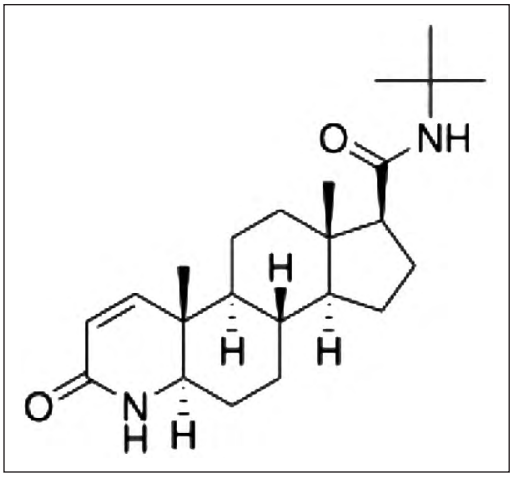

Fig. 1 *Finasteride.*

Finasteride is a synthetic 4-azasteroid compound. Most importantly, it is an inhibitor of type II 5-alpha-reducatse, which transforms testosterone into its active form, DHT, .i.e. 5-alpha-dihydrotestosterone(3,4). In rats, mice, apes and humans two isoenzymes (I and II type) have been discovered, with differing expression in the tissues. In humans, 5-alpha-reductase (type I) is present is liver and in sebaceous glands in the skin, whereas type II is present in such organs as: prostate, epididymides, seminal vesicles, and hair follicles (5). The mechanism of action of finasteride rests upon inhibiting the activity of isoenzyme type II. The conversion of testosterone into 5-alpha-dihydrotestosterone is blocked. The activity of finasteride has an impact on the inhibition of the key factor in the development of androgenic alopecia in individuals who are genetically susceptible. In the first year of treatment the number of hair follicles that react to the finasteride treatment is established. The further therapy aims to maintain the effects (6). Since 1992 finasteride has been administered orally in the 5 mg dose as a drug for prostatic hyperplasia. The drug containing finasteride, *Propecia*, is also used in androgenic alopecia in men (7).

Leyden et al. conducted a study in 15 locations in the United States. Randomly selected individuals were administered 1 mg oral dose of finasteride vs. placebo once daily for 12 months, and all participants took part in further research throughout the following year. The hair number was assessed in a blind study in the 6th, 12th and 24th month. The photographs of the frontal (anterior/mid) scalp in the 6th, 12th and 24th month, respectively, were evaluated by a team of dermatologists at the end of the treatment (8).

Finasteride proved to be efficacious in the treatment of men with head top hair loss. At present, research is devoted to the efficacy of the treatment in the frontal/mid scalp. Whereas the above treatment referred to the head top primarily, over a half of the subjects had a problem with hair loss in the frontal and mid part of the scalp. After the 12-month treatment the mean hair count per 1cm^2 scalp increased by 12 hairs. It was established that there is a correlation: the lower hair thickness at the onset of the therapy, the higher hair increase in response to finasteride. All methods used to evaluate the results revealed the supremacy of finasteride over placebo.

At first, finasteride slows down the miniaturisation of hair follicles and stimulates hair growth. In the following phases of the treatment hairs become longer and thicker. There have not been discovered any considerable adverse side effects of the treatment with finasteride (7).

TABLE I

Finasteride efficacy study results.

Finasteride action	Finasteride dosage applied	Results after 12 months' treatment
HAIR COUNT	1 mg/day	Hair count in the group treated with finasteride increased by 9.6 items/1cm^2 in comparison with the control group, where the hair number decreased by 2.0 items/ 1 cm^2.
ENHANCEMENT OF HAIR APPEARANCE	1 mg/day	In 37% patients treated with finasteride an improvement in hair appearance was noticed, as related to the control group with only 7% improvement.
HAIR LOSS INHIBITION	1 mg/day	70% patients treated with finasteride did not notice further hair loss, whereas 30% individuals noticed further hair loss.

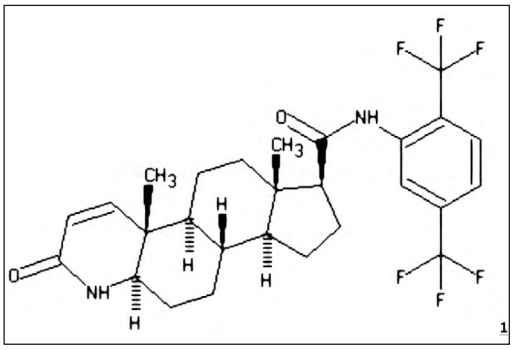

Fig. 2 *Dutasteride.*

Dutasteride is a powerful, selective oral inhibitor of 5-alpha-reductase type I and II in humans. It lowers the level of dihydrotestosterone in the blood serum(9). Stough et al. conducted a study on monozygotic twins in order to evaluate the impact of phenotypical and environmental factors on androgenic alopecia. Twins are a perfect object of study of the efficacy of a drug for hair loss whose expression is determined genetically. Twins possess the same genetic code and thus it is easy to determine the impact of both substances on the therapy when one of them is administered a drug and the second one a placebo (10). The main objective of the study was to determine the efficacy of dutasteride as a hair loss inhibitor. The secondary objective was to evaluate its impact on the hair count and hair appearance assessment (self-assessment). Throughout the study phenotype changes in genetically identical twins were being monitored[10].

In this study dutasteride considerably contributed to the improvement of hair growth throughout one year, and none of dutasteride-treated patients noticed the deterioration in hair growth, unlike patients in the placebo group.

Asarum europaeum

Asiasari radix serves as a natural herbal medicine used in China to treat oral cavity and gingiva inflammations. The plant is known for its analgesic and anti-inflammatory properties, as well

as its protective activity against brain cell damage. It also promotes hair growth (11). For the purposes of the research the root of *Asarum europaeum-Asiasari radix* was used. It proved to be efficacious in the telogen-to-anagen conversion of hair follicles. It was revealed that the *Asiasari radix* extract had contributed to cellular proliferation, protein synthesis and promoted cellular growth. From 45 plants used in oriental medicine, *Asiasari radix* proved to have the most hair growth-promoting potential. Seok-Seon Rho et al. conducted a 45-day experiment on mice that were topically treated with *Asiasari radix* extract on their shaven backs. The effects were additionally evaluated in vitro on keratinocytes and human dermal papilla cells (11).

TABLE II

Finasteride efficacy study results.

Dutasteride action	**Dosage applied**	**Results after 12 months**
Hair length growth	0,5 mg/day	Due to dutasteride activity hair length increased on average by 35 mm in comparison with the control group
Increased hair count	0,5 mg/day	In the treatment group the hair count i ncreased by approximately 16.5 hairs/1cm^2 in comparison with the control group, approximately 3.8 hairs/1cm^2 more.

TABLE III

Results of the research on the Asarum europaeum root extract.

Asiasari radix extract action	**Applied dosage (ethanol mixture)**	**Result**
CELLULAR PROLIFERATION	0.0001 %	Visible cellular proliferation growth reaching 115.6 % in comparison with the control object.
POTENTIAL TO UPTAKE CYSTEINE IN HAIR FOLLICLE	0.0001 %	Cysteine uptake in the hair follicle was increased by 129% in comparison with the control object.
TELOGEN-TO-ANAGEN CONVERSION OF HAIR FOLLICLES	0.0001%	The picture showing a histological examination of the skin sample clearly shows the evoked anagen phase, with hair follicles thicker and more deeply-set in comparison with the control object.

To sum up, the Asiasari radix extract proved to be effective in promoting hair growth. It also contributes to promoting the phase of a rapid hair growth, the anagen phase; cellular proliferation of the dermal papilla and to the increased ability to absorb cysteine in the hair follicle, which results in protein synthesis in the hair follicle. However, no impact on the activity of 5-alpha-reductase was discovered. Potentially, treating androgenic alopecia with this vegetable extract could be a valuable alternative to commonly used hair loss inhibitors, such as minoxidil, finasteride, or dutasteride. So far no research has been conducted on the hair loss inhibition of Asiasari radix on humans.

