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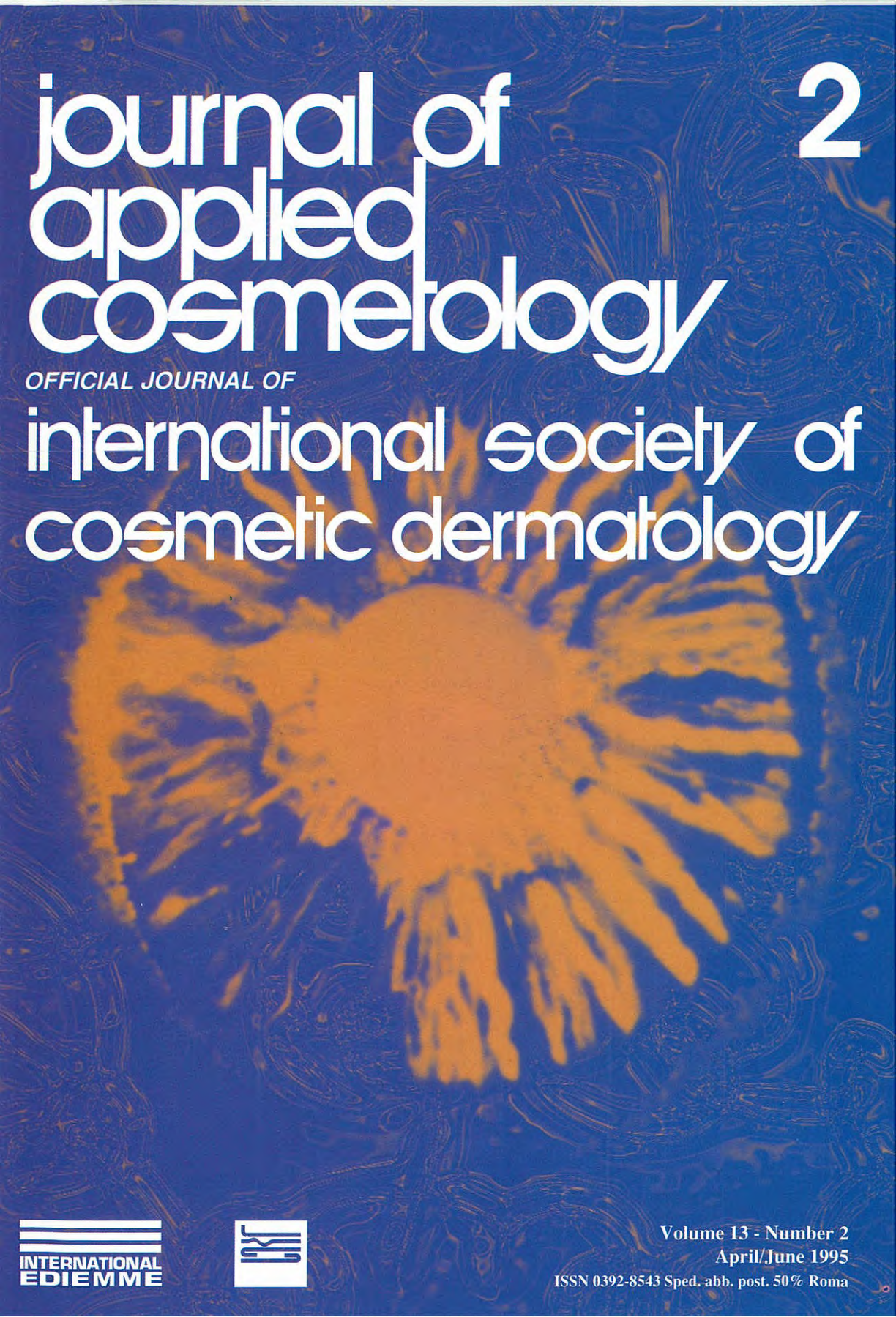

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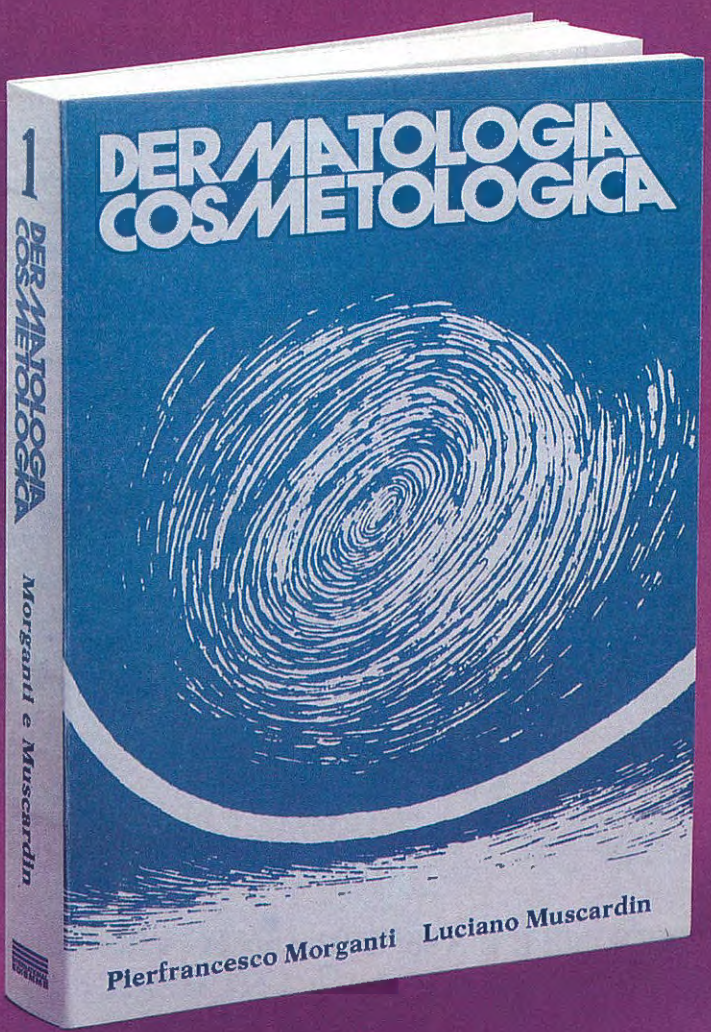

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A cura di P. Morganti e L. Muscardin
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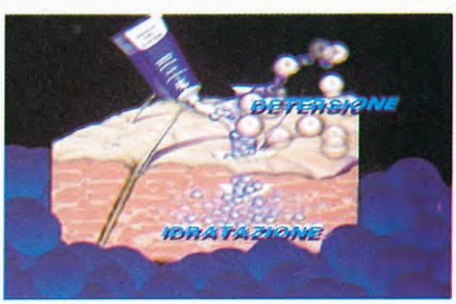

