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AN *IN VITRO* SELECTION OF NEW COSMETIC ACTIVE COMPOUNDS: FROM SCREENING TESTS ON MONOLAYERED FIBROBLAST CULTURE TO EFFICIENCY STUDY ON 3-D DERMAL EQUIVALENT

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Synopsis

The requirement for documentation of efficiency with technical data in support of claims attributed to cosmetic products has been recently introduced by the European Community directive concerning cosmetics. The use of monolayered fibroblast culture enables the fast acquisition of information, however, this model is oversimplified compared with the in vivo architecture where the cells are dispersed in the Extracellular Matrix (ECM) network. Consequently, the use of 3-D Dermal Equivalent model including a collagen-GAGs-chitosan matrix, major components of ECM, populated by fibroblasts is a better way of studying the effects of new molecules on fibroblast metabolism and on ECM synthesis. We present here an in vitro selection of promising new active compounds produced by biotechnology. Four biopeptides were preselected after screening the effects of 200 biopeptides on the stimulation of cell proliferation on monolayered fibroblast cultures. Then, these four biopeptides were tested on monolayered fibroblast culture, both for glycosaminoglycan and protein synthesis. The most effective one, Milk derived biopeptide, was used for an efficiency study using a Dermal Equivalent (DE) model. This Milk biopeptide was tested on DE by systemic application (1.25 % (v/v) in culture medium) for 8 days. Then, total protein, collagen, glycosaminoglycan and elastin synthesis were measured respectively by radioactive proline incorporation, electrophoresis followed by densitometry and colorimetric assays. These in vitro techniques have lead to the selection of an active cosmetic compound which could have interesting properties, due to the significant activation of glycosaminoglycan neosynthesis it is able to induce, for the treatment of aged and dehydrated skin.

Riassunto

L'esigenza di una documentazione che attesti l'efficacia dei prodotti cosmetici, insieme a dati tecnici che ne supportino la validità è stata recentemente introdotta dalla direttiva della Comunità Europea sui cosmetici. L'uso di colture monostrato di fibroblasti permette la rapida acquisizione di informazioni. Tuttavia questo modello è eccessivamente semplificato se paragonato all'architettura in

vivo, dove le cellule sono sparse nella rete della Matrice Extracellulare (ECM). Di conseguenza, l'uso del modello 3-D Dermal Equivalent, che include una matrice collagene-GAG-chitosano quale maggiore componente dell'ECM, popolata da fibroblasti, è un modo migliore per studiare gli effetti di nuove molecole sul metabolismo dei fibroblasti e sulla sintesi della ECM. Viene presentata qui una selezione in vitro di promettenti nuovi principi attivi prodotti dalla biotecnologia. Sono stati preselezionati quattro biopeptidi dopo aver analizzato gli effetti di 200 biopeptidi sulla stimolazione della proliferazione cellulare su colture monostrato di fibroblasti. Questi quattro biopeptidi sono stati poi testati su una coltura monostrato di fibroblasti, sia per la sintesi dei glicosaminoglicani che per la sintesi proteica. Il più efficace, un biopeptide derivato dal latte, è stato usato per uno studio sull'efficacia utilizzando un modello Dermal Equivalent (DE). Questo biopeptide da latte è stato testato sulla DE attraverso applicazioni sistematiche (1.25% (v/v) in un medium culturale) per 8 giorni. Il totale proteico, il collagene, i glicosaminoglicani e la sintesi di elasticità sono state poi misurate rispettivamente attraverso incorporazione di prolina radioattiva, elettroforesi seguita da saggi di densitometria e colorimetria. Queste tecniche in vitro hanno portato alla selezione di un principio attivo che la neosintesi dei glicosaminoglicani è capace di indurre, per il trattamento della pelle invecchiata e disidrata.

INTRODUCTION

The increasing interest in cosmetics in recent years has led to considerable development of new technologies. The formulation of a new cosmetic product involves complex technologies such as rheology, surface-active chemistry, emulsion science and biotechnology.