Zizyphus jujuba

Over one thousand plants have been studied recently to assess their impact on hair growth promotion. Despite the fact that vegetable extracts are not that commonly used in the treatment of androgenic alopecia, alternative medicine is becoming increasingly more popular. The plant that has proved to be effective as an androgenic hair loss inhibitor, is *Zizyphus jujuba*. *Zizyphus jujuba* is a plant widely distributed in Europe and Southeast Asia. It reveals medicinal properties, including analgesic and anti-diabetic. Indigeneous tribes used bark from this tree for birth control purposes (12).

The experiment was conducted on 5-week old mice with shaven backs so that *Zizyphus jujuba* seed essential oil could be applied topically. The treatment lasted for 7 days, and its efficacy was evaluated immediately after the therapy and after 14 and 21 days after the treatment. Ten randomly extracted hairs were examined with respect to their length, thickness and mass[12].

TABLE IV

Experiment results testifying to the efficacy of the essential oil from Ziziphus jujube seeds.

Zizyphus jujuba essential oil action	**Dosage applied**	**Results after 21 days**
HAIR LENGTH	1 μL	Hair growth increased on average up to 10.02 mm with reference to the control group; on average 8.94 mm.
HAIR THICKNESS	1 μL	Hair thickness increased up to approximately 4.8 mm in comparison with the control group, with 4.1 mm.
HAIR MASS	1 μL	Hair mass increased up to approximately 54 mg/cm^2 in comparison with the control group, with 50 mg/cm^2.

Summing up, essential oil from *Zizyphus jujuba* seeds has a beneficial effect on hair growth. After 21 days of experiment, hair count on the backs of the treated mice considerably outnumbered the hair count on the backs of the mice that had not been treated with this essential oil. Such a comparison makes it possible to verify that the number of hairs in the phase of the rapid hair growth, i.e., anagen, increased. Hair mass and thickness increased as well. Unfortunately, the exact mechanism of action has not been discovered yet; nevertheless, the experiment substantiates the statement that the oil used undoubtedly stimulates hair growth. So far this kind of study with the use of *Zizyphus jujuba* has not been conducted on humans.

Fallopia multiflora

Increasingly more studies are being conducted in order to evaluate the activity of traditional herbal medicines that prevent hair loss. One of the plants from this group is *Fallopia multiflora*, known as Fo-Ti and *Polygonum multiflorum*. It is commonly used in Eastern Asia in patients suffering from hair loss and as lipid plasma level lowering food supplement (13). Hye-Jin Park et al. decided to conduct research on the impact of *Fallopia multiflora* on hair growth[14].

Study findings confirmed the efficacy of fallopia extract in promoting hair growth. In the group treated with the vegetable extract a considerable increase in the hair length occurred in comparison with the control group. The plant had contributed to the increase in hair length. The experiment proved that the intense hair growth phase was triggered through the activation of beta-catenin, which plays a crucial role in regulating hair follicle growth. Before, experiments concerning the action of *Fallopia multiflora* on humans had not been conducted.

TABLE V

Results confirming the efficacy of Fallopia multiflora extract.

Fallopia multiflora action objective	**Extract concentration used**	**Results after 4 weeks of application**
NUMBER OF HAIR FOLLICLES	4.7 mg/ 12 cm^2	In every following week the number of hair follicles increased. After 4 weeks of application the average number of follicles was 15, in comparison with the control group, with 11 follicles on average.
HAIR LENGTH	4.7 mg/ 12 cm^2	In every following week hair length increased. After 4 weeks it was 65 mm on average in comparison with the control group, with the average 55 mm.

Minoxidil

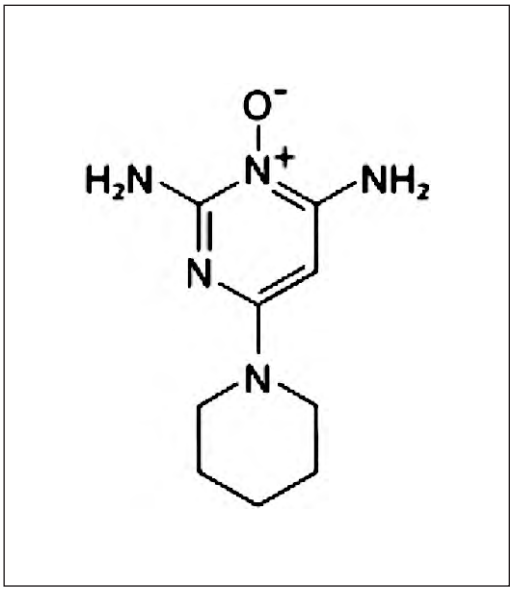

Fig. 3 *Minoxidil.*

Minoxidil is a 2,4-diaminopyrimidine derivative. It was introduced to therapeutics in 1970 as an oral drug for severe hypertension in the cases of resistance to other medications. During minoxidil therapy there appeared a side effect, namely excessive hair growth. The phenomenon raised interest and this led to the decision to use this substance in androgenic alopecia treatment. Since 1986 minoxidil has been classified as a substance promoting hair regrowth[1]. Despite over 20 years of research still the knowledge of precise mechanisms of action of minoxidil on hair growth is limited. There are numerous possible explanations for the influence of the medicine on hair, e.g., by promoting its growth rate; shortening the telogen phase, while prolonging the intense growth phase, i.e., anagen; or by increasing hair thickness and length[15].

Mi Hee Kwack et al. decided to undertake research in order to assess how minoxidil affects hair. The recent experiments on mice have revealed that beta-catenin is active in anagen hair, whereas when a premature catagen phase occurs, its activity is inhibited. The findings indicate that due to the presence of beta-catenin the intense hair growth phase may be prolonged. A topical treatment with minoxidil promotes hair growth in the androgenic hair loss of the male type, which suggests that it can have an impact on the prolongation of the anagen phase. The study was conducted on a hairy scalp sample, obtained from men suffering from androgenic alopecia in order to isolate hair follicles needed for the experiment. In order to evaluate if minoxidil initiates the anagen phase, an in vivo study was performed on 7-week old mice. Mice's backs were shaven and the hair growth was synchronized to the telogen. The therapy with 3% minoxidil lasted for 10 days. A profound delay in the catagen phase and the accumulation of beta-catenin in hairs were observed (15).

The mechanism of action of minoxidil rests upon its impact on the hair growth cycle; it shortens the resting phase, and at the same time it prolongs the hair's intense growth phase. This occurs as a result of the activation of beta-catenin in the hair follicle. Minoxidil turned out to be efficacious in promoting hair growth. Due to that, it can be used to treat androgenic alopecia.

Aminexil and SP94

Aminexil and SP 94 are actives present in L'Oreal market products recommended for hair loss. According to the manufacturer's claim, the products simultaneously strengthen the hair shaft and increase the number of hairs. Aminexil is 2,4-diaminopyrimidine 3-N-oxide.

SP94 is 6-O-linoleyl-D-glucose, which can take part in ceramide synthesis and provide structural ingredients for a strong, structurally homogeneous and thick hair shaft. L'Oreal experts conducted a study on Aminexil efficacy in a single blind trial. The study lasted for 3-6 months[16].

Chromatography analysis revealed that the product's mechanism of action rests primarily upon the transformation of a SP94 molecule into lipids (including ceramides - EOS and NS), which are responsible for the cohesiveness of the molecules that build the hair follicle and prevent hair sheath fibrosis [16].

Independent clinical trials with the use of products containing a mixture of Aminexil and SP 94 were conducted on a group of 180 people by Camacho et al. findings obtained in the trial confirm the inhibition of hair loss; however, the regrowth of lost hair was not confirmed 17. The actives present in the preparation may constitute an addition to the everyday hair care regime for the hair with the symptoms of androgenic alopecia.

TABLE IV

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CONCLUSION

The progress of androgenic hair loss can be stopped primarily through 5-alpha-reductase inhibitors. There are pharmacological preparations available on the market. They are formulated for topical use and promote the improvement of hair condition in the case of male pattern androgenic hair loss. A therapy with the use of such substances as finasteride dutasteride, and minoxidil is time-consuming and requires regularity in its application. The interest of scientists in traditional, natural methods of preventing hair loss, including herbal medicines, is increasing. Herbal preparations based on vegetable extracts from the plants, such as Fallopia multiflora, *Asarum europaeum* and Zizyphus jujube may constitute a perfect alternative to pharmacological preparations, considering the risk of adverse side effects associated with taking the latter. There are also typically cosmetic methods to limit hair loss in balding, e.g., using preparations containing 2,4-diaminopirymidine-3-N-oxide.