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Volume 2
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Editors: P. Morganti, F.J.G. Ebling

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October 26/29, 1995 Montecatini Terme - Pistoia (Italy)

INTRODUCTION

Free radical-mediated lipid peroxidation has been proposed to be critically involved in several desease states including cancer, rheumatoid arthritis, drug associated toxicity, and postischemic reoxygenation injury, as well as in the degenerative processes associated with aging (1-3).
Higher organisms place a considerable emphasis on defense against oxidative damage. The defense mechanisms involve both enzyme and small molecules. The former are represented by superoxide dismutase, catalase and glutathione peroxidase. The latter include vitamin E and β-carotene, which act as free radical scavengers in membranes, and vitamin C, uric acid and albumin bound bilirubin, which perform this function in the acqueous phase (4). Protein sulfhydryl groups and glutathione have also been suggested to play a pivotal role in cellular antioxidant defenses (5). Skin is an highly differentiated and complex organizated structure, and with age or in particular conditions become more vulnerable to free radical induced damage (i. e . hypersensitivity, degenerative alterations, photocarcinogenesis) (6-7). In view of the hypothesis that antioxidants may be particularly indicated to protect skin from oxidative stress, we investigated lipoperoxidation in skin lipids after topical treatment with different fractions of lemon oil and compared them to those obtained with vitamin E.

MATERIALS AND METHODS

Treatment and Sampling

The study was limited to 10 adult male volunteers. The age of the subjects ranged from 18-52 years old. The mean age in the group was 33 ± 11. The subjects were requested to avoid the use of hair lotions or other oil containing ointments during the duration of the experimental period.
The experimental procedure was designed to evaluate the effectiveness of the lemon oil extract (L.O. -22) as compared to vitamin E in preventing lipid peroxidation of skin surface lipids. The study was carried out in the Dermatology Clinic at the University Hospital. The 10 volunteers were divided randomly into two groups (A and B) of 5 individuals. During the first week, group A was asked to apply vitamin E on days 1, 3, and 5 whereas the group B was asked to use L.O.-22 on days 1, 3 and 5. At the end of the first week of treatment, the volunteers were asked to return to the Dermatology Clinic one week later. Upon re-entry, the groups were subjected to a cross over trial. Group A was instructed to use L.O.-22 on days 1, 3 and 5 whereas group B was asked to use vitamin E on the corresponding days.

Experimental procedure

At the beginning of the experiment a sample of skin surface lipid was obtained from the forehead of each individual. The surface lipids were obtained by swabbing an area of the forehead (3 cm x 3 cm) with a cotton swab. The procedure was standardized such that the area was swabbed three times horizzontally and three times vertically for each individual. The two groups where then asked to wash the forehead thoroughly with neutral soap and then group A was given a vial containing 3 ml of 5% alpha-tocopherol solubilized in 20% ethanol to apply freely on the forehead. The application of vitamin E or L.0.-22 was repeated on days 3 and 5. Samples of skin lipids obtained by the swabbing technique were taken at 1, 2, 4, 6, 8 and 24 hours after application of vitamin E or L.0.-22. This experiment was carried out on days 1, 3 and 5.

Extraction of lipids

The cotton swabs were extracted for their lipid content with 3 ml of chloroform:methanol mixture (1:2.5). The extraction procedure was al-

lowed to continue at room temperature for 2 hours in the presence of heneicosanoic acid (10 µg) as internal standard. The cotton was removed and re-extracted with fresh chloroform:methanol solution (1 ml). The extraction solutions were combined and 1% NaCl in 0.01 M HCl was added to the mixture and centrifuged. The chloroform layer from both extractions was pooled and washed with 3 ml methanol:water (1:1). The solution was centrifuged and after phase separation, the chloroform layer was recovered and evaporated to dryness under a stream of nitrogen gas in the dark. The dried lipids were dissolved in 3 ml chloroform:methanol solution (2:1) and the solution was stored at 20°C in the dark until analysis of lipid content and lipid peroxidation studies.

Peroxidative stress of skin surface lipids

The sensitivity of skin surface lipids extracted from individuals in the two experimental groups was evaluated by measuring the susceptibility of the lipids to oxidative stress in the presence of ferrous ions-EDTA complex (0.2 M) at pH 7.5. Oxidative damage to lipids generally leads to the formation of malonaldehyde (MDA) which has been investigated extensively as a marker for lipid peroxidation processes in vitro and in vivo.

Assay for MDA

MDA was measured using a micromethod modified from Slater and Sawyer (8): to 0.5 ml of the mixture of reaction were added 0.5 ml of 20% (w/v) thrichloroacetic acid; after centrifugation, 0.9 ml of the snpernatant fraction was added to 1 ml of 67% thiobarbituric acid (Sigma) dissolved in 0.026 M Tris-HCl buffer pH 7.0. The samples were heated in boiliug water for 10 min. After cooling, the absorbance was determined at 532 nm on a Perkin Elmer mod.559 spectrophotometer. Extraction blanks were prepared and treated in the same way as the experimental samples but an equal volume of buffer was substituted for reaction mixture. MDA was quantitated using MDA standard (Aldrich) and expressed in nanomoles per nanomoles of phosphate.

Assay for phosphorus

Phosphorus was measured using an ultramicro modification from Bartlett (9).Aliquots (1.5 ml) of lipid extract were combusted adding 0.3 ml of 10 N sulphuric acid and heating the mixture at 200 °C for 3 hours. Two drops of 30% hydrogen peroxide were added and the solution was heated for 1.5 hours more at 200 °C to complete the combustion and to decompose all the peroxide. After combustion, 0.65 ml of distilled water, 0.2 ml of 5% ammonium molybdate and 0.05 ml of the Fiske-Subbarow reagent were added, and the solution was heated for 7 minutes at 100°C. The optical density was read at 830 nm. Inorganic orthophosphate was used to prepare the standard curve.