According to the 6th amendment and following uptades of the European Community directive on cosmetic products (93/35/EEC), the manufacturers of marketed cosmetic formulations must keep information regarding: a) the assessment of the safety to human health, b) existing data on undesirable health effects resulting from the use of the product, and c) proof of the effect claimed for the cosmetic product. This implies the need to identify and codify experimental tests that are able to demonstrate biological cosmetic activity.

In vitro studies carried out in order to demonstrate the biological effects of an active cosmetic compound are usually performed on monolayered cultures. Even if today normal human fibroblasts, keratinocytes, melanocytes and endothelial cells can be maintained in monolayered culture, these conventional models are oversimplified and do not fully reproduce the in vivo situation because the cells are not in their physiological three-dimensional environment, in which each cell are subjected to cell-cell and cell-matrix interactions.

The development of three-dimensional models allows us to assess these kinds of interactions but also allows topical applications of the tested formulation, mimicing the standard conditions of use of the finished product. Today, a number of such three-dimensional models have been developed, and are able to reproduce in vitro either a multilayered epidermis cultured on various substrates (1, 2, 3), a dermal equivalent (4, 5) or a reconstructed skin (6,7,8). The Dermal Equivalent (9) developed by our laboratory for pharmaco-toxicological applications includes a Dermal Matrix (10) made of collagen-glycosaminoglycans-chitosan populated by normal human fibroblasts which neosynthesise an organised extracellular matrix, with striated collagen fibers surrounding the cells (11, 12, 13).

As biotechnologies allow the production of a large number of molecules, the most promising of them must be preselected prior to evaluation in in vivo cosmetology trials.

We present here in vitro techniques used for selecting new active cosmetic compounds. Four biopeptides were preselected after screening the effects of 200 biopeptides on the stimulation of cell proliferation on monolayered fibroblast cultures. This paper reports the results of tests using these four biopeptides on both for glycosaminoglycan and protein synthesis in monolayered fibroblast cultures. The most effective, Milk derived biopeptide, was used for an efficiency study using a Dermal Equivalent (DE) model.

MATERIALS AND METHODS

Tested Biopeptides

Four biopeptides issue from biotechnologies were obtained by fermentation of several proteins (Wheat, Milk, Soya) by different micro-organisms (Coletica, France). Various hydrolysates were obtained according to the micro-organism used for the fermentation: Wheat 1, Milk 1, Soya 1 and Soya 2.

Test procedure on Monolayered Fibroblast Culture

Cell proliferation: normal human fibroblasts (10 000 cells/cm^2) were seeded in 12-wells plate (Costar, USA) and were cultured with culture medium containing Dulbecco's Modified Eagle's Medium DMEM (Life technologie, France) supplemented with 2% foetal calf serum (Boehringer, Mannaheim), 50 µg/ml streptomycin, 2 mM L-Glutamin, 100 UI/ml penicilin (Biomérieux, France) supplemented with 1.25 % (v/v) of each biopeptide. Control fibroblast cultures were done in the same conditions in the

absence of biopeptides. After 2 to 4 days of culture, fibroblasts (n=3 wells) were detached by incubation with trypsine-EDTA (Sigma, USA) and counted using a Coulter Counter (Coultronics, USA). Total proteins assay (14): fibroblasts (23 000 cells/cm^2) were cultured in 75 cm^2 flasks for 7 days with a culture medium containing 10% foetal calf serum (FCS). After this proliferation step, the cells were cultured for 7 additional days with 5% FCS culture medium supplemented by 1.25 % (v/v) of each biopeptide except for the control. Then, the culture medium was removed, the fibroblast layers rinced with PBS (Sigma, USA) and harvested by scraping. The cells were lysed with 1% Tryton X 100 prior to Micro BCA method to determine the amount of proteins in the suspension. Glycosaminoglycans assay (15): the fibroblasts were cultured in monolayer for 14 days as described above. In the collected culture medium, the glycosaminoglycans were extracted by ethanolic precipitation, separated by electrophoresis on cellulose acetate gel in a 0.1 M pH 5 barium buffer solution and stained with GAGs-specific alcian blue. The GAGs were identified by comparison with standards (Hyaluronic Acid, Chondroïtin-4-Sulphate, Dermatane and Heparane Sulphate) and quantified by integrating peak surfaces. The assay was done in triplicate.

