J. Appl. Cosmetol. 9, 65-71 (July - September 1991)

AN IN VIVO BIOCHEMICAL SYSTEM TO ASSESS THE ANTIOXIDANT, ANTI-AGING EFFECTS OF COSMETIC PRODUCTS.

Calabrese V., Randazzo S. D*., and Rizza V.
Institute of Biochemistry, Faculty of Medicine, University of Catania; Viale Andrea Doria N°6 ; 95125 Catania - Italy.
* Dept. of Experimental Dermatology, Faculty of Medicine, University of Catania - Italy.

Received: *March 3, 1991*

Key words: *Malonaldehyde; Lipid Peroxidation; Skin Aging; Free Radical Scavenger.*

Synopsis

The purpose of this work was to develop an "in vivo" biochemical system capable of assessing the ability of cosmetic products to control free radical induced lipid peroxidation and tissue damage that accompanies the aging process. There is extensive support in the literature regarding the involvement of free radicals in the etiology of the skin aging process. Our system employs both indirect and direct measurement of Fe++EDTA induced lipoperoxidation. The measurement of malonaldehyde (MDA) presence on skin surface is an indirect method of measuring lipoperoxidation, while direct fatty acid analysis is done by using gas liquid chromatographic techniques.
Reduction or inhibition of MDA production, as well as changes in the ratio of unsaturated/saturated skin fatty acids in treated subjects may be an indication of the effectiveness of certain cosmetic products in anti-aging treatment

Riassunto

Lo scopo del nostro lavoro é stato quello di sviluppare un sistema biochimico "in vivo" capace di valutare l'abilità di un cosmetico a controllare la perossidazione lipidica indotta dai radicali liberi e le alterazioni tessutali che accompagnano il processo dell'invecchiamento. Numerosi dati della letteratura indicano il coinvolgimento dei radicali liberi nell'etiologia dell'invecchiamento della pelle. Il sistema da noi adottato utilizza sia la misurazione diretta che indiretta della lipoperossidazione, indotta incubando con Fe-EDTA. La determinazione della malonaldeide presente nel film lipidico superficiale della cute é un metodo indiretto di misura della lipoperossidazione, mentre mediante gas cromatografia é stata effettuata un'analisi diretta degli acidi grassi cutanei.
La riduzione o l'inibizione della produzione di MDA, così come modifiche del rapporto insaturo/saturo degli acidi grassi cutanei, nei soggetti trattati, possono fornire indicazioni sull'efficacia dell'impiego di un cosmetico nel trattamento "anti-aging della pelle.

Introduction

In recent years, free radical generation has been increasingly implicated in a variety of physiological processes in living systems (1), as well as in the etiology of several human diseases (2). Molecular oxygen can maximally accept four electrons to produce two molecules of water. The one, two and three-electron reduction of oxygen results in the production of toxic and reactive intermediates: superoxide anion ($O_2^{\cdot-}$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($OH^{\cdot}$) respectively.

The major toxic effects, however are probably due to $OH^{\cdot}$ formation from O_2 and H_2O_2, which generally leads to membrane lipid peroxidation and other cellular lesions (3-4). Lipofuscin accumulates in cells with age and, in general, shows a linear increase over the life span.

This heterogenous polymer, referred to as aging pigment, is apparently the result of an aldehyde, such as malonaldehyde, conjugating with primary amine groups of other lipids, nucleic acids, and proteins, to form Shiff's base type compounds.

This process may be related to the concept that skin aging involves oxidative mechanisms with the participation of oxygen free radicals, subsequent lipid peroxidation of cell membranes, and production of reactive by-products like malonaldehyde.

Polyunsaturated fatty acids, targets of free radical attack, and production of its by-product malonaldehyde, may be viewed as the dynamic parameters in evaluating the rate of age-related changes that occur at tissue levels.

This background information prompted our design of an in vivo biochemical model which could be used to assess the effects of topical treatments (i.e. cosmetics) as antioxidants which protect against lipoperoxidative changes of the skin.

Materials and methods

Treatment and sampling:

The study was limited to adult males, who were required to avoid the use of hair dressing and other sources of lipid contamination.

The surface lipids were preleved from all areas of the forehead according to a standardized procedure which consisted of rolling a cotton flock over the forehead three times horizontally and then three times vertically. The collection site was washed thoroughly with neutral soap 4 hours prior to the removal of surface lipids which began at 12,00 noon.