Fatty acids analysis

Lipids contained in half of the extract were transesterified in 1 ml of 2% sulphuric acid in methanol:benzene (1:1 v/v) for 4 hours at 65°C. Methyl esters so obtained were brought to dryness under a gentle stream of nitrogen and resuspended in 2 ml of hexane plus 1 ml of methanol. The exane layer was then transferred in 3 ml vials, provided with teflon-lined screw caps, dried and resuspended in 100 µl of exane. Fatty acid analysis was carried out with a Carlo Erba gas- chromatograph (mod. Fractovap 4200). SE 30 (3% on 80/100 mesh Chromosorb WHP) was used as the stationary phase in 2 m x 2mm ID glass column with nitrogen as carrier gas. The temperature was programmed from 160°C to 260°C at a rate of 8°C/min. The detector and injector temperatures were set at 260 °C. Quantitative measurements and calculations were effe-

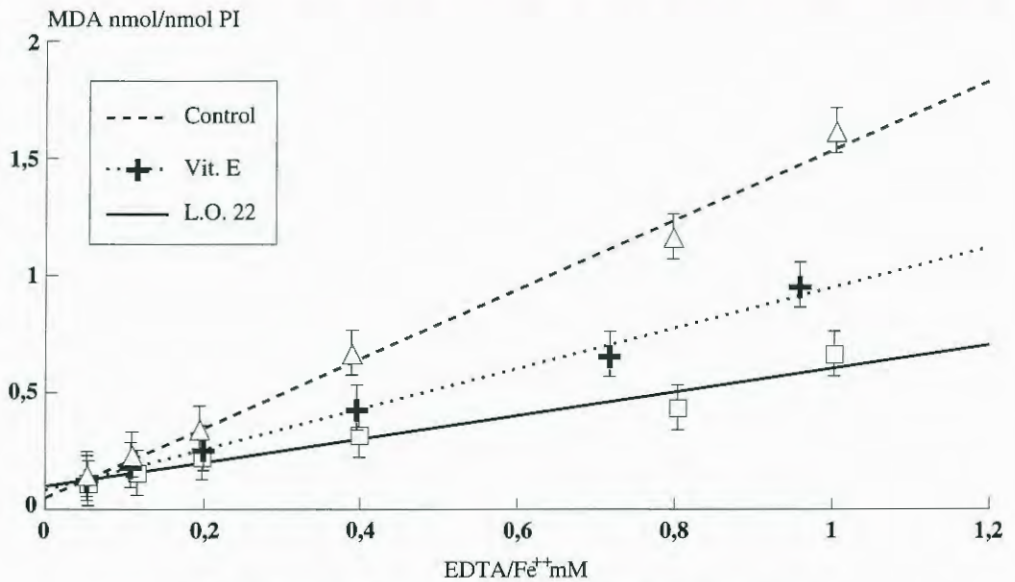
cted by comparing peak height ratios between acids and internal standard of samples with those of standard solutions of comparable concentrations. Results were expressed as µmoles of fatty acids per nmoles of inorganic phosphate. Student's t test was used for estimating the significance of difference between group means.

RESULTS

The antioxidant activity of vitamin E has been widely recognized over the past years and hence has provided a useful guideline for evaluating the antioxidant activity of other natural products. In our study, we have compared vitamin E to a natural antioxidant denominated L.0.-22. This product was fractionated from a lemon oil extract via steam distillation followed by column chromatography of the distillate on phasesorb and eluted with ethanol:water mixture (3:1). The chemical characteristics of this fraction suggest the presence of an isoprenoid - quinoid compound wich exhibits strong antioxidant activity in in vitro assay system as shown in figure 1. As can be seen, the addition of increasing concentration of Fe-EDTA complex to a lipid extract increased the production of malonaldehyde (MDA). However, this increase was significantly reduced when the incubation medium contained 5% vitamin E or when the incubation medium was made 5% with respect to L.0.-22. As can be seen from figure 1, at the same concentration of vitamin E and L.0.-22, the latter product was significantly more effective than the vitamin E compound in inhibiting lipid peroxidative formation of malonaldehyde. A

Figure 1

MDA PRODUCTION AS FUNCTION OF EDTA-Fe^{++} CONCENTRATION



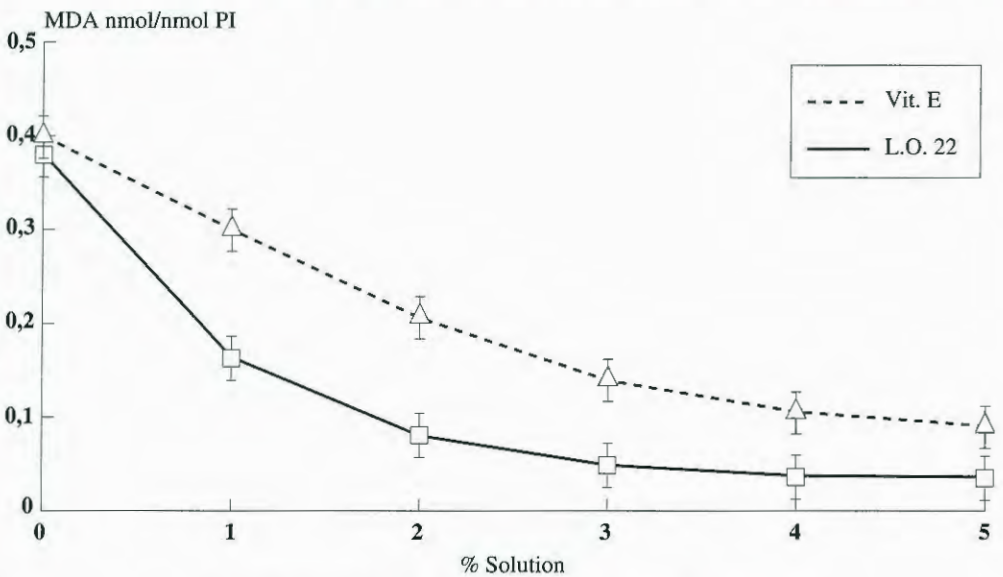
Results are the mean ± S.D. of 5 experiments.

dose dependent response was also performed for the in vitro assay system, figure 2. The data show that at concentration ranges of 0.01% to 1% the L.0.-22 antioxidant activity was approximately 30-50% higher as compared to a similar dose range of vitamin E. At higher concentration ranges (1%-5%), L.0.-22 inhibited the production of MDA by 15-20%. The differences in the dose-response activity of L.0.-22 suggests a possible aggregation phenomenon at higher concentrations and hence reduces the effective antioxidant activity of the product. In contrast, vitamin E did not display this dose dependent inhibition of MDA production.

The susceptibility of lipids toward peroxidative stress were evaluated using lipid sample collected in the study with male volunteers. As can be seen from figure 3, the L.0.-22 treated subjects, as compared to vitamin E group, consistently exhibited a higher resistence to lipid peroxidative stress reactions as shown by the decreased production of MDA. Similar results were obtained during the cross over trial of groups A and B, figure 4. This study demonstrated that treatment with L.0.-22 significantly inhibited peroxidative damages to skin lipids. In order to investigate further the mechanism of action of L.0.-22, we analyzed the various lipid classes in each sample of skin lipid and, as reported in figure 5, we found that peroxidative damage was decreased significantly for short chain fatty acids. Hence treatment with L.0.-22 protects against peroxidative damage of lipid fatty acids in general and in particular the short chain fatty acids.