Test procedure on Dermal Equivalent

Human foreskin fibroblasts (200 000 cells/cm^2) were seeded into freeze dried Dermal Matrix made of 72% bovine collagen types I and III, 8% ovine chondroïtin-4-sulphate and 20% chitosan. DEs were cultured in DMEM supplemented with 10% neonatal calf serum, 25 mg/l gentamycin, 100 000 UI/l penicillin, 1 mg/l amphotericin B and 40 mmol/l L-glutamine and 50 mg/l ascorbic acid (Sigma, USA) for 3 weeks and the medium was changed twice a week. Mature DEs were cultured for 8 days in a culture medium containing 1.25% (v/v) of selected biopeptide, except for control DEs. At the end of the systemic application period, synthesised GAGs, total proteins and collagenic proteins and elastin were assayed on collected and freezed culture media and into DEs. Histological control: three DEs were fixed in Boin's solution and embedded in paraffin; 5 µm sections were stained with hematoxylin-phloxin-saffron and examined under a ZEISS IM 35 microscope. MTT Test (16): DEs were incubated with 2 ml of a solution of 1 mg MTT/ml of PBS. The reduced MTT dye was extracted with 4 ml of HCl 0.04N acidified isopropanol (Merk, USA) and mechanical agitation at room temperature for 30 minutes. A 100 µl sample of each extraction was placed in a 96 wells-plate and the absorbance was read at 550 nm on a plate reader (DYNATECH MR 4000) with 100 µl isopropanol as a blank. Total and collagenic proteins assay (17): briefly, on the 7th day of biopeptide culture, 12 DEs were cultured with culture medium supplemented with 5 µCi/ml of (5-3H)-proline for 24 hours. Half of them were used for determining the total incorporated radioactivity using a ß scintillation counter Packard Tri-carb 2100 TR. The other half (n=6 DEs) was used in order to measure the radioactivity incorporated in the non-collagenic proteins, after a specific degradation of collagen by collagenase (Advance Biofacture, USA). Substracting one from the others gave the amount of radioactivity incorporated in collagen expressed in radioactivity units (disintegrations per minutes (dpm) per DE). Glycosaminoglycans assays: the quantity of glycosaminoglycans synthesised after 5 days of treatment was assayed as described above in the collected culture medium. Elastin assay (18): the soluble fraction of elastin was assayed using Fastin elastin kit (Biocolor, Ireland) in the DE culture medium, sampled on the last day of biopeptide application (n=6 fractions).

RESULTS

The study of new biopeptides on the fibroblast metabolism was divided in 3 steps: (1) Preselection screening of 200 biopeptides on fibroblasts proliferation cultured in monolayer (2) Evaluation of 4 preselected biopeptides on GAGs and total proteins synthesis by fibroblasts cultured in monolayer (3) Efficiency study of the most effective biopeptide on three-dimensional Dermal Equivalent including measurements of the synthesised total proteins, collagen, GAGs and elastin.

Evaluation of 4 biopeptides on Monolayered Fibroblast Culture

Proliferation rate of fibroblasts cultured in monolayer in the presence of various biopeptides added to the culture medium (1.25% v/v) was determinated by cell counting after 2 and 4 days culture (n=3) and are presented in Fig.1a.

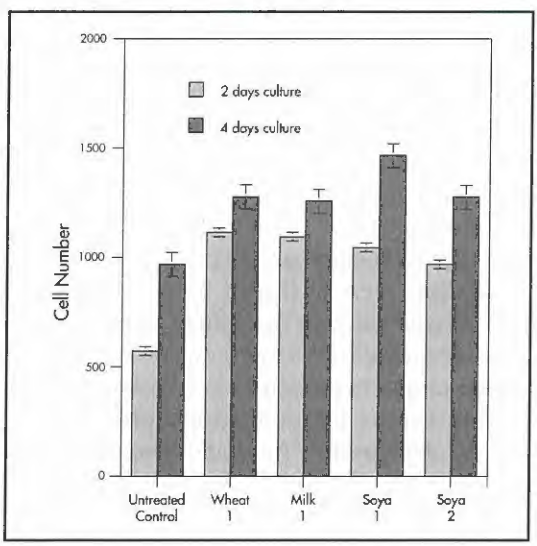

Fig. 1a. Proliferation Rate of fibroblasts cultured in monolayer according to the different biopeptides added in the culture medium (1.25% v/v) determinated by cell count after 2 and 4 days culture (n=3).