Treatment with Vitamin E was made by α–pplying a 3 ml solution containing 5% α–tocopherol in 20% ethanol over the total skin surface area of the forehead using the method described above. Samples were taken at different time intervals after treatment.

Specimens were stored in test tubes with teflon-lined screw caps at -20° C, and analysed within a week.

Extraction of skin surface lipids

Specimens were transferred to a mixture of methanol (2.5 ml) and chloroform (lml). The mixture was kept at room temperature for lh. The lipid residues were extracted once more with 1.0 ml of chloroform-methanol (1:1 v/v) for 30 min and then pooled with the first extract. Heneicosanoic acid (10μg) was added to the combined extracts as an internal standard, and 2 ml of 1% NaCl in HCl 0.01 M, was then added to the extract.

Following centrifugation the upper layer was discarded and the lower layer was washed with 3 ml methanol-H_2O (1:1 v/v).

The phases were separated again by centrifuga-

tion and the chloroform phase was evaporated. The lipids were then dissolved in 3 ml chloroform-methanol (2:1 v/v), and the solution was stored at -20°C until analysis.

System for inducing peroxidative stress on skin surface lipids:

Aliquots (1.5 ml) of lipid extract were brought to dryness under a stream of nitrogen.
In order to stimulate peroxidative reactions Fe^{2+}-EDTA (0.2 mM) containing 0.1 M phosphate buffer pH:7.5 was added to the reaction mixture, (final volume of reaction of 0.5 ml) and maintained at 37°C for 1 hr. At the end of the incubation period, MDA was measured.

Assay for MDA

MDA was measured using a micromethod modified from Slater and Sawyer (5): to 0.5 ml of the reaction mixture was added 0.5 ml of 20% (w/v) thrichloroacetic acid; after centrifugation, 0.9 ml of the supernatant fraction was added to 1 ml of 67% thiobarbituric acid (Sigma, St. Louis, MO) dissolved in 0.026 M Tris-HCl buffer (pH:7.0).
The samples were heated in boiling water for 10 min. After cooling, the absorbance was measured at 532 nm, on a Beckman 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 quantified using MDA standard (Aldrich, Milwaukee, WI) and expressed in nanomoles of MDA per nanomole of phosphate.

Assay for phosphate

Phosphate was measured using an ultramicro modification of Bartlett (6). To the aliquots (1.5 ml) of lipid extract was added 0.3 ml of 10 N H_2SO_4 and the mixture was heated to 200°C for 3 hours. Two drops of 30% H_2O_2 were added and the solution was heated for 1.5 hours more at 200°C to complete the reaction by decomposing all the peroxide. 0.65 ml of H_2O, 0.2 ml of 5% ammonium molybdate, and 0.05 ml of the Fiske-Subbarow reagent were then 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-henzene (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 hexane layer was then transferred into 3 ml vials, covered with teflon-lined screw caps, dried, and resuspended in 100ul of hexane. 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 a 2 m x 2 mm 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 linearity was checked using commercially available mixed standards. Peak height measurements were used for quantitation, and expressed as micromoles of fatty acid per nanomole of inorganic phosphate.

Results

Figure 1 illustrates MDA formation per nmole of lipidic material as related to different concentrations of lipoperoxidation inducing material Fe-EDTA. The data show that MDA formation is linearly related to increases in concentration of Fe-EDTA.

Figure 2 illustrates the effects of a topical treatment of Vitamin E (α-tocopherol) on skin surface peroxidation. According to the data, skin lipids incubated in Fe-EDTA mixture exhibit notable resistence to lipoperoxidative damage after treatment with Vitamin E, as noted by the decrease in MDA formation. In relation to time, the protective effect of Vitamin E begins 4 hours after treatment and persists until 24 hours.