Figure 2

MDA PRODUCTION IN THE PRESENCE OF ANTIOXIDANTS



Results are the mean ± S.D. of 5 experiments.

Figure 3

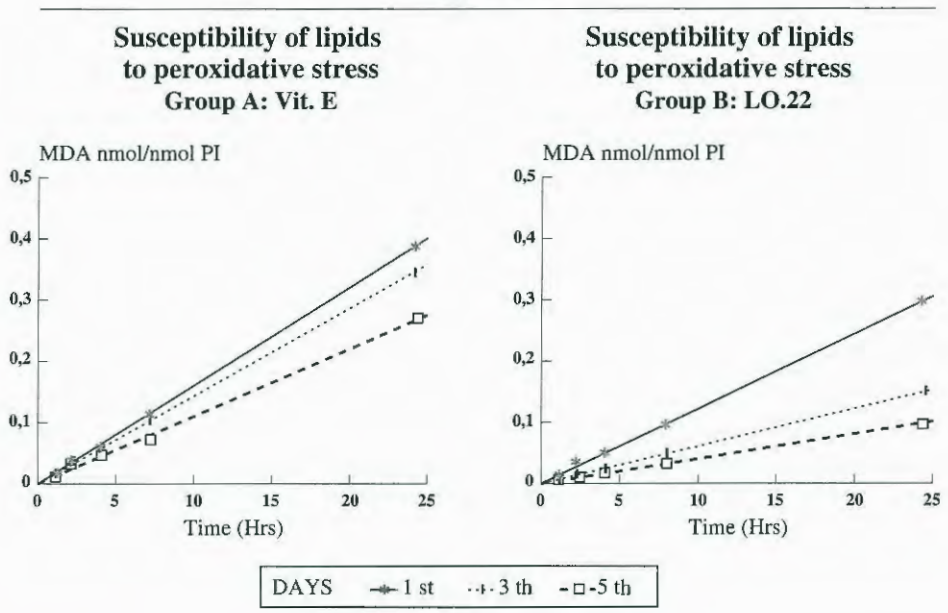
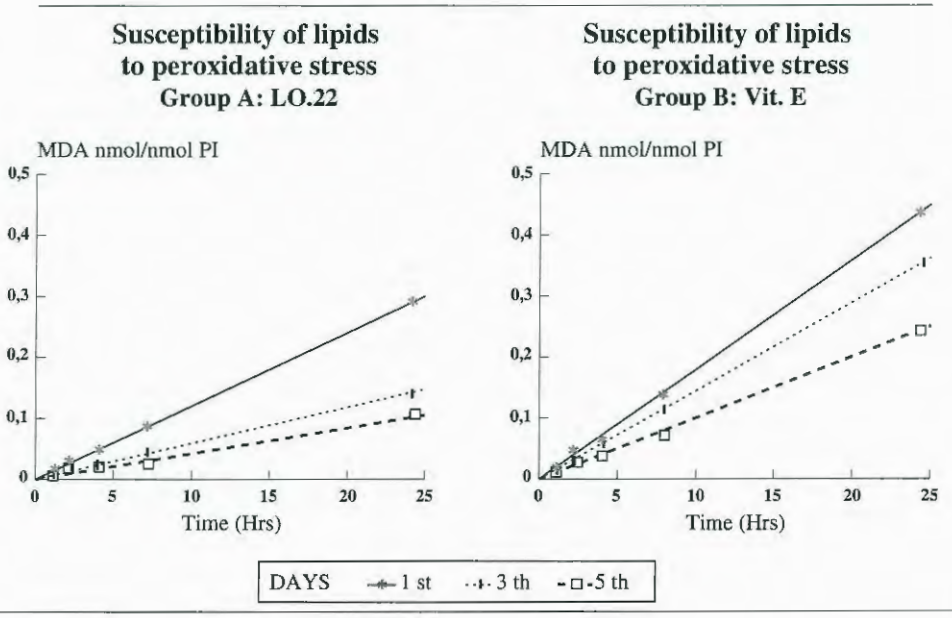


Figure 4



DISCUSSION

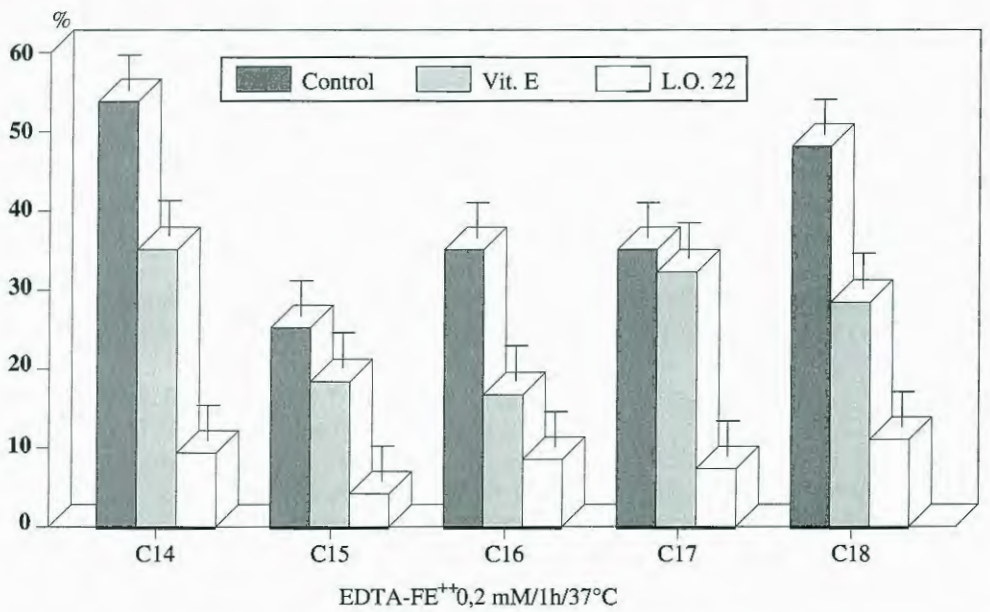
Damage to biological system caused by generation of active oxygen species is often referred to as "oxidative stress" (11). There is now growing evidence for the involvement of oxygen radicals and other oxygen-derived species as causative agents in aging and several human diseases. Skin is an highly differentiated and certainly complex organizational structure which, with age, becomes particularly vulnerable to a cohort of degenerative disorders. These disorders have been correlated not only to immunological changes - altered differentiation of lymphocytes, involution of thymus - but also to a free radical-mediated induction of hypersensitive and photocarcinogenetic processes (6-7). In the recent years, various experimental models have been developed in order to investigate the molecular mechanism by which active oxygen species cause cell and tissue damage and to find suitable treatments capable of preventing such damage.