For all the tested biopeptides, we observe a significant enhancement of the fibroblast proliferation rate versus the control culture (Student's test, $p<0.01$). This effect is more significant after 2 days culture than after 4 days culture as shown by the respective activation percentages: 81% and 36% for Wheat 1, 76% and 34% for Milk 1, 64% and 50% for Soya 1 and, 50% and 36 % for Soya 2.

The synthesised glycosaminoglycans released in the culture medium by fibroblasts treated with biopeptides versus control were measured by electrophoresis followed by densitometry. The

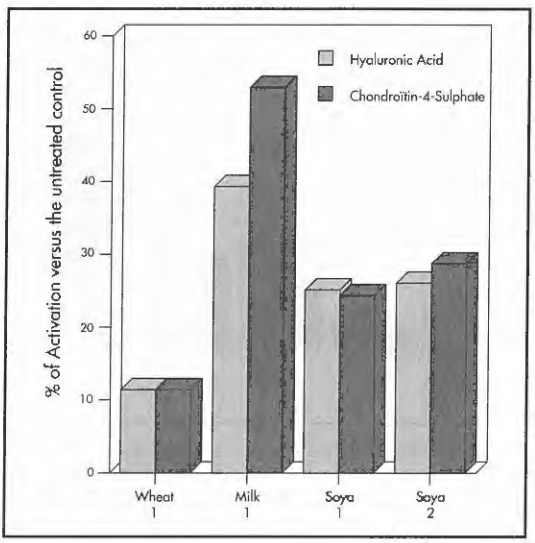

Fig. 1b. Synthesised Glycosaminoglycans evaluated by electrophoresis of the culture medium of biopeptide-treated monolayered fibroblast culture versus control and expressed as activation % versus control (n=3).

results, presented in Fig.1b, were expressed as percentages of activation versus untreated control for each glycosaminoglycan detected in the culture medium: Hyaluronic Acid (HA) and Chondroïtin-4-Sulphate (C4S). The amounts of GAGs measured in all the biopeptide-treated culture media are significantly increased versus the untreated control (Student's test, $p<0.01$). Milk 1 biopeptide is the most effective substance tested regarding the activation percentages of both HA (39%) and C4S (53%).

The synthesised total proteins were evaluated

using the Micro BCA method performed on biopeptide-treated monolayered fibroblast cultures and untreated controls, and are illustrated in Fig.1c. We observe that all the tested biopeptides stimulate the total proteins synthesis; however, only Milk 1 and Soya 2 biopeptides induce a significant activation, respectively of 7% and 20%, compared with the untreated control (Student's test, $p<0.01$).

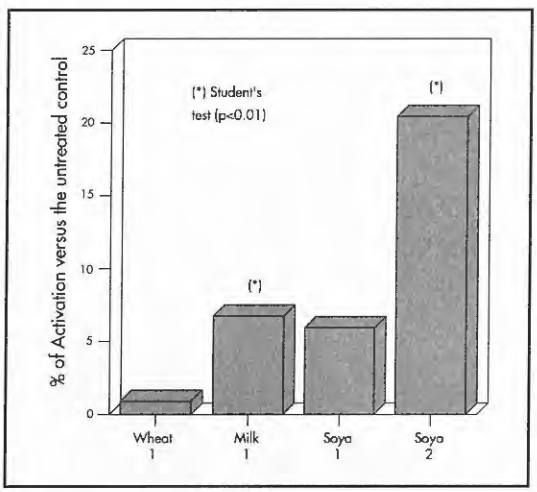

Fig. 1c. Synthesised Total protenis evaluated after Micro BCA method performed on biopeptide-treated monolayered fibroblast culture versus control and expressed as activation % versus control.