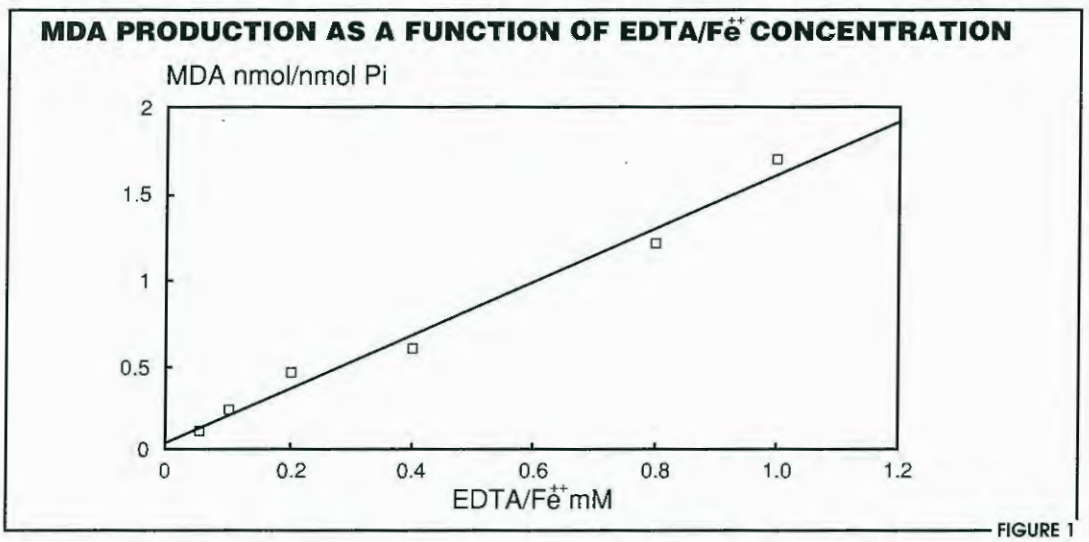

FIGURE 1

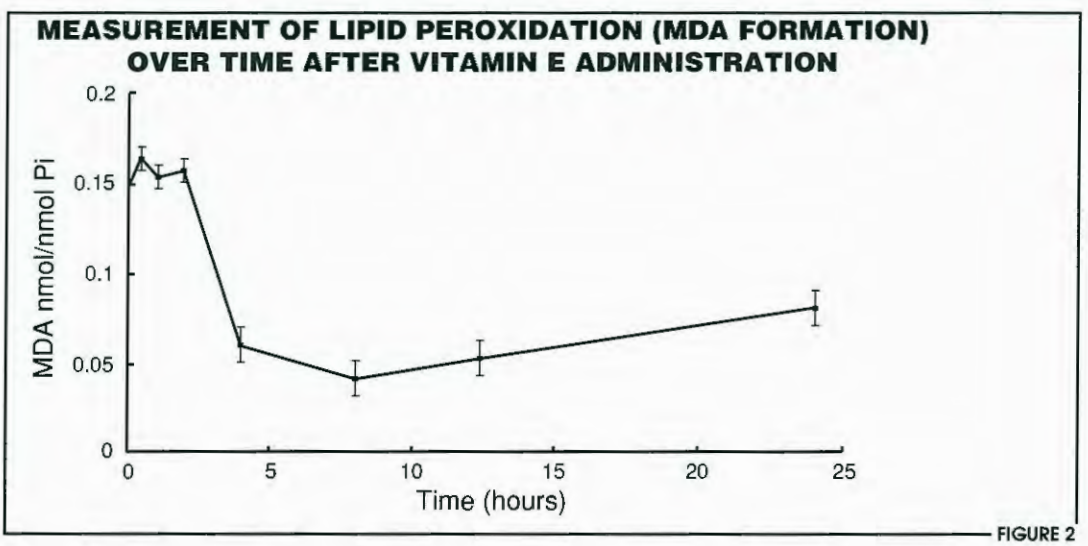

FIGURE 2

Figure 3 illustrates the effects of different concentrations of Fe-EDTA on skin surface fatty acid peroxidation. Lipoperoxidation, directly measured as % decrease in unsaturated/saturated fatty acid ratio is higher after incubation with 1 mM Fe-EDTA as compared to concentrations of 0.5 and 0.2 mM. Furthermore, this decrease is greatest for even-chain-length fatty acids C-14 and C-18 as compared to other even-chain-length fatty acids. Interestingly, odd-number carbon chain C:15 reveals the lowest percentage of unsaturated/saturated fatty acid breakdown at all concentrations of Fe-EDTA mixture.

Figure 4 shows the effects of "in vivo" treatment of skin surface lipids when Vitamin E is topically applied as 3 ml of 5% α-tocopherol, as significantly protective against free radical induced lipoperoxidation. Of the different fatty acids, C:15 appears to be the best protected by Vitamin E treatment, while C:14 is the most susceptible to peroxidative damage.