In our study we employed a peroxidative system to mime pathogenic condition supposed to be involved in the aging of the skin. In this system, skin surface lipids of normal subjects are stressed in presence of EDTA-Fe, with production of malonaldehyde, one of the most studied by-products of cellular peroxidative damage. MDA formation linearly increases as a function of EDTA-Fe concentration.

Also, we studied lipoperoxidation through direct analysis of skin fatty acids, founding a strong correlation between decrease in the unsaturated/saturated fatty acid ratio (which is an in-

Figure 5

% PERIOXIDATION OF SKIN LIPIDS COLLECTED AT 24 HRS OF 5th DAY



Results are the mean ± S.D. of 5 experiments.

dex in the peroxidative breakdown of unsaturated fatty acids) and MDA formation in presence and in absence of different antioxidants (10). Topic treatment with vitamin E, or different fractions isolated from lemon oil extract, provided significant protection against oxidative stress. Among the compounds investigated, the fraction indicated as L.0.-22 exhibited the best protective effects against free radical induced injury, as compared to vitamin E. This study indicates that a fraction isolated from lemon oil extract, may be used to prevent lipoperoxidative changes of the skin, pointing out the importance of a natural antioxidant technology in the anti-aging treatment of the skin.

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J. Appl. Cosmetol. **13**, 35-40 (April - June 1995)

STELLATE SCARS IN PHOTOAGED SKIN. A HISTOLOGICAL AND ULTRASTRUCTURAL STUDY

RENATA STRUMIA
Clinica Dermatologica Università di Ferrara, Italy - Direttore: Prof. A. Califano

Received: *October 25. 1994*

Key Words: *Stellate Spontaneous Pseudoscar; Photoaging.*

Synopsis

The A. reviews the literature concerning stellate spontaneous pseudoscars (SSP) and performs a histological and ultrastructural study. The conclusion is that SSP are really scarring lesions which may appear on a skin which has become particularly delicate due to age, drugs and photoexposure. So the A. suggests they be named "stellate scars in photoged skin" and not stellate spontaneous pseudoscars.

Riassunto

Dopo aver inviato i dati della letteratura riguardanti le pseudocicatrici stellate spontanee (SSP) l'autore conduce uno studio istologico ed ultrastrutturale su questo tipo di lesione.
La conclusione di tale studio é che tale lesione corrisponde ad una vera e propria cicatrice che può verificarsi su cute particolarmente delicata a causa dell'età, di farmaci o di fotoesposizione.
L'autore suggerisce quindi di denominarla " cicatrice stellata di cute fotoinvecchiata" e non "pseudocicatrice stellata spontanea".

INTRODUCTION

In 1967 Colomb et Al (1) used the term stellate spontaneous pseudoscars (SSP) (pseudo-cicatrices stellaires spontanees) to describe some peculiar white cutaneous lesion which had a scar appearance and singular forms localized particularly on the backs of the hands and forearms of elderly patients. Their almost constant association with Bateman's senile purpura, which has notoriously the same locations, suggested the role of repeated microtraumatisms. However, after the careful study of a large number of cases, Colomb was convinced that these lesions were truly "spontaneous and independent of any sore or loss of substance" (2).

Intensive solar radiation seemed to have a predisposing role and might partially explain the preferential site of the disease on photoexposed areas (3).

Analogous lesions were observed also in younger subject who presented atrophy and premature aging of the skin due to prolonged corticosteroid treatments or excessive solar exposition. These ones were called "presenile forms" (2).

The histologically appearance of all clinical forms was stereotyped and described as constant and characteristc. The whitish color of the pseudoscars was ascribed to the poor vascularized collagenous disc viewed through an atrophic covering skin and not to the absence of melanin in the epiderms. All the A.A., in fact, reported a normal or even increased presence of melanocytes and pigmented basal cells in correspondence to the clear zone.

To the best of Knowledge, no other description of these lesions was reported in the literature until 1990: in this year, in a report on the pigmentary changes of the ageing skin, Ortonne included them among "hypomelanosis" together with idiopathic guttate hypomelanosis. He emphasised the need for further histologic studies to determine the natnre of these lesions (5).

In the past few years, we have noticed a great increase in the frequency of SSP, particularly of the presenile forms. The cause of this phenomenon is certainly linked to the greater use, for various reasons, of prolonged carticosteroid treatment. Transplant organ recipients, for instance, present lesions of this kind even in very young subjects as do epathic patients.

So we, therefore, consider it of some interest to report our histological and ultrastructural observations on some classic SSP in an attempt to obtain new phathogenetic informations particularly on the achromic component of these lesions.

MATERIAL AND METHODS

In three male, 63, 64, 74 year-old subjects with classical SSP on their forearms, cutaneous biopsies were performed to include the whitish scar area and the surrounding pigmented area. A portion of the specimen was fixed in 10% buffered formalin for the following stains: hematoxilin-eosin, PAS, orcein, Masson-Fontana and Lillie. Another portion was fixed in 3% glutaraldeide for ultrastructural examination.

Result

Conventional microscopy

All three cases presented the same histological apparence. The hematoxilin and eosin stain showed hyper-orthokeratosis particularly over follicular units. In the pseudoscar the dermis had a fibrosclerotic apparence with fibroblasts and vascular neogenesis. The fibrous mass had well-delineated sides and base. The lateral margins of the pseudoscar were characterzed by evident elastosis of the collagen which appeared basophilic. Extravasation of blood and hemosiderin were also present.

The PAS stain showed a marked thickness of vessel walls without occlusion. In the lateral areas, there was some loss of microvesseles and venules appared dilated.

The orcein stain showed the absence of elastic fibers in the central area; few filiform fibrils

persisted in the deeper dermis. In the surrounding zones, the dermis was occupied by homogeneous orceinophilic accomulations.
On Masson-Fontana stain the achromic central zone presented irregular distribution of melanin in the basal cells of the epidermis with wide clear areas alternated with pigmented ones. In the surrounding epidermis, on the contrary, basal cells and suprabasal keratinocytes showed regular presence of melanin on their upper poles (Fig.1).

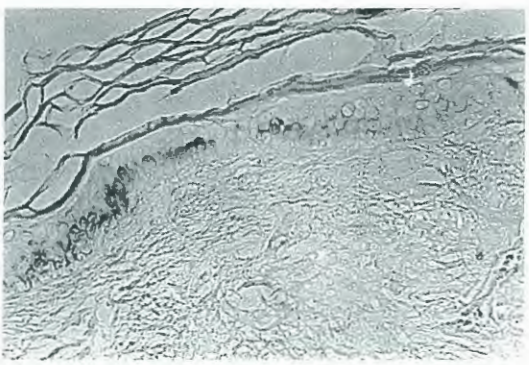

Fig. 1 - Right: absence of melanin in bosal cells and suprabasal keratinocytes in the achromic scarring zone. (Masson-Fontana stain 40x)

The Lillie stain showed extravasation of blood on both sides of the fibrous mass and an almost complete absence of epidermal melanin in the central area.