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Cosmetic Regulation of Sebaceous Gland Activity

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Key words: *Sebum; Sebaceous gland; Hormones regulation;*

Summary

There is over one hundred thousands of sebaceous glands in humans. They are uneven distributed all over the skin. Sebaceous glands belongs to holocrine type glands. Their activity is regulated by both internal and external factors and active substances. To the endogenous factors belongs hormones mostly androgens and progestagens. Estrogens play the minor role inhibiting the sebum secretion. Other hormones like propiomelanocortin and somatotropin have some activity but are not very important for the sebaceous glands secretion. The development of those glands is strongly determined on the presence of several cytokines like IL-1, TGF-β, EGF and IGF-1. Also the PPAR (α,β and γ) receptors are engaged in the sebum composition and releasing control.
Among the exogenous substances on the top of list are retinoids, particularly 13-cis retinoic acid which inhibits the activity and size of sebaceous glands even by 90%. Of importance are also compounds inhibiting 5-α-reductase like organic acids zinc salts, zinc oxide, azelaic acid and vitamins of the B group the proven activity have pyridoxal phosphate (vitamin B6) .
Indirect action provide also some skin microrganisms producing many substances which stimulate sebaceous gland and antioxidants inhibiting sebum trasformations.

Riassunto

Il corpo umano è ricoperto in modo irregolare da migliaia di ghiandole sebacee che, distribuite nelle diverse zone cutanee, svolgono l'attività secretoria regolata da fattori endogeni ed esogeni. Tra i fattori endogeni, i più importanti sono gli ormoni androgeni e progestinici, mentre gli estrogeni svolgono un ruolo secondario di inibizione della secrezione del sebo. Altri ormoni quali la propiomelanocortina e la somatotropina, pur svolgendo una certa attività, non hanno un ruolo determinante per la secrezione sebacea.
Lo sviluppo delle ghiandole sebacee è fortemente influenzato dalla presenza di molte citochine quali:

IL-1, TGF-Beta, EGF e IGF-1. Anche i recettori PPAR (alfa, beta e gamma, sono coinvolti nella composizione e nella secrezione del sebo.
Tra le sostanze esogene il primo posto è rappresentato dai retinoidi, in modo particolare dall'acido 13-cis retinoico che inibisce al 90% sia l'attività che le dimensioni delle ghiandole sebacee. Rivestono una certa importanza anche i composti che inibiscono l'attività della 5-alfa reduttasi quali i sali di zinco, l'ossido di zinco, l'acido azelaico e le vitamine del gruppo B, soprattutto il piridossal fosfato (vit B6).
Una azione indiretta è svolta anche da alcuni microorganismi che producono molecole che stimolano la produzione delle ghiandole sebacee, o antiossidanti che ne inibiscono la trasformazione in sostanze maleodoranti.

The sebaceous gland

The sebaceous glands are simple or compound clustered structures (acini), which develop embryologically in the upper part of a hair follicle. Their development starts in the third month of a human fetal life. There are two cell types in sebaceous glands – the lipid producing sebocytes and keratinocytes – the duct builders. Their holocrine lipid secretion is released into a common duct with an orifice to the hair follicle, placed at the funnel level 200 - 500μm under the skin surface (1), (2), (3). The sebaceous glands, well developed in the embryonic period, produce vernix caseosa, which plays an essential role in protecting the skin and the organization of the epidermal barrier of the neonate. Among others, along with sebum, onto the surface of the skin photoprotective, inhibiting oxidation processes and anti-irritant substances are secreted. A significant role is also played by one of the main components of sebum - sapienic acid (C16:1, n6) with antimicrobial properties, like antimicrobial peptides whose secretion is induced by the presence of bacteria, (3), (4). After birth the activity of sebaceous glands diminishes and maintains at the lowest level up to period of pubescence. The enlargement of the sebaceous glands is one of the first changes occurring during puberty. All of the above changes are hormonally controlled (1).

The physiology of the sebaceous glands

In man there is about 100000 sebaceous glands distributed all over the skin. The largest number is located on the scalp, and the T-zone (forehead, nose, chin) and the front and back of the gutter sweat torso. These areas are prone to oily skin. One follicle is usually surrounded by 3-5 glands, having one common wire lead-out. On the scalp follicles and glands are large. The size of the gland influences, inter alia, the amount of sebum produced and secreted to the skin surface (1), (5).

The sebaceous gland belongs to holocrine glands, composed of rapidly dividing flat peripheral cells. As the cells approach the central part of the gland, they lose the ability to divide and undergo disintegration. The time of transition of a descendant cell, already proliferating, to the phase of a mature cell, i.e. the differentiation period, lasts for over a week. After 8 days a sebocyte turns into sebum. The pressure exerted by the liquid accumulated in a vesicle leads to the final phase of a natural apoptosis. As is easy to calculate, sebocyte's turnover time is approximately 3 weeks. The vesicle bursts and the secretion gets into the duct lumen. Then, sebum is released onto the surface of the epidermis (1).

The cells in an outer layer of the acinus, which rests on an external membrane, are the reproductive cells. The synthesis of lipids takes place in a smooth endoplasmatic reticulum. Next they are packed to Golgi apparatus, forming the drops of fat inside the cell. After differentiation, the nucleus of sebocyte diminishes and disappears. The remaining cellular structures are also eradicated. Finally the mature sebocyte explodes releasing its entire content to the gland duct. The liquid mixture of lipids floats through the sebaceous duct consisting of multilayered cuticle up to the funnel and hair channel, reaching skin surface (1), (2).

The sebum biosynthesis

The most visible function of sebaceous glands is sebum secretion. The humans are born with a certain number of sebaceous glands, which reveal different activity and efficiency in various stages of life. The sebaceous glands fully develop by the 6 month of life. The intensification of sebum secretion takes place few hours after birth and reaches maximal values during the first

week and gradually slows down. It rises again at the age of 9 years, together with an increased activity of adrenal glands and lasts up to the age of 17 throughout the whole period of pubescence. (6), (7).

Chemically sebum is a mixture of the few hydrophobic groups of compounds with the prevailing glycerides. The sebum includes mono-, di- and triglycerides (depending on age: about 45-50%), conditioning the viscosity of sebum, waxes (20-25%), free fatty acids (about 16%, including 10% of unsaturated linoleic acid), ensuring adequate sebum liquidity, squalene (approximately 10-12%), cholesterol esters (3.5-4%), free cholesterol (about 1-1.5%), other sterols (1-2%). Daily secretion standard is about 1-2g (8), (9), (10).

The hydrolysis of human sebum delivers a mixture of numerous fatty acids with branched chains. The predominant forms are iso and anteiso isomers (with a methyl substituent group at the third from the end or penultimate carbon atom in chain), although also acids with branched chains and substituent methyl groups in different positions are present, as well as the acids with two or three methyl substituents. Almost half of the fatty acids of human sebum consist of the monounsaturated acids with double bond in n-6 position. The model of n-6 unsaturation seems to be characteristic for a human. For the other analyzed mammalian species, which were investigated n-9 unsaturated chains are characteristic(1).

The analyses proved that the composition of sebum fatty acids changes with age. The sebaceous fatty acids differ during fetal period and before pubescence from the fatty acids of the adults. The differences include length of chains, proportion of monounsaturated fatty acids n-6 to n-9 and with a number of various branchings. The sebum of children before puberty is different from the one of the adults with a larger amount of cholesterol, its esters and a smaller amount of wax esters. The holocrine nature of sebum secretion reveals a mechanism, which may cause the changes of sebum composition without any changes during synthesis of sebaceous lipids. Because it takes sebocyte disintegration to release sebum, the product incorporates the synthesized lipids as well as the ones from the structure of sebocyte cell membranes. The lipids from both sources have different composition. The synthesized endogenous sebaceous lipids contain mainly squalene, wax esters and n-6 unstaturated fatty acids, while the exogenous lipids from the sebocyte cell membranes abound in cholesterol, n-9 fatty acids and linoleic acid. During the pubescence period, the amount of endogenous sebaceous lipids increases in sebum, while the exogenous types proportionally diminish (1).