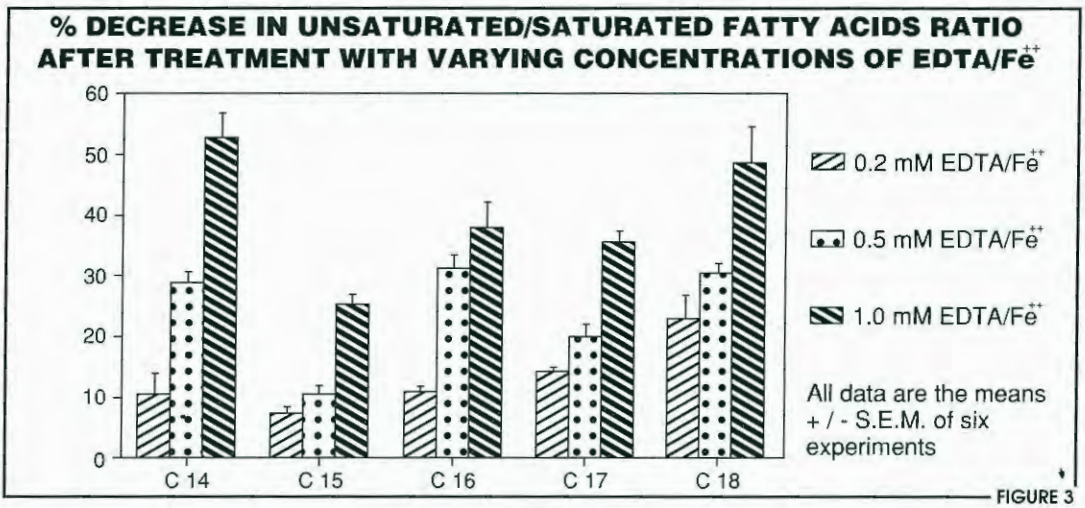

FIGURE 3

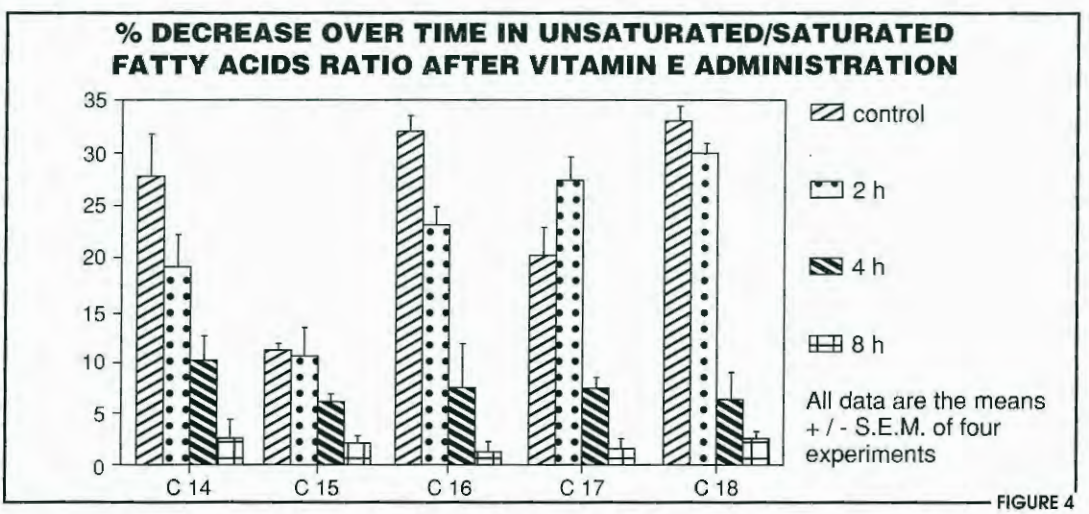

FIGURE 4

Figure 5 plots MDA formation against the percent decrease in the unsaturated/saturated fatty acid ratio obtained over time (8 hours total) for differing chain-length fatty acids.