Electron microscopy

We examined basal cells and suprabasal keratinocytes of the central achromic area and the surrounding pigmented skin. In the central zone, areas characterized by absence of melanosomes persisted (Fig. 2). In the surrounding pigmented skin we observed a regular presence of single and in complexes melanosomes on the upper poles of the cells (Fig. 3).

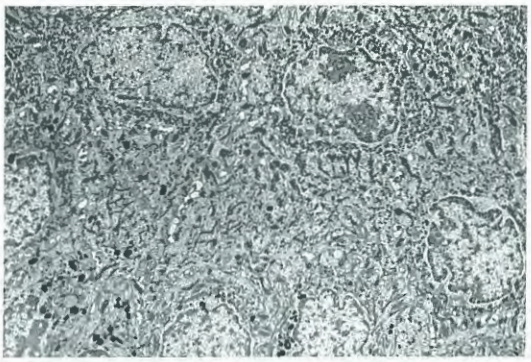

Fig. 2 - Electron micrograph of central zone of the stellate scar: only few singles melanosomes persist in basal cells and suprabasal keratinocytes. (3500x)

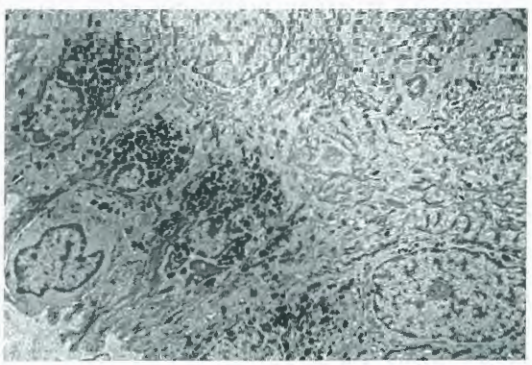

Fig. 3 - Electron micrograph of the surrounding pigmented area: numerous melanosomes on the upper poles of the cells. (3500x)

DISCUSSION

Our histological observations are analogous to those previously reported in the literature with regard to the dermis but differ as far the epidermis findings are concerned. All the AA., in fact, had described the normal presence of melanin over the pseudoscar. In our cases, on the contrary, the Masson-Fontana stain showed an irregular distribution of epidermal melanin with alternance of clear and pigmented areas over the pseudoscar.
The ultrastructural study which, to the best of our knowledge, had never been performed pre-

viously, supported the results of conventional microscopy.

A defect within the epidermal melanin unit may be hypothesized which results in a focal loss of skin pigmentation.

Similar findings have recently been reported in idiopathic guttate hypomelanosis (6). In this condition, the total number of melanocytes is considerably less than that in normal epidermis and their distribution is irregular. Under the electron microscopy, melanocytes present fewer or no dendrites; these findings are compatible with focal degenerative process which results in a defect within the epidermal melanin unit.

On our opinion, in SSP achromia could be attributed to the "real scarring" nature of the lesions. All the patients we studied and, in particular, young organ transplant recipients, who being aware the side-effects of immunosuppressive drugs are very attentive to any changes in their skin conditions, have reported the appearance of Bateman's purpura after microtrauma; they did not however remember any previous wounds in the areas in which SSP were present. It is likely that trauma of some kind may induce Bateman's purpura while others, which also cause minimal loss of substance, can stimulate an abnormal scarring reaction. In fact, the fibroblast in photoaged skin are numerous and metabolically active; this is not true in chronologicallly aged skin (7). It is well known that melanocytic proliferation over the wound begins later than that of keratinocytes and Langherans cells. The onset of melanocytes coincides with the epithelial closure of the incisional gap and their movement is directed into performed sheets of epidermis (8). A light electron microscopic study of the skin regenerated from cultured epithelial autografts on full-thickness burn wounds has demonstrated the absence of melanocytes until a year or more after transplantation (9). Hypomelanosis in SSP might therefore be a consequence of a scarring process.

In the light of the present knowledge of cutaneous photoaging, it would seem that the perispherical mass of orceinophilic material, which was considered to be an integrant part of the istological pattern of SSP, may be aspecific and consistent with the common aspect of photoaging. Also dilated venules, perivascular flogistic infiltrate and thickness of vessel walls are characteristic of photoaging (6).

In conclusions, we believe that SSP are really scarring lesions which appear on a particularly delicate skin due to age, drug treatment and photoexposure and would suggest they be named "stellate scars in photoaged skin".

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BENEFICIAL EFFECTS OF TOPICAL APPLICATION OF FREE RADICAL SCAVENGERS.

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Key Words: *Free Radicals; Lipid Peroxidation; Sequestering Agents; EDTA; Photoaging; BHT; β-carotene; Vitamin E; Phospholipidis.*

Synopsis

It is increasingly evident that free radicals play a key role in determining the over-all well-being and general appearance of the skin. It is clear also that a stressful environment can cause an over-abundance of these reactive and potentially harmful species and that to mitigate the effects of such an excess, the natural defenses of the skin may not suffice and may have to be supplemented. An understanding of the nature of free radicals, how they are formed and the role they play in some key reactions such as lipid oxidation, has led to a multi-faceted approach to their control.
This includes topical application of sun-screens, chelating agents, excited state quenchers and free radical scavengers, etc.... To optimize the effectiveness of the various means of protection which are available, attention must be paid to the presence or absence of synergists and trace metals, of the strength of the chelating agents and their concentrations. For instance the effectiveness of tocopherol is greatly enhanced by ascorbic acid, that of BHA by citric acid. Ascorbic acid may act as a pro-oxidant but such an effect can be eliminated by a strong chelating agent such as EDTA.
The incorporation into cosmetic formulations of judiciously selected means of controlling the proliferation of free radicals in the skin is proving to be beneficial both short and long term.