The endogenous control of the sebaceous gland

The development and function of sebaceous gland is regulated by the number of factors such as transcription factors, hormones, hormone nuclear receptors, retinoids, ILGF and cytokines. The hormones significantly influencing the activity of sebaceous gland are the androgens, estrogens, growth factor, corticoliberin, insulin and glicocorticoids. They mostly stimulate secretion of sebaceous lipids, while the retinoids, cytokines and hormone nuclear receptors seem to be the promising inhibitors of sebum synthesis (2), (11).

The factors regulating development of sebaceous glands still long for detailed investigation. The important mediators of gland development are the epidermal growth factor (EGF), homeotic HOX genes and sonic hedgehog genes (SHH), which when inhibited, cause sebocyte growth disorders.

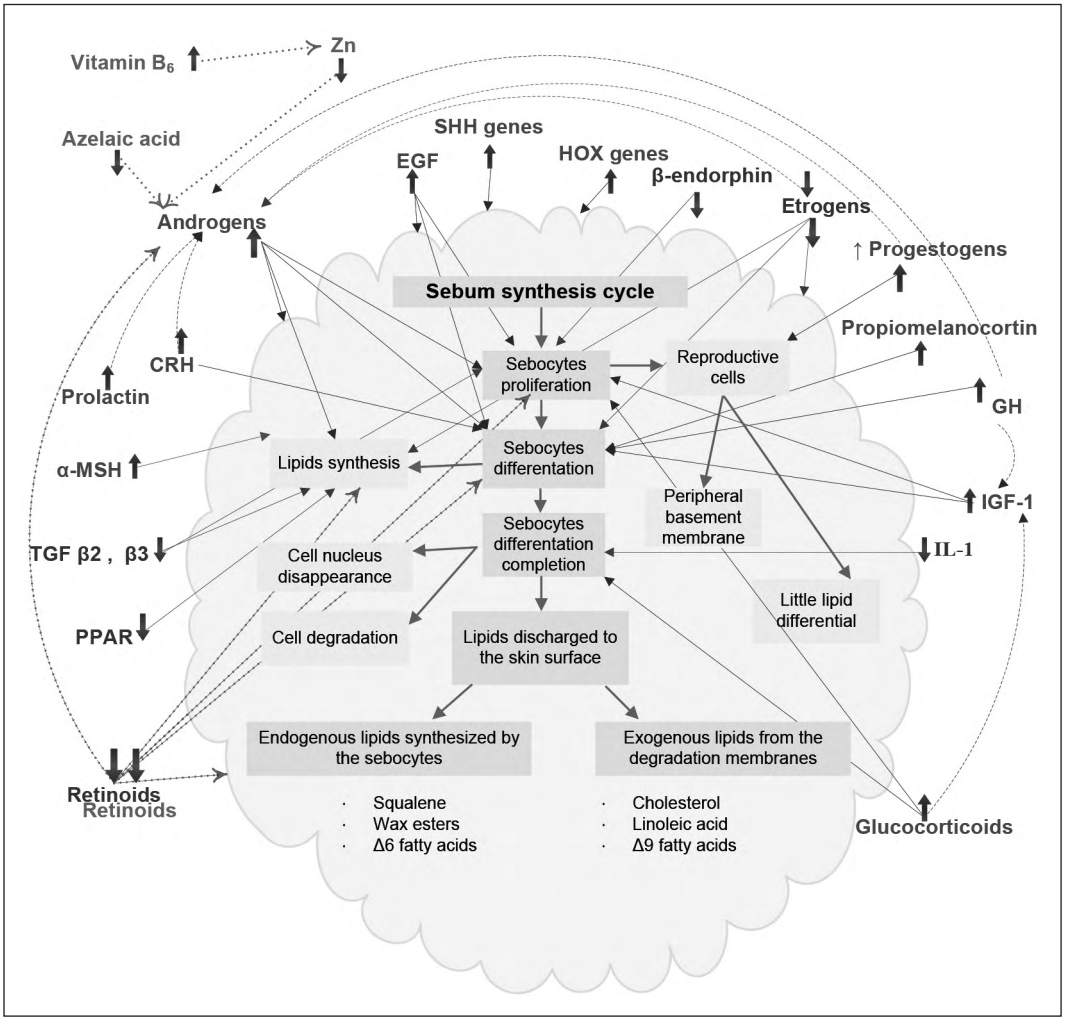

Fig. 1 *Development and control the sebaceous gland. Somatotropin (GH); Corticotropin-releasing hormone (CRH); Insulin-like growth factor-1 (IGF-1); Epidermal growth factor (EGF); Transforming growth factor (TGF); Interleukin-1 (IL-1); Peroxisome proliferator activated receptors (PPAR); α-melanocyte-stimulating hormone (α-MSH).*

The androgens

The ability of sebaceous gland to respond to androgens is determined in fetal period. The growth of sebaceous glands and their differentiation requires the influence of androgens and a few other biological factors (2).

The androgens influence the sebaceous glands through mitosis and lipogenesis stimulation. The human sebaceous glands seem to respond to testosterone and other androgens. Both dehydroepiandrosterone (DHEA) and androstenedione stimulate sebum secretion in the areas retarded by estrogens. However such effects were not observed in case of androsterone. The most effective androgens are those with 17β-hydroxy

group, like testosterone, 5α-dihydrotestosterone (DHT), 5α-androstane-3β,17β-diol. Androgene-sensitive skin areas like sweat glands, hair follicles and sebaceous glands, metabolise androgens in accordance with characteristic pathways. Although the skin is unable to synthesize androgens *de novo* from cholesterol, it incorporates all the indispensable enzymes to transform prohormones DHEA and androstenediones into testosterone and the strongest androgen - dihydrotestosterone (DHT). The metabolic pathway, illustrating formation of active androgens from dehydroepiandrosterone sulfate (DHEAS) is shown in the Figure.4 (1), (6), (12), (8).

The activity of the enzymes participating in metabolic pathway in skin is different depending on the body area. In the non-seborrheic areas prevails the oxidizing action of 17β-dihydrogenase (17β-HSD) (the transformation of estrogens and testosterone into less active precursors). Meanwhile in sebaceous glands in the acnegenic regions, like face skin and scalp the activity of 5α-reductase (5α-R) is 2-4 times larger than 17β-dihydrogenase. The isozyme of the first type of 5α-reductase participates in transformation of testosterone into dihydrotestosterone (DHT), the strongest among androgens stimulator of sebum secretion. (13), (12), (2).

The estrogens

The estrogens undoubtfully influence the human sebaceous glands, by inhibiting their activity. On the contrary to the androgens, which stimulate mitogenesis and sebogenesis, the estrogens influence only sebum secretion and don't participate in the cells proliferation. It is an interesting problem whether the estrogens react circumferentially or locally as inhibitors of secretion of endogenous androgens. It seems less likely because the inhibiting reaction is observed also in the presence of supplementary testosterone. On the other hand it was demonstrated in the foreskin glands of mice and rats, that estradiol inhibits a metabolism of testosterone, which proves that estrogens can directly influence androgens performance. There are numerous studies on the circumferential and local inhibiting action of androgens in relation to the differentiation of sebaceous lipids (2), (1).

The progestagens

We can observe a profound impact of progesterone on the amount of produced sebum. Theoretically, progesterone should reduce seborrhea as it inhibits the activity of 5-alpha –reductase, which regulates the amount of dihydrotestosterone (DHT), thus lowering oil secretion. Unfortunatelly, it is not that simple (6), (14).

The amount of produced sebum is not affected by the mere presence of progesterone, but in fact, its ratio to estrogens. If the progesterone concentration is higher than that of estrogens, and the amount of progesterone in the body is higher than the amount of estrogens, a rapid sebum production occurs. The adverse effect of progesterone activity is attributed to the inhibition of the beneficial effects of estrogen activity.