Efficiency study on Three-Dimensional Dermal Equivalent (Fig. 1c)

After the selection of Milk 1 biopeptide (Hydrakine®, Coletica, France) according to the activation of GAGs synthesis, we wanted to confirm and validate these results using a 3-D DE model.

Histological control of the Dermal Equivalent used for this study is illustrated in Fig.2. Fibroblasts (F) have migrated, proliferated and invaded the porous structure (P) of the Dermal Matrix. We observe a production of neosynthesised human Extracellular Matrix (ECM) surrounding each fibroblast.

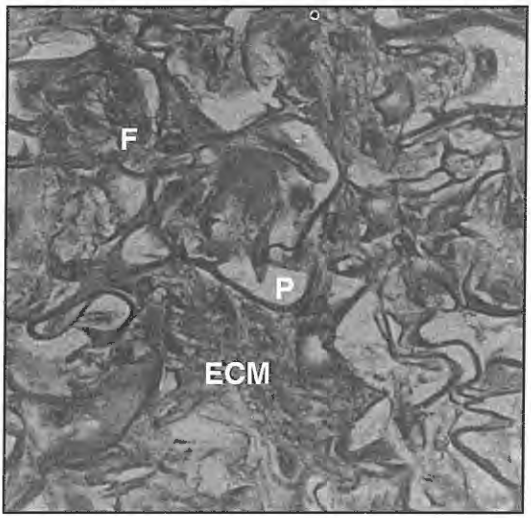

Fig. 2 Histological control of a mature Dermal Equivalent (x 320)
(F) Fibroblast (ECM) Extracellular Matrix (P) Pore of Dermal Matrix

The cellular viability correlated with the number of cells was evaluated by a MTT test performed on control untreated DEs and on Milk 1 biopeptide-treated DEs (n=6). The control DEs absorbances were not significantly different from those of Milk 1 biopeptide- treated DEs (Student's test, $p<0,01$), that means that there is the same number of cells in control and biopeptide-treated DEs.

The glycosaminoglycans released into the culture medium were evaluated by electrophoresis and densitometry of the culture media of biopeptide-treated DEs and control DEs (n=6). These results expressed as percentages of activation versus the control are presented in Fig.3a. Hyaluronic Acid and Chondroïtin-4-Sulphate are detected in the culture media of both treated and control DEs. Milk 1 biopeptide induces a significant increase of HA (114 %) and of C4S (54 %) versus control DEs culture media (Student's test,$p<0.01$).

Total and Collagenic Proteins synthesised by the fibroblasts into the DEs were evaluated after (5-^{3}H)-proline incorporation and specific degradation by collagenase in biopeptide-treated DEs

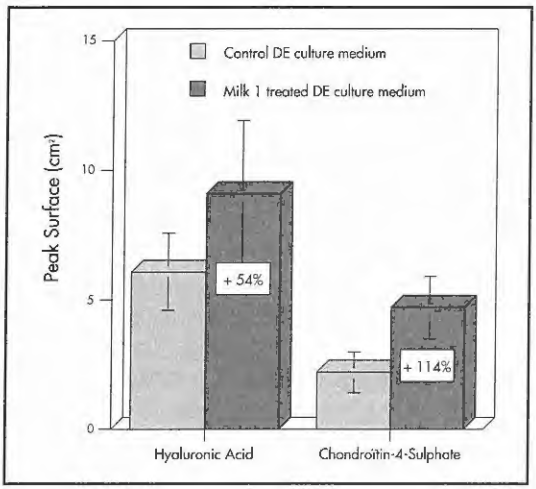

Fig. 3a. Synthesised Glycosaminoglycans evaluated by electrophoresis of the culture medium of biopeptide-treated DEs versus control and expressed as activation % versus control (n=6).