Riassunto

Vi sono ormai molti studi che pongono in rapporto diretto il ruolo chiave svolto dai radicali liberi nei riguardi dell'invecchiamento cutaneo. E' stato anche dimostrato come gli inquinanti ambientali rappresentino la forma primaria della formazione di tali radicali liberi, che non possono essere totalmente neutralizzati dagli antiossidanti naturali prodotti dai sistemi biologici cutanei.
Tali antiossidanti debbono perciò essere supplementati con l'alimentazione. La comprensione sulla natura dei radicali liberi, sul come e sul perché della loro formazione e del ruolo che essi svolgono, ad esempio, nell'ossidazione dei lipidi, ha permesso la messa a punto di sostanze adatte alla loro neutralizzazione, quali i filtri solari, gli agenti chelanti, ecc... Per ottimizzare l'efficacia di tali sostanze ne deve essere attentamente valutata la concentrazione, la tipologia e la presenza di sostanze che ne possano sinergizzare l'attività. Per esempio, l'attività del tocoferolo é notevolmente potenziata dalla presenza dell'acido ascorbico e quella del BHT dall'acido citrico.
L'acido ascorbico può, però, agire anche come pro-ossidante ma tale attività può essere facilmente eliminata con l'aggiunta di un forte agente chelante, quale l'EDTA. L'inclusione nelle formulazioni cosmetiche di principi attivi attentamente selezionati per controllare a livello cutaneo la proliferazione dei radicali liberi sembra, perciò, essere utile per apportare benefici effetti sia a breve che a lungo termine.

radical, itself becomes a free radical. However because of the chemical structure of this radical, its lone electron is more highly delocalized and hence it is less reactive. The increased life-time makes it more susceptible to termination and also to the kind of synergism observed for instance between tocopherol and ascorbic acid. Thus the effectiveness of tocopherol as an inhibitor of peroxidation is enhanced because the tocopheryl free radical exists long enough to react with ascorbic acid and be reduced by it back to tocopherol.

Because of the high reactivity of the more potent free radicals such as the hydroxyl radical, they react with all synthetic or biologic molecules at diffusion controlled rates. Thus damage which they might cause will have occurred in less than a nanosecond after their formation. In that time they will have diffused no more than 10nm (3). To be effective the scavenging reaction must therefore take place within that cage and in that time frame. It is, therefore essential that the scavenging molecule be present at the required concentration close to where the radical is produced. Damage due to free radicals may of course extend beyond the immediate vicinity of where they are produced (4). It is possible for example, for toxic aldehydes which may be produced in lipid peroxidation to migrate to more distant sites where they can cause oxidizing stress.

Acceptance of an electron by molecular oxygen results in the formation of a superoxide anion,

$O_2 + e \rightarrow O_2^-$

a species which very rapidly converts either spontaneously or enzymatically to hydrogen peroxide (H_2O_2).

In the presence of transition metals (iron, copper, titanium) this hydrogen peroxide undergoes a Fenton-type reaction to generate the highly reactive hydroxyl radical.

$H_2O_2 + Fe^{2+} \rightarrow \bullet OH + OH^- + Fe^{3+}$

Ferric ion produced in this reaction can be reduced back to the ferrous state

$Fe^{3+} + O_2^- \rightarrow Fe^{2+} + O_2$

The coupling of these two reactions is the so-called metal catalyzed Haber-Weiss cycle. Thus in a biological environment which inevitably contains iron, the relatively innocuous superoxide ion is readily converted to the much more lethal hydroxyl free radical. Hence it is important to block this catalytic effect of metals by suitable sequestration. This almost certainly occurs in biological systems, iron in heme, magnesium in chlorophyll are examples of chelate complexes. It may well be that OH• scavengers such as mannitol, salicylate, thiourea, deoxyribose to some extent also operate by this mèchanism since they all have metal-ion binding ability. Thus these compounds reduce the concentration of hydroxyl radical not so much by direct scavenging or removal but by preventing them from being formed via a metal catalyzed reaction.

When supplied with appropriate amounts of energy (~36 kcal/mole), molecular oxygen in its normal triplet ground state is raised to its excited singlet configuration through reversal of the spin of one of its electrons.

↑·Ö:Ö·↑ --> ↑ ·Ö:Ö· ↓ --> :Ö:Ö

triplet singlet

Singlet oxygen is not a free radical since it no longer has any unpaired electrons. It does however possess an entirely empty orbital. Hence it reacts readily with most organic structures. This oxidation however is not a chain reaction as is the case when free radicals are involved, an oxygen molecule simply adds to a double bond in a Diels-Alder type reaction.

Lipid oxidation involving singlet oxygen can be suppressed by suitable quenchers which can remove the excess energy the excited oxygen possesses and return to its less reactive ground state. Quencher molecules such as xanthine, melanin, guanosine, ß-carotene to name a few may not necessarily also act as free radical scavengers although some like melanin and tocopherol do (5).

What are chelating or sequestering agents?

They are organic molecules which readily share pairs of electron with transition metals such as the ubiquitous iron and copper in biological systems to form co-ordination complexes. The nature of the ligands determines the kind of complex which is formed and how many co-ordination sites on the metal are occupied. The electro-static field due to the ligand interacts with the d orbitals of the metal which are of equal energy in the gas phase and splits them so that they now acquire different energies determined by the intensity of the ligand field. In the case of iron for example, the coordination complexes can be either "low spin" in which all the 6 electrons in the 3 d orbital of iron are left paired or they can be "high spin" in which case only 2 of these electrons are paired. Clearly with 4 unpaired electrons, a "high spin" complex will more readily promote radical reactions such as the conversion of hydrogen peroxide, a species of limited chemical reactivity to the highly reactive and dangerous hydroxyl radical. Besides the nature of the ligand, factors such as temperature and concentration also determine the extent of saturation of the co-ordination sites and whether the chelator eventually acts as a promoter or an inhibitor of oxidation.

Ethylenediamine tetraacetic acid (EDTA), a "high spin" chelator frequently used in cosmetic preparations has 6 co-ordination sites with which to saturate the 6 sites on ferrous iron. It is therefore a powerful inhibitor of lipid peroxidation. Yet to be fully effective it still has to be present at a concentration at least 10 times that of the ferrous iron. If the concentration of EDTA is substantially less than that of iron, it acts as a pro-oxidant (6).

Vitamin C is also a "high spin" ligand but it has only 2 co-ordination sites and is a reducer. The result is that ascorbic acid promotes Fenton-type oxidation. To counteract this effect i.e. for it to be an oxidation inhibitor, it must be present at concentrations at least two orders of magnitude greater than that of the ferrous iron. More effective sequestration of trausition metal ions (iron, copper, titanium) than provided by the ascorbate moiety is clearly essential if the anti-oxidant properties of ascorbic acid are to be fully realized. This of course, is important in cosmetics where iron compounds are frequently used as pigments.

Free radicals are involved in photoaging

The widely accepted hypothesis to account for the various effects of U.V. Light postulates that actinic radiation induces the formation of free radicals in the skin where they may be nullified by the skin's own anti-oxidant defenses (7,8). If however the dose of U.V. Light is high, these natural defenses will be overwhelmed and will no longer suffice to maintain the concentration level of these free radicals below a critical threshold beyond which they will damage proteins, lipids, etc. present in their immediate vicinity.