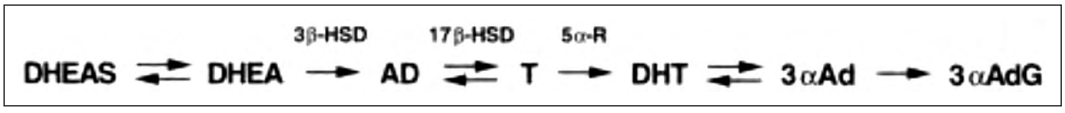

Fig. 2 *A schematic representation of androgen metabolism in the skin. The enzymes 3β-hydroxysteroid dehydrogenase (HSD), 17β-HSD, and 5 -reductase (5 -R). Dehydroepiandrosterone-sulfate (DHEAS); dehydroepiandrosterone (DHEA); androstenedione (AD); testosterone (T); 3 -androstanediol (3 Ad); 3 -androstanediol glucuronide (3 AdG)[6].*

In females, the quantitative ratio of these hormones is strictly dependent upon the luteinizing hormone, called lutropin (LH), needed for the proper course of the menstrual cycle. The peak concentration of this hormone is observed in the last days of the follicular phase of the menstrual cycle. Lutropin is responsible for the luteinizing of the corpus luteum, i.e., for the transformation of the granulosa cells of the ruptured ovarian follicle (the follicle surrounding the oocyte) into lutein cells (producing progesterone). The level of these hormones is strictly connected with the phases of the menstrual cycle and with the woman's current physiological condition. As is known, the progesterone level also rises in pregnant women, because the hormone is responsible for supporting gestation, and, adequately, in the second part of the menstrual cycle it prepares the body for embryo implantation; that is, in the period of increased sebum production (11), (13), (7).

The prolactin

It is produced in large amounts during the pregnancy and stimulates sebaceous glands. During hiperlactemia it participates in development of hirsutism and seborrhea in women (11), (13).

The corticoliberin (CRH) – corticotropin releasing hormone

The Pro-CRH transformed into CRH seems similar regardless its location (whether circumferential or locally present – including skin structures). The particular neuropeptides, hormones, cytokines function as signal transmitters in communication between the three cooperating systems the pituitary gland - hypothalamus – adrenal glands. Reacting to stress, the skin can also produce similar mediators. The research shows that human sebocytes have the expression of functional receptors, for a hormone releasing corticotropin (CRH), melanotropines (α-melanocyte-stimulating hormone; α-MSH), β-endorfins, vasoactive intensinal polypeptide (VIP), neuropeptide Y and calcitonin gene-related peptide (CGRP). After establishing ligands bonds, the receptors initiate secretion of inflammatory cytokines, proliferation, differentiation, lipogenesis and androgens metabolism in sebocytes (5), (15).

The CRH is active in human sebocytes and may for them an autocrine hormone with homeostasis of differentiation activity, it directly causes even double increase of lipid synthesis, without stimulation of proliferating cells and growth of expression of mRNA hydroxysteroid 3β-dehydrogenase (the enzyme, which converts dihydroepiandrosterone to testosterone in human sebocytes). These observations confirm participation of CRH in clinical development of acne, skin aging, excessive cornification and other skin disorders related to the changes of secretion of sebaceous lipids (5), (15).

The propiomelanocortin (POMC)

The propiomelanocortin (POMC) is released by the pituitary gland and functions as a precursor hormone for the substances influencing activity of sebaceous gland, like adrenocorticotropin (ACDH), melanocyte-stimulating hormone (α-MSH) and β-endorfin. The melanocortins, especially ACTH and MSH are important mediators of stress reaction of classical HPA line (hypothalamus-pituitary gland-adrenal gland). It was discovered that these both hormones are also produced by skin keratinocytes, melanocytes and cultured sebocytes. The melanocortins cause various biological effects by bonding and activating their receptors on a plasmatic membrane (16).

The POMC peptides stimulate differentiation of sebocytes.

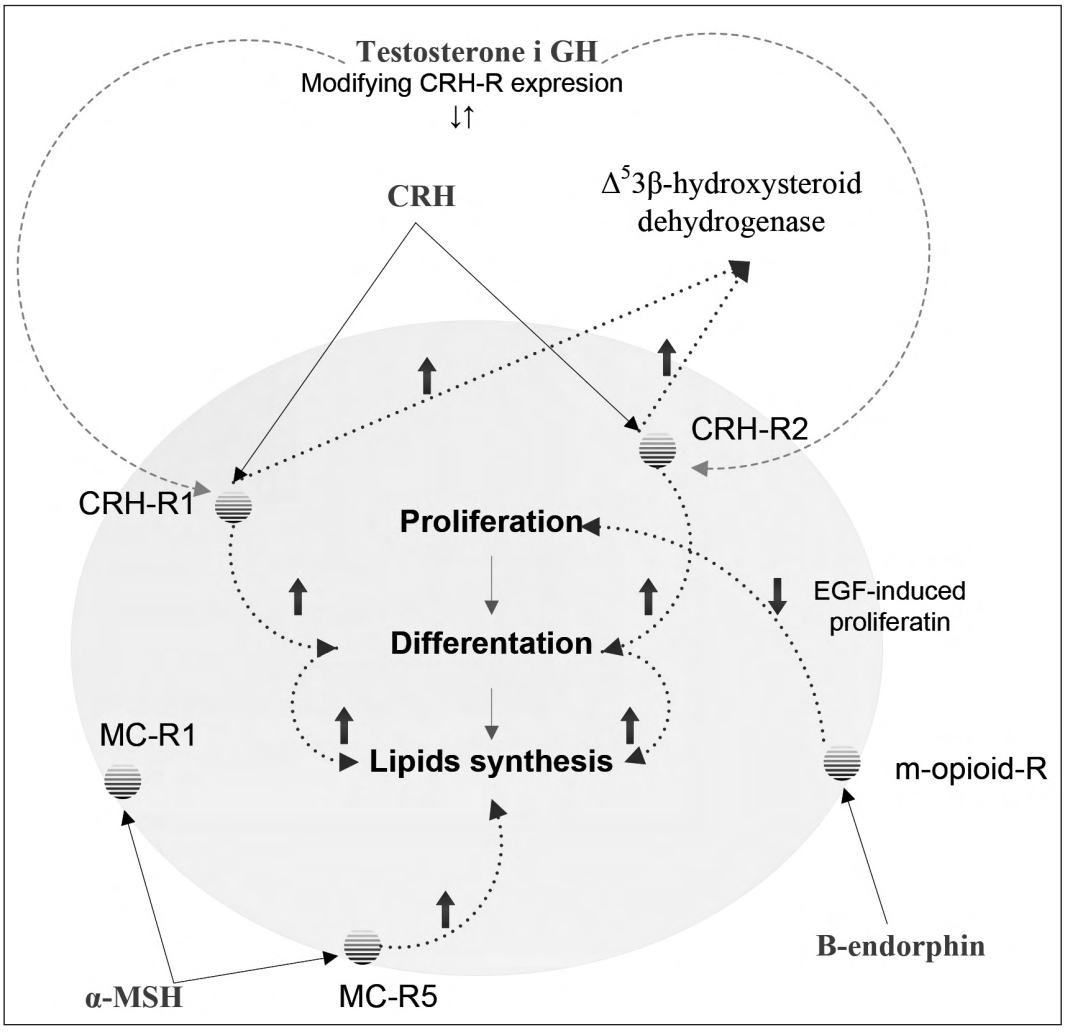

Fig. 3 *Functioning of sebocyte. Corticotrophin receptors (CRH-R1, CRH-R2); Melanocortin receptors (MC-R1, MC-R5); opioid receptor (m-opioid-R); corticotropin-releasing hormone (CRH); α-melanocyte-stimulating hormone (α-MSH); growth hormone (GH);*

The secretion of human sebaceous lipids may increase as a result of α-MSH hormone influence on melanocortine receptor MC5-R. This hormone influences also squalene synthesis, which is an important sebum ingredient. The increased squalene production and induction of MC5-R takes place only during sebocytes differentiation, which suggests an active role of MC5-R in sebum synthesis (16).

The β-endorfin is a paracrine neuropeptide for human sebocytes, which inhibits their proliferation, caused by the epidermal growth factor (EGF), when bounded with opioid receptor(5).

The somatotropin (GH)

The strong expression of a growth hormone (GH) is visible on human skin. This hormone is engages in the development of sebaceous gland. It stimulates differentiation of sebocytes and increases the influence of a 5α- dihydrotestosterone on the synthesis of sebaceous lipids and induces IGF. The GH doesn't stimulate DNA synthesis in sebocytes (2), (13), (5).