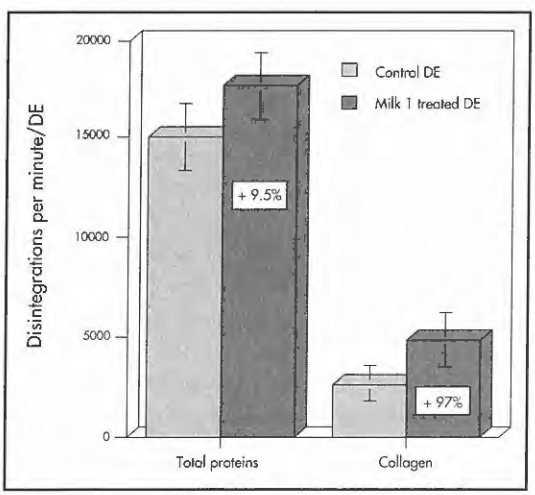

Fig. 3b. Synthesised Total and Collagenic Proteins evaluated after (5-3H)-proline incorporation and specific degradation by collagenase in biopeptide-treated DEs versus control DEs (n=6).

versus control DEs (n=6). The results are expressed as percentages of activation versus untreated control and are illustrated in Fig.3b. Milk 1 biopeptide has a significant activation effect on total protein synthesis and especially on collagenic proteins synthesis as shown by respective activation percentages of 9.5 % and 97 % versus control DEs (Student's test, $p<0.05$). Elastin synthesis was evaluated using an Elisa kit, in biopeptide-treated DEs culture medium, and control DEs culture medium (n=6) and is illustrated in Fig.3c. A significant increase of 64% in elastin synthesis is observed in the presence of the biopeptide (Student's test, $p<0.01$).

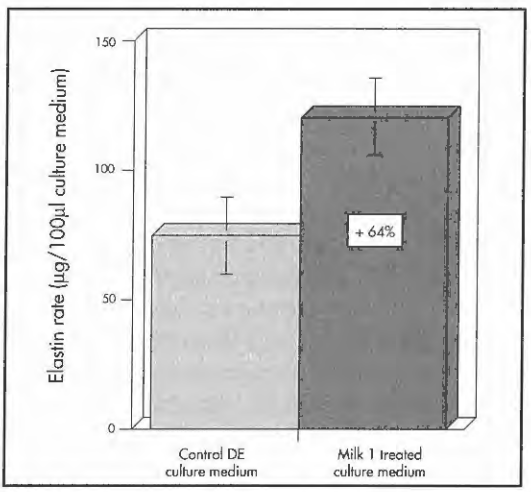

Fig. 3c. Synthesised Elastin evaluated by colorimetry in biopeptide-treated DEs culture medium and control DEs culture medium (n=6).

DISCUSSION

Manufacturers will increasingly have to take into account a recent directive from the European Community which requires that the claimed properties of the cosmetic products be supported by technical data and documentation demonstrating efficacy. *In vitro* techniques will play an increasing role in the development of active cosmetic compounds, because they represent an interesting alternative to tests on animals or humans. On the other hand, biotechnology now allows the production of numerous molecules that could have important effects in cosmetology. However, these new molecules should be selected using a well defined strategy.

In this paper, we present the in vitro techniques used for selecting biopeptides, produced by biotechnology, which are able to stimulate neosynthesis of ECM components, and especially GAGs. As a matter of fact, the decrease in concentration of those molecules during ageing is specially linked with dehydratation of aged skin. A stimulation of their neosynthesis might be an interesting way of reducing some ageing effets on skin structure and properties as the polyanionic nature of these macromolecules are responsible for skin turgescence due to their capacity to retain water (19).

The choice of the two in vitro models, monolayered fibroblast culture and Dermal Equivalent was motivated by the crucial role of fibroblasts in cutaneous ageing mechanism and dehydrated skin phenomena. Briefly, cutaneous ageing is characterized by a decrease in the synthesis of proteins, such as collagens and proteoglycans. Also, a spontaneous and progressive degradation of the elastic fibers take place (20) and a low cellularity develops (21). Consequently, the most visible changes occuring in ageing skin are in the dermis where the destruction of the relationship between fibroblasts and the interstitial matrix occurs (22). Fibroblasts play a pivotal role in the morphogenesis and dynamic remodelling of the dermis including the synthesis of ECM components and specific enzymes involved in ECM degradation. Consequently research on skin ageing should focus on fibroblasts, whose role consists in maintaining the matrix structure and fonctionality.