LIPID PEROXIDATION AFTER VITAMIN E ADMINISTRATION OVER TIME: PLOT OF MDA VS. % DECREASE IN UNSATURATED/SATURATED FATTY ACID RATIO

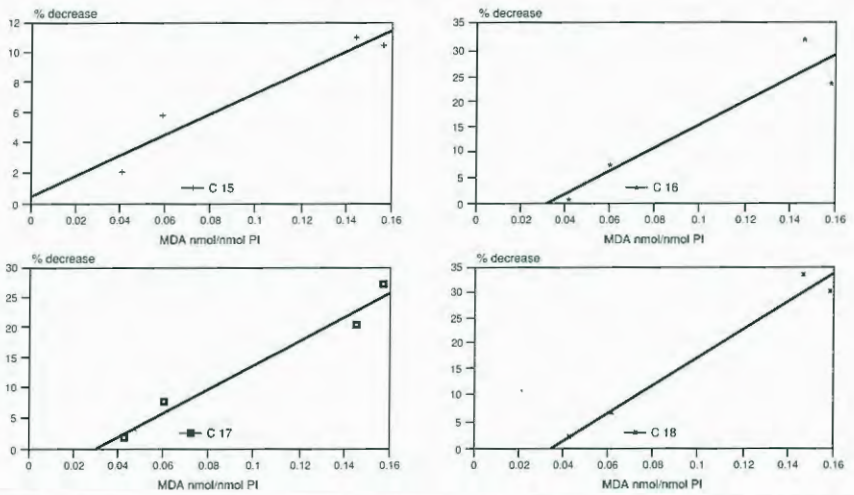

FIGURE 5

Discussion

Skin is an highly differentiated and certainly complex organizational structure. A number of degenerative skin disorders appear late in the life of humans suggesting that, among other factors, aging may act as primer or as an adjuvant factor in the expressivity of the skin pathology. Furthermore, as a result of immulogical changes that come naturally with human aging such as the involution of the thymus and altered differentiation of lymphocytes, aged skin becomes hypersensitive to photocarcinogenesis (8).

Peroxidative damage in cells results from an imbalance between production of free radicals (oxygen singlet, hydroxyl radical, hydrogen peroxide) and their neutralization by the cellular antioxidant defense system (superoxide dismutase, glutathione peroxidase, catalase, glutathione and tocopherol). When the production of superoxide predominates, cells accumulate products of lipid peroxidation. One of the most useful indicators for measuring peroxidative damage or oxidative stress is the presence of malonaldehyde.

Using Fe-EDTA, we created a situation of oxidative stress which was intended to reproduce the pathogenic condition which is believed to underlie the skin aging process and cancerous cutaneous processes. When EDTA-Fe is added to skin surface lipids of normal subjects, MDA production linearly increases. Direct analysis of lipoperoxidation of skin fatty acids, by gas liquid chromatographic techniques, revealed a strong correlation between

decrease in the unsaturated/saturated fatty acids ratio (i.e. peroxidative breakdown of unsaturated fatty acids) and MDA formation.
Treatment with Vitamin E, as an antioxidant, provided significant protection against oxidative stress, as the measured decrease in MDA formation, and as the unsaturared/saturated fatty acids ratio maintained closer to control value, suggests. The above data suggest that our method may be a particularly useful tool for investigating the antioxidant and even antiaging effects of cosmetic products.

References

1. **Prior W.A. (1977):** "Free radicals in Biology". The involvement of radical reactions in aging and carcinogenesis. In W.A. Prior (Ed.), Medicinal Chemistry, Vol. 5, Elsevier Scientific Publications, New York, pp. 331-359 .
2. **Slater T.F. (1972):** "Free Radicals Mechanisms in Tissue Injury" Pion Ltd. London.
3. **Fridovich I. (1975):** "Superoxide Dismutase." *Annu. Rev. Biochem.* **44:** 147-159 .
4. **Halliwell B. (1981):** "Free radicals, oxygen toxicity and aging." In R.S. Sohal (Ed.), Age Pigments, Elsevier North Holland Biochemical Press, Amsterdam, pp . 1-62 .
5. **Slater T.F. and Sawjer B.C. (1971):** "The stimulatory effects of carbon tetrachloride and other halogenoalkanes on peroxidative reactions in rat liver fractions in vitro" *Biochem J.*, **123:** 805-814.
6. **Bartlett J.R. (1959):** "Phosphorus assay in column chromatography" *J. Biol. Chem.*, **234:** 466-468.
7. **Harman D. (1984):** "The aging process" *Proc. nat. Acad. Sci. U . S .A.* **78:** 7124-712 6 .
8. **Giannetti A. (1986):** "Immunological changes in aging skin." In P. Morganti, W. Montagna (Eds.), *Cosmetic Dermatology*, Vol. 1, International Ediemme, Rome, Italy, pp. 139-143 .