The insulin-like growth factor-1 (IGF-1),

The main activity of IGF-1 (Insulin-like growth factor-1) is directed to stimulate sebocyte proliferation, while additionally it plays an important role in their differentiation.

The epidermal growth factor (EGF),

The EGF (Epidermal growth factor) strongly determines development of sebaceous glands and additionally regulates sebocytes differentiation (2).

The transforming growth factor (TGF)

The TGF β_3 and β_2 but no β_1, (transforming growth factor) reduce cell proliferation and lipogenesis in the human sebaceous glands.

The interleukin-1 (IL-1)

The interleukin-1 (IL-1) is an important cytokine in skin, where it plays a role of mediator in inflammatory reactions. It occurs in sebaceous glands and their ducts. The increased expression of IL-1 reflects in blackheads in the skin. IL-1 also retards sebocytes differentiation, but don't influence their proliferation(2).

The glycocorticosteroids

They perform stimulating activity on the proliferation and differentiation of sebocytes and increase the performance of IGF-1 on the sebaceous cells(13).

The peroxisome proliferator activated receptors (PPAR)

The transcriptive factors (PPAR peroxisome proliferator- activated receptor) belong to the superfamily of hormone nuclear receptors, which contains also steroid receptors, thyroid hormones receptors, vitamin D receptors and retinoic acid receptors. These receptors are engaged into control of gene coding proteins, responsible for lipids metabolism and are activated i.e. by physiological concentrations of fatty acids as well as by the medicines diminishing the excess lipids in blood. In human sebocytes there is an noticeable expression of PPAR-α, PPAR-β and PPAR-γ.
The activators of PPAR-α and PPAR-γ regulate differentiation of sebocytes, slowing down sebaceous lipogenesis and reducing synthesis of specific sebaceous lipids: squalane and triglicerides, while the activators of PPAR-β influence lipids formation in sebocytes and keratinocytes (2), (11).

The retinoids

Of all the retinoids the strongest action on the sebaceous gland has an 13-cis-retinoic acid (isotretinoin). It inhibits the activity of sebaceous glands and reduces their size even by 90%. Sebaceous glands undergo involution, with resulting limited lipid production. Isotretinoin has bacteriostatic properties, inhibiting the development of *Propionibacterium acnes*. It stabilizes the natural flora on the surface of the skin. It normalizes the keratinisation process due to the inhibition of the proliferation of sebocytes, and it

probably restores the proper process of cell differentiation. This substance considerably dries out the skin, contributing to its fine texture. It reveals teratogenic properties. It may lead to fetus deformation. It reduces telomerase activity, which promotes the death of epidermal cells, and it reveals pro-apoptotic activity. Thanks to this, pathologically changed epidermal cells die rapidly, whereas new and healthy ones take their place. Isotretinoin inhibits seborrhea and its long-term efficacy is estimated at 70-85%. Isotretinoin also acts as an inhibitor of 3-alpha-hydrosteroids oxidation by retinol dehydrogenase, leading to the diminished amount of androstendione and dihydrotestosterone. Additionally, retinoic acid induces a rapid and short-term expression of the transforming growth factor (TGF). Isotretinoin is present in such preparations as Isotrexin for topical use, as well as Izotek and Roaccutane for oral application. It cannot be used in cosmetic preparations (17).

The exogenous inhibitors of activity of sebaceous glands

Many exogenous factors may stimulate action of the endogenous substances, which in case of sebaceous glands leads to the increased seborrhea. Moreover, the increased amount of lipids on skin, causes growth of skin bacteria. The larger number of microorganisms may cause irritations, that finally intensify seborrhea (7).

The most accurate method of improving skin condition and appearance in case of excess sebum secretion, should be topical application of formulas containing substances inhibiting activity of sebaceous glands.

One of the potential reduction possibilities of sebaceous glands activity is diminishing concentration of DHT in hair follicle area and sebaceous gland by inhibition of 5-α-reductase (7). The substances approved for use in cosmetics and performing in the described way are the zinc salts (mainly organic acids salts, glutaminic and pirolidonecarboxilic acids), pirydoxine (B_6 vitamin) and azelaic acid. The zinc performance on reduction of sebum secretion concerns both inhibition of action of 5-α-reductase and also inhibiting microbial lipases, disintegrating sebum triglicerides to the free fatty acids. Zinc in large concentrations may completely reduce activity of 5-α-reductase (7), (16), (18).

In case of azelaic acid the diminishing performance on 5-α-reductase is already observed in its small concentrations (0,2mol/L), while at 3mol/L almost complete reduction of enzyme activity is achieved(19). Additionally, azelaic acid like zinc, decreases number of the skin bacteria and the number of free fatty acids remaining from the disintegration of lipid compounds(20), (21).

The mechanism of action of B_6 vitamin is slightly different, for it doesn't inhibit directly 5-α-reductase. However it boosts up zinc performance as inhibitor of 5-α-reductase, doesn't influence activity of azelaic acid(19). Because of the large sensitivity, the B_6 vitamin is incorporated into cosmetics in a form of biological extracts i.e. yeast (7).

The different sebostatic mechanism present retinoids when applied topically. The compounds of retinoids like retinal, approved for cosmetics, or all-trans-retinoic acid used in dermatological preparations don't directly influence sebum secretion. Some indirect action may occur after conversion into isotretinoin in skin. Because of the fact that biochemical processes in skin will lead not only to the 13-cis-retinoic acid, but also other compounds, its activity will be much weaker that isotretinoin action (7).