Two kinds of aging are now recognized: intrinsic and extrinsic (9,10)

Intrinsic aging refers to the changes which occur when an organism progresses in chronological age. Extrinsic or premature aging results from exposure to external insult, more particularly U.V. Light, which leads to actinic damage. The difference between exposed and unexposed skin is very obvious when examined on the same individual. Other, if somewhat Less evident changes, also occur. Collagen is significantly affected by U.V. Light and by free radicals (11). For instance there are fewer collagen bundles and there is an increase in soluble collagen (12). Aged skin is thinner, whereas photoaged skin is coarse and pigmented. The melanin content and hence of melanin radicals is increased. Over-exposure to U.V. Light also leads to the formation of many long term changes in the skin such as wrinkles and loss of elasticity. These changes may require many years of exposure to manifest

themselves. It is therefore difficult to perform systematic studies on humans. Fortunately according to L. Kligman (13) the hairless mouse has been shown to be a relevant animal model in the study of photoaging and can be considered a reliable guide to what happens on human skin.

Beneficial effects of topically applied free radical scavengers.

Prior application of tocopherol and scavenging anti-oxidants such as ascorbic acid and propyl gallate has been found to be effective in the prevention of chronic skin damage evaluated by skin wrinkling and sagging and the presence of tumors (14).

Kheltab et al (15). have shown that the increase in the concentration of free radicals observed following U.V. irradiation could be prevented by the topical application of 0.25% vitamin E, 0.02% BHT, 0.2% ,ß-carotene or 0.02% vitamin A. In this study free radicals were detected as thiobarbituric acid reacting substance, TBARS.

Record et al.(16) assessed skin damage by thymidine incorporation into DNA and by lipid peroxidation. They found that such damage could be significantly reduced by the topical application of 1% tocopherol 1 hour or even 24 hours before irradiation.

Erythema induced by exposure to U.V. light is significantly reduced by the topical application of anti-oxidants (17).

The concentration of anti-oxidant naturally present in skin, is found to be decreased on irradiation.This is to be expected since the natural defenses of the skin have to cope with an increased concentration of free radicals. As a result lipid peroxidation is increased tremendously. Supplementation of the skin's defenses with δ-tocopherol prevents this damage.
Thus L. Packer and his associates (18) found a 50% reduction in the concentration of vitamin E and complete depletion of ubiquinol in skin irradiated with a dose of U.V. light equivalent to about 5 hours of exposure to natural sunlight. Concomitant with this decrease in the skin oxidative defenses they observed a very large increase in lipid peroxidation. This sensitive marker of damage to lipids, proteins and nucleic acids clearly reveals that when the skin's anti-oxidant system is destroyed or decreased, damage to important biomolecules can occur. Even a single dose can overwhelm the skin's anti-oxidant defenses and cause significant damage but this can be prevented by supplementation with anti-oxidant either systemically or by topical application.

Taking the inhibition of UVA-induced photobinding of 8-methoxypsoralen (8-MOP) to epidermal macromolecules as a measure of photoprotection, Beijersbergen et al (19) found from in-vivo studies in rats, that even one application of 0.01% tocopherol provides observable protection and that the concentration level of tocopherol in the skin for optimum protection is only a few times higher than that which is normally present. On this basis they concluded that the protection observed with tocopherol acetate, a compound which lacks a free-phenolic •OH group and hence is not a free radical scavenger, is in fact due to the minute amount of tocopherol produced on the skin by the slow and inefficient enzymatic hydrolysis of the acetate.

These findings agree with those of an in-vivo study which indicates that free radical scavengers inhibit erythema in human skin exposed to UVB (20). Color was measured by reflectance spectrophotometry. This study further confirmed also the low rate of hydrolysis of tocopherol acetate. As in animal studies, protection against erythema in humans also requires a very small amount of tocopherol.

To be effective repeated treatment of the skin with tocopherol acetate is required.
Evidence that Vitamin E reduces fine lines and wrinkles has recently been obtained (21). The study involved 20 women who applied a placebo to one side of the face and the same water-in-oil vehicle with 5% tocopherol to the other side. Comparison of skin replicas taken after 4 weeks of treatment showed that more than 50% of the panelists had a significant decrease in skin roughness.

Free radical scavengers in cosmetic formulation

The stratum corneum consists of dead i.e non-reproducing cells, the corneocytes, which are embedded in a lipid matrix. It is the presence of this lipid which together with water gives the stratum corneum its flexibility and is responsible for the smooth appearance of the skin. Besides decreasing the barrier function of the stratum corneum, loss of these lipids also leads to what is described as dry skin i.e. skin which feels taut and which looks rough and dull. Thus skin which has been exposed to detergents with attendant removal of lipids from the stratum corneum has an unpleasant feel and appearance. This is also the case when the stratum corneum has been delipidized by dissolving away some of the lipids with acetone, ether or some other solvent or when the concentration of lipids in the stratum corneum has been reduced by oxidation with chemicals capable of generating free radicals such as hydrogen peroxide, benzoyl peroxide, chlorine, etc. Lipid peroxides obtained from sebum or cosmetic preparations irradiated with U.V. Light have been shown to be irritating to human skin (22).

Thus the prevention of the oxidatiou of lipids in the stratum corneum by removing free radicals which initiate this process has cosmetic benefits in that it helps mitigate the symptoms of "dry skin" by helping prevent the loss of lipids in the same way that an occlusive cosmetic prevents the loss of moisture. It helps maintain unimpaired the functions of the stratum corneum and hence the well-being and the appearance of the skin.

CONCLUSION

It has become increasingly evident that free radicals can adversely affect the ultimate appearance as well as the general well being of the skin and that the skin's own natural defenses which might be overwhelmed by an over-abundance of these highly reactive species caused by a stressful environment needs additional help to ensure adequate control. The lipids in the stratum corneum are particularly prone to undesirable peroxidation.
When the primary act which sets off the oxidation chain reaction is the absorption of a photon, it can be prevented from occurring by means of appropriate sunscreens. Should initiation result from free radicals generated by electron transfer involving heavy metal ions, it can be averted by passivating these ions by suitable sequestration. However, the chelating agents must be selected with care and be present at sufficiently high concentrations to ensure that they act as anti- and not as pro-oxidants. The oxidation chain reaction can be interrupted by providing a sacrificial molecule i.e. an anti-oxidant such as α-tocopherol (vitamin E) which is capable of converting chain propagating free radicals into much less reactive ones, thus allowing the termination steps to dominate.
The effectiveness of anti-oxidants can be enhanced by synergists such as organic acids, amines, amino acids, nucleic acids, phospholipids (lecithin) etc. To ensure maximum protection, the free radical scavenger should be combined with a synergist and an adequate concentration of a chelating agents. Furthermore the scavenger must be present at the very site where the oxidation reaction is taking place.

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alla sera

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