The model used for screening should give rapid results using relatively easy and inexpensive analytical techniques. This is the reason why monolayered fibroblast culture was used for a preselection of the most promising biopeptides among about 200 products of initial interest (Coletica, France) using the criteria of the cellular proliferation rate. Four of these biopeptides were able to give very interesting results on fibrohlast renewal, when cultured in monolayer. In this paper, we have carried out investigations on these four selected biopeptides by checking if this proliferation stimulation effect is accompanied by an increase of the synthesis of extracellular matrix components such as proteins and glycosaminoglycans. At this stage, two biopeptides appears to have potential cosmetic properties: Soya 2 biopeptide for its effect on proteins synthesis stimulation which is the subject of another study (23), and Milk 1 biopeptide for its significant activation of GAGs synthesis (39% increase of HA and 53% C4S). The latter was selected for further efficiency study. The aims of such study are to comfirm and to improve on the previous results using more sophisticated analytical methods for measuring dynamic synthesis of ECM components in a DE, where fibroblasts are in a more physiological environment. In this three-dimensional model, the fibroblasts are non proliferative and surrounded by their own human neosynthesised extracellular matrix, with a very similar organization to that in normal dermis (12). After treatment of DEs by Milk 1 biopeptide, no variation on the cell number using the MTT viability test was found versus control, and we conclude that the increase in ECM proteins and GAGs are due to a real activation of synthesis. The collagen synthesis was significantly increased (97%) by Milk 1 biopeptide treatment. The effect of Milk 1 biopeptide on GAGs synthesis was confirmed as we obtained an increase of 114 % of Hyaluronic Acid and 54 % of Chondroïtin-4-Sulphate synthesis compared with the control DE. Moreover, the presence of the Milk 1 biopeptide in the culture medium induced an elastin synthesis activation of 64%. Elastin is a macromolecule characterized by its high physical and chemical strength giving suppleness and plasticity to the skin. During dermal ageing, there is a spontaneous and progressive degradation of the elastic fibers. This phenomenon is attributed to a reduced synthesis of elastin molecules and also to a concomitant increase in the fiber degradation susceptibility by proteases (24).

Cutaneous ageing is an insidious and progressi-

ve degenerative process, inevitable in course and predictable in outcome. However, cosmetic active compounds can slow down the normal and photoinduced ageing phenomena and can improve external aspects of aged skin. Indeed, modern cosmetology proposes various active compounds such as alpha-hydroxy acids, ceramides or actives extracted from seaweed or plants with different mechanisms of action. Today, biotechnologies open a way to the discovery of a wide range of powerful and innovative cosmetic active compounds like the Milk 1 biopeptide studied in this paper. New processes based on fermentation by different micro-organisms characterized by their highly developed enzyme systems, give rise to radically different peptides than those obtained with conventional chemical method. The biopeptides produced in this way are highly specific and are structural analogues of cell mediators like cytokines. They might be recognised by biological receptors and can induce biological effects. Moreover, the biopeptides selected for this study are very small molecules that penetrate deeper in the skin than macromolecules such as those are present in conventional cosmetic filmogene substances. Consequently, Milk 1 biopeptide is a radically innovative cosmetic moisturizing agent, because it induces the endogenous restauration of the moisturizing potential of the skin by stimulating the neosynthesis of glycosaminoglycans.

CONCLUSION

Modern cosmetology requires the detailed testing of efficacy together with the use of new and original active compounds. Biotechnologies are very effective in the production of innovative active compounds. However, the wide range of products need to be evaluated with fast and accurate methods allowing the selection of the most promising molecules. Consequently, tbe use of in vitro models are going to be increased in the future to ensure the safety (25) and to study and prove the efficiency of new cosmetic molecules.

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