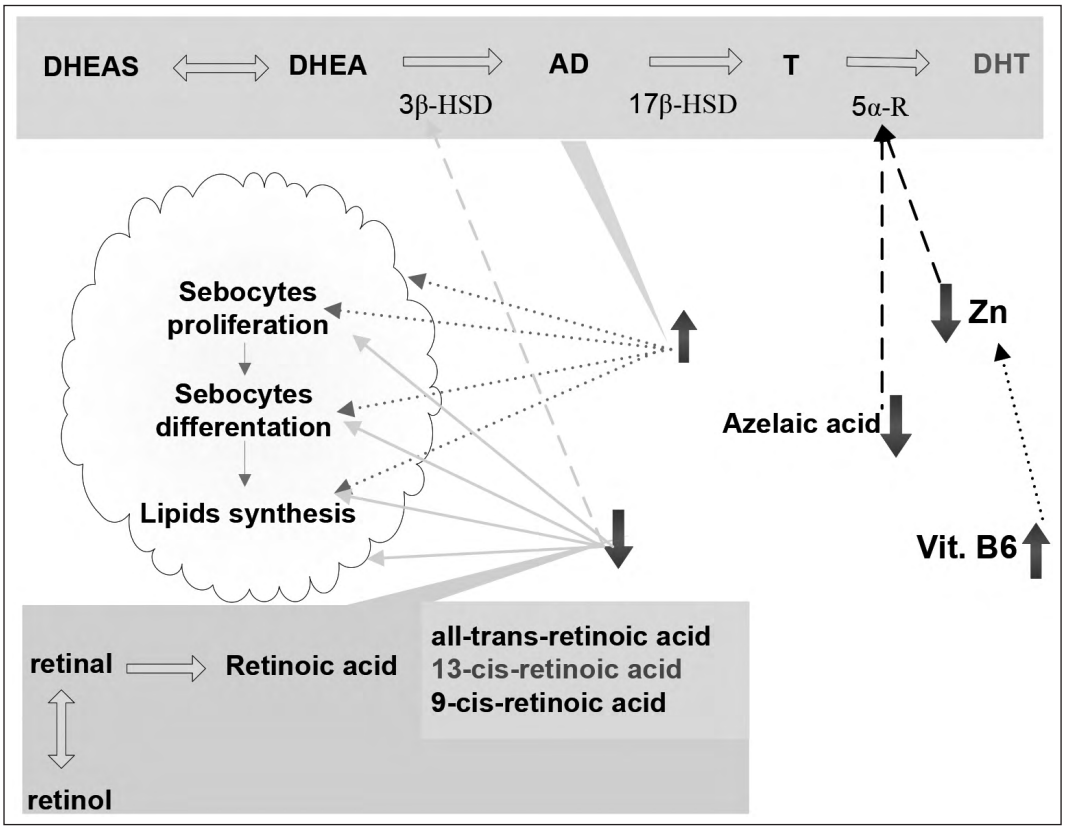

Fig. 4 *Sebaceous gland activity inhibition...*

Role of micro-organisms in stimulating sebaceous glands

Natural bacterial flora contains *Propionibacterium acnes*, a Gram-positive bacterium, which may turn into a parasite in the case of immune system deficiency and bacterial overgrowth. It also contains *Corynebacterium acnes, Brevibacterium acnes, Staphylococcus epidermidis*, physiologically producing exfoliatin, which stimulates exfoliation of the epidermis. In pathological cases the toxin leads to excessive epidermal exfoliation. Additionally, the flora contains a yeast species, *Malassezia furfur (Pirytrosporum ovale)* (10), (7). Its role is not known, though. However, it has been observed that the amount of yeast diminishes due to the antifungal treatment, resulting in the improved clinical results (sebaceous gland activity diminishes), and vice versa – in the periods of increased oil production the amount of yeast increases. *Malassezia's* metabolism changes sebum content. Certain substances are transformed into compounds that irritate the skin (22), (23).

Antioxidant role in the inhibition of sebaceous gland activity

Sebum content changes not only due to Malassezia furfur activity, but also during the

process of oxidation. In order to prevent this, a number of antioxidants are used. Antioxidants, such as vitamin E, coenzyme Q10, β-carotene and ascorbic acid belong, among others, to such substances. Their objective is to neutralize free radicals. These substances protect fats found in sebum against oxidation.

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Memories of a Cosmetically Disturbed Mind

by Johann W.Wiechers

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It is difficult for me to review this book written from a friend of mine not more living among us.
Many times I discussed some of the reported topics with Johann during meeting hold in different countries, underlining the same criticisms on the meaning *of natural*, the *consumer* perception on chemicals, the real *cosmetic activity*, or where Cosmetic Science is going.
I remember the discussion during the In-Cosmetics in Barcelona about some experimental data reported from a paper having not statistical significance. It was an *in vivo* study made with no more than 10 people divided in two groups! The obtained result was an 18% increase in moisturization with an evident *anti wrinkling activity!!!* This was one of the afternoon papers reporting all marketing claims without real results.
For this reason Johann declares on the book that "it is a disgrace for people who pay money to attend these talks to have such cosmetic rubbish being poured over them"! I agree with him that the cosmetic industry has to sell products with effectiveness and safeness supported by *in vitro* and *in vivo* statistically effective data, recovered by studies, organized from well known scientists. Moreover according to Johann, sustainability of environment and economic profit has to go in the same direction. An alternative paradigm of viewing the interaction between human activities, social conditions, and the environment is, in fact, the *green economy*, known as *bio economy* or *ecological economics* also. It is an approach based on integrating economic development, environmental sustainability, and social equality. Thus signalling pathways within plants and between plants and their environment are becoming to be elucidated to optimize their interaction with the biotic and abiotic environment. The expected rapid development in biosciences will greatly facilitate, therefore, the transition to a renewable, resource-oriented economy in the area of energy, materials and chemicals.
In the cosmetic field also, the combination of plant and fishery's breeding and industrial Bionanotechnology could serve as the basis of a Knowledge-based Bio-Economy (KBBE), according to the 2030 EU programs. This is why R&D has to be the pillar of the future sustainable cosmetic industry by a more secure energy supply, efforting to counter greenhouse gas emissions, minimised production of waste, as well as the conservation of ecosystems, rural social structures and employment, all to be obtained cost effectively.
The high standards of health care and the necessity to maintain wellbeing of an increased aging population, in fact, are the today's key drivers of Cosmetic and Medical research as the need for treatment of beauty appearance and degenerative and lifestyle-related diseases. Thus, the integration of molecular and cellular biology into clinical medicine will certainly make a change in Cosmetic

Dermatology also, playing a more prominent role in beauty, health, and preventive medicine. At this purpose, probably research on stem cells will result in the enhanced efficacy and fewer side-effects of future innovative cosmetic products and drugs.

On the other hand, advanced in Bionanotechnology will enable the development of innovative cosmetics, biomedical implants and man-made machine interface, providing the technological basis for smart textiles, which will be available to protect the body and compensate somatic deficiencies.

In conclusion, novel technologies, innovative cosmetics and tools will provide safe new products with added value for consumers. Going towards this direction plants will be highly potential sources of new enzymatic functions applicable to microbial biotechnology, so that our better understanding of microbial metabolism will give more opportunities to produce new products and develop biocatalysts for novel applications.

The model of a cell containing only a subset of genes and able to efficiently produce a defined product, employing metagenomics or protein engineering, may not be far a way. By all these new technologies we will obtain innovative ingredients more active at level of SC' layers. But for being effective these ingredients have to be delivered through the skin layers. According to Johann, "the clinical efficacy of a product is the result of the intrinsic activity of the active ingredient and its delivery to the site of action", so that it is necessary "to *deliver* the active ingredients to *have* active products. The solution to this problem can be defined in the *Four R's Skin Delivery:* to deliver the *Right* chemical to the *Right* site in the skin at the *Right* concentration for the *corRect* period of time". Thus," the delivery of an active molecule is predominantly determined by its physicochemical properties and by the vehicle into which it is included". As a consequence, there is no efficacy of a cosmetic product without penetration of the active ingredient. "Without penetration no delivery, and without delivery no active product"!

But according to the EU and international Rules, Cosmetics has to work exclusively on the skin surface because only pharmaceutical ingredients can penetrate. This is the reason of the *Mediocre Mediacracy* reported in the book.

According to Johann and to my paper published some years ago [EU borderline cosmetic products: review of current regulatory status. *Clinics in Dermatology* (2008) 26: 392-397] the final part of article 1, regulating Drugs or Medical Devices, as well Cosmetics declares the same functions "to restoring, correcting or modifying physiological functions". Thus, according to the European Directive which regulates their production and sale, cosmetic products, must not have therapeutic effects or claims, so that they should neither interact with the physiological mechanisms of the skin nor, more importantly, should penetrate it. This is the reason why the conclusive remark of the article was that: Cosmetics perform their specific functions *miraculously*. In fact, it is Interesting to underline that the simplest cosmetic emulsion, composed of oils and water, induce modifications on the skin physiology, as well as "petrolatum penetrates into the lipid-rich intercellular spaces of the SC, enhancing its barrier properties and making the horny layer pliable so that it does not crack when deformed", according to AM Kligman' studies.

Moreover it has been reported that petrolatum promotes wound-healing and prevents ultraviolet-induced tumors, even though it is not classified as a drug, but it is used as normal component of cosmetic formulations also.

In conclusion, on one hand the ultimate goal of Bionanotechnology is an understanding of the Impact of the environment on gene activities as well as the pathways of decentralised communication as in

plant and centralised communications as in human and animals. On the other hand it is necessary to define the mechanism of action of cosmetic products to eliminate the overlapping with drugs and medical devices. While the rules attribute to drugs a pharmacological activity and to medical devices a mechanical activity, cosmetics could have a *biological* mechanism of action. This means that it will be possible to solve the pathological problems by the use of drugs and medical devices both effective at level of cells affected by pathological diseases, while cosmetic products could have the primary function to "keep in good conditions" the cell suffering of minor disorders or mild skin abnormalities, such as dryness, eccessive sebum secretion, UV rays protection, photoaging, age-spots reduction, acne juvenilis etc.

In conclusion, taking into account the advancement of science in the skin biology and in the delivery system of the active ingredients, we believe that, without being considered as drug, the modern cosmetic product could support/restore physiological functions by a biological activity. Therefore a cosmetic product could have the possibility to solve the problem of the xerotic skin, restabilising the physiological skin equilibrium and, consequently, the ability to re-balance the biological process that regulate the turnover of keratinocytes with the right production of lipids and NMF. In this way it will be possible to discuss about the delivery of the active ingredients, the effectiveness of a cosmetic product that could really have a preventive activity, contributing to reduce the excessive consume of drugs and the increasing worldwide cost of all the Public Health Ministries.

I think this was the spirit of my friend Johann W. Wiechers in writing all the articles reported in this book. On one hand Biotechnology will have a fundamental role to play for sustainable chemicals manufactoring, providing a solution to overcome the most urgent global challenges as well as to address limited raw material resources, energy, water and global warming. On the other hand cosmetic industry has to give the right messages to mass-media underlining the necessity we have of a *clean Chemistry*.

For this reasons all the cosmetic chemists and dermatologists, as well as the biologists, pharmacologists, physiologists and marketing managers working in the cosmetic field has to be interested to have this book in their library for every day consultation with the scope to better understand what are the real rules governing an effective and safe cosmetic product and where Cosmetic Science is going, so well reported from a great scientist, unfortunately no more among us.

P. Morganti
Editor-in-Chief

Health Challenged Skin: The Estheticians' Desk Reference

by Morag Currin

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This Interesting book, especially written for aestheticians, gives them the possibility to analyze the skin correctly for helping their clients to obtain the desired end benefits, also when affected by diseases and particular skin manifestations. It provides an overview of the different skin types, the organization of the skin layers with the different cells involved, reporting a briefly description on the more known pathologies and therapies used to day.

While 27 pages of the book are focused to describe the elements comprising the skin' horny layer (stratum corneum) with its corneocytes embedded into the lipid lamellae, the majority of the book reports and describes one by one and alphabetically, many diseases/disorders impacting the daily work of the aestheticians.

"Understanding the structure and function of the stratum corneum (SC) is, in fact, of primary importance being the key of a healthy skin, associated with attractive appearance". Thus, the aestheticians have to know the fundamental role the keratinocytes play as well as the function corneocytes and lipid lamellae have to make and maintain the skin barrier as main protection from the environment and microorganisms' aggression.

SC, in fact, prevents not only the penetration of toxic substances and pathogens, but regulates also water loss and skin hydration. These particular skin layers are composed of corneocytes, dead squamous cells filled with keratin filaments enclosed by an envelope of cross-linked proteins and surrounded by a semi-continuous matrix of lipids. How are made the corneocytes? Born within the epidermis, at level of the basal layer, keratinocytes progressively move toward the skin surface, transforming themselves in corneocytes by a series of reactions. During this turnover process, the phospholipids, fundamental components of keratinocytes, is the cell membranes are broken down during their transformation to terminally differentiated corneocytes, producing glycerine and natural moisturizing factors (NMF). This glycerine, regulated by Aquaporin-3 will penetrate into the cells, for acting as skin moisturizer. By these reactions lipids and NMF also produced, constitute the skin hydrated barrier.

Lipids such as cerebrosides, ceramides, fatty acids and cholesterol, are organized in lamellar fashion, while NMF is principally represented from sugar-like compounds, urea, lactate aminoacids, and other polar hygroscopic molecules. In addition, the lipid layer surrounding the corneocytes helps seal them to prevent loss of NMF.

Other important protein-plays contribute to form the structure of SC,such as loricrin and involucrin, having the role to link lipids with the key elements of the corneocytes structure. Moreover, the *connections* that hold the corneocytes together are a specialized protein structure called corneodesmosomes.

An other essential protein of the SC is filaggrin, which plays an important role for the production of NMF, so that its genetic induced lock, seems to be at the origin of allergies and pathologic diseases, such as ichthyosis,atopic dermatitis, psoriasis, and eczema. The NMF components, in fact, adsorb water from the atmosphere and combine it with their own water content, allowing the SC to stay hydrated despite exposure to the environment aggression. Thus, if these proteins are dysfunctional or absent, the lipids can't stay anchored onto the cells and the barrier function is impaired, resulting in dry, sensitive skin and/or severe dryness, skin lesions, etc. Everything in SC is, therefore, linked in a flexible manner to give the skin its structural and functional strength.

Additionally keratin, the main component of the corneocyte, needs to hold enough water to keep this hard cell flexible enough as well. Other link factors such as stress, depression, and anxiety, affect our emotional well-being further increasing skin problems.

According to the book suggestions it is necessary that the aestheticians understood the basic necessities of the client's body and mind in order to treat them in the most beneficial and safe way. Thus the necessity to select the best cosmetic products, supporting and moving also towards the Mind-body-spirit philosophy of the consumer. Looking after a client from a Mind-body-spirit is, in fact paramount. This is the reason why the majority of the book has been dedicated to give all the news necessary to the aestheticians for recognizing and differentiating many of the diseases affecting the skin, managing also the overall health and mental well being with the best available treatments, without trying to be a physician.

In conclusion this Interesting book has to be considered as the new Bible for all the aestheticians that could like to have a deeper and clear knowledge on the skin activities, and on the more frequent skin diseases, giving them a simple successful guidance for their daily work in today's Office, SPA, and Salon.

P. Morganti
Editor-in-Chief

11th International Congress of Cosmetic Dermatology
September 26-28, 2014, Beijing

Under the Auspices of:
International Society of Cosmetic Dermatology
Organized by:
Chinese Society of Dermatology
Chinese Medical Association
Co-organized by:
Peking University People's Hospital
No.1 Hospital of China Medical University

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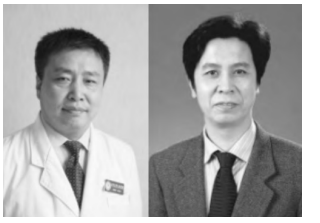

WELCOME MESSAGE

The 11th International Congress of International Society of Cosmetic Dermatology will be held in Beijing China from September 26 to 28, 2014. We extend our sincere invitation to our colleagues and friends worldwide to this event.

Many famous professors will attend this meeting and will give lectures, among whom are Prof. Pierfrancesco Morganti, Prof. Henry W. Lim, Prof. Philippe G. Hembert, Prof. George Cotsarelis, Prof. Sewon Kang. The new concept and new technology in cosmetic dermatology will be the hot topics. These include advances in laser treatment, use of retinoic acid, skin rejuvenation, creotoxin, filler, beauty from inside out, progress in dermatologic surgery; photoprotection, dialogue between the east and the west on antioxidant usage, skin imaging and skin bioengineering, effects of traditional Chinese medicine, complications and pitfalls in cosmetic dermatology, cosmetic procedures(live demo); active ingredients in cosmetics. We believe that all these topics will make this congress of high academic level and of great success.

ISCD, focusing on the international frontiers and hot spots of cosmetic disorders, will deliver to you in-depth understanding of the frontier theories and techniques in this field.

This congress will also provide a opportunity for participants to meet old friends and making new friends.

Beijing is a modern and beautiful city with over 3000 years' history. We sincerely hope that you will enjoy the meeting and Beijing as well.

Welcome to Beijing!

Presidents

Jian-Zhong Zhang, MD.

Xing-Hua Gao, MD, Ph.D.

HONORARY PRESIDENTS

TENTATIVE SPEAKERS

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	Before Jun.31st	Before Aug.31st	After Sept.1st
Full Participant	200	350	400
ISCD Member	150	300	350
Resident/Student/Nurse	100	250	300
Accompanying Person	100	100	100

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HOT TOPICS

1) What's new in laser and other light devices
2) Ablative and non-ablative photo rejuvenation
3) Pigmentary disorders
4) Vascular disorders
5) Photodynamic therapy
6) Comprehensive therapy in scar
7) Injectable
8) Pearls in dermatologic surgery
9) What's new in photoprotection
10) Pollution and skin aging
11) Anti-oxidants
12) Skin imaging and skin bioengineering
13) Secret behind the beauty-Traditional Chinese Medicine
14) Nanotechnology in cosmetic dermatology
15) Complications and solutions in cosmetic dermatology
16) Stem cells and growth factors in cosmetic dermatology

In copertina / Front cover

Elettrofilatura di Nanofibrille di Chitina (CN).
Foto al microscopio elettronico a scansione (SEM). Su gentile concessione del CNIS, Centro di ricerca per le Nanotecnologie applicate all'Ingegneria della Sapienza, Università La Sapienza, Roma - Italia

Electrospinning of Chitin Nanofibrils (CN).
Scanning Electron Microscopy (SEM) micrographs. On kind permission of CNIS, Research Center on Nanotechnology Applied to Engineering of Sapienza University, apienza University of Rome - Italy

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