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VALIDATION TESTS AND CELL CULTURES IN COSMETOLOGY: THE PRESENT AND PROSPECTS

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Synopsis

The interest increasingly attracted by cosmetics in recent years has led to considerable development in the related technologies. This progress, however, has not been followed up by adequate legislation. The recent 93/95 Directive of the European Economic Community established that from January 1st 1998 the safety of cosmetic products can no longer be tested on animal models, and that alternative methods must be employed, if available. Direct experimentation **in vivo** is prevented by ethical reasons, while animal models are frequently poorly predictive. For these reasons **in vitro** culture is now of outstanding interest in cosmetology. The authors review the cell culture types that can be used in cosmetological studies

Riassunto

Il grande interesse di cui sono stati oggetto i cosmetici negli ultimi anni ha determinato un notevole sviluppo delle tecnologie legate alla loro produzione, uno sviluppo che non è stato tuttavia accompagnato da adeguati interventi legislativi. La recente Direttiva 93/95 della Comunità Economica Europea vieta dall'1 gennaio 1998 l'utilizzo di modelli animali nei test di valutazione della sicurezza dei prodotti cosmetici, che da quella data dovranno essere effettuati con metodi alternativi, se reperibili. Motivi etici impediscono la sperimentazione sulla specie umana, mentre i modelli animali sono spesso scarsamente predittivi. Ecco perché in cosmetologia la coltura in vitro è attualmente di grande interesse. Gli Autori riassumono i principali tipi di colture cellulari che possono essere utilizzati negli studi cosmetologici.

That our skin is the mirror of our organism is a recognised concept that is proved consistently by the difference between our chronological and biological age. In this sense Cosmesis must be recognized as much more than the simple attempt at improving the aesthetic quality of our image. Indeed, today the cosmetic industry devises products that do not only aim at improving but also at protecting the skin - for instance against the damage wrought by sunlight - and more and more frequently at assisting dermatological medical treatment (1). The interest increasingly attracted by cosmetics in recent years has led to considerable development in the related technologies. This progress has however not been followed up by adequate legislation. After formulation and production, cosmetics should be analysed in technically advanced laboratories. The field of the evaluation of the safety and activity of cosmetic products appears to be full of interesting prospects (2, 3). Over the last 20 years, a series of directives of the European Economic Community (EEC) have required the definition of a series of experimental models for the study of the toxic properties of chemical substances, based mainly on animal tests (4). This research has highlighted the limitations of such experimental models. Moreover, it has shown that the results obtained **in vivo** with traditional models can be complemented and improved by data obtained **in vitro** on cell populations, which allows to study also the development of the toxicological processes induced by chemical substances. The recent 93/95 EEC directive establishes that from January 1st 1998 the safety of cosmetic products can no longer be tested on animal models, if the alternative methods are available. Alternative methods must be devised to identify the toxic potential of ingredients. Several laboratories are working to provide cosmetic industries with validated and reproducible tests that do not require experimentation on animals. Directive 76/768 moreover requires that by 1997 every finished cosmetic product marketed in the EEC be accompanied by exhaustive information on the whole history of the product, from its quantitative formula and production method to the toxicological files of every component, the allergologic tests where necessary, and the clinical tests of activity if they are mentioned in the label. The 93/95 directive requires these files to take "into consideration the general toxicological profile, the chemical structure and the exposure level of all ingredients". Direct experimentation in vivo is prevented by ethical reasons. On the other hand, animal models are frequently poorly predictive, nor are they directly applicable to man because of species and individual differences, as well as differences in doses and exposure time.
In vitro culture of the various components of the skin is at present widely used in genetic, immunological, neurological and, last but not least, cosmetological studies (5). Research is now focusing on **in vitro** reconstruction of the fibroblast and keratinocyte differentiation to obtain a predictive model on the toxicity and topic efficacy of the various substances or finished products on human skin.

Cell culture types

Cell cultures have provided and continue to provide interesting data, since they are simplified systems constituted by organized living cells. Their main advantages are their reproducibility, affordable cost, the fact that they yield results faster than animal models, and that they allow to study a sufficient amount of specimens to obtain statistical significance. The main problem can be the difficulty in correlating the active concentrations **in vivo** with those **in vitro**, considering also that the cells' features vary over time (4). There are different types of cell cultures. Those that are utilised more frequently in validation tests are:
- **cultures in suspension**, where cells can live and proliferate without an adhesion surface;
- **monolayer cultures,** where cells grow **in vitro** attaching to the surface of a culture dish. They need to attach to the substrate for their life and proliferation. Their growth results in monolayers,

i.e. confluent surfaces constituted by a single cell layer. Monolayer cultures are usually primary cultures, i.e. they derive directly from cells explanted from donors. This type of culture allows cells to maintain many functional and specialization characteristics observed in vivo (Fig. 1);

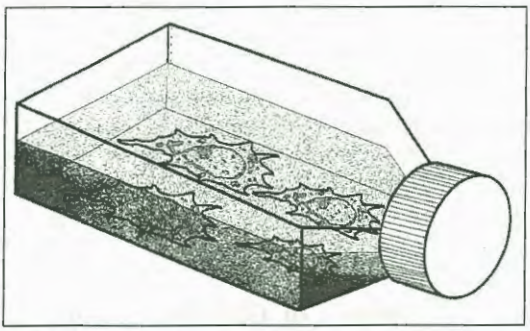


Figure 1: *monolayer culture: cells (fibroblasts) grow in vitro attaching to the surface of culture flask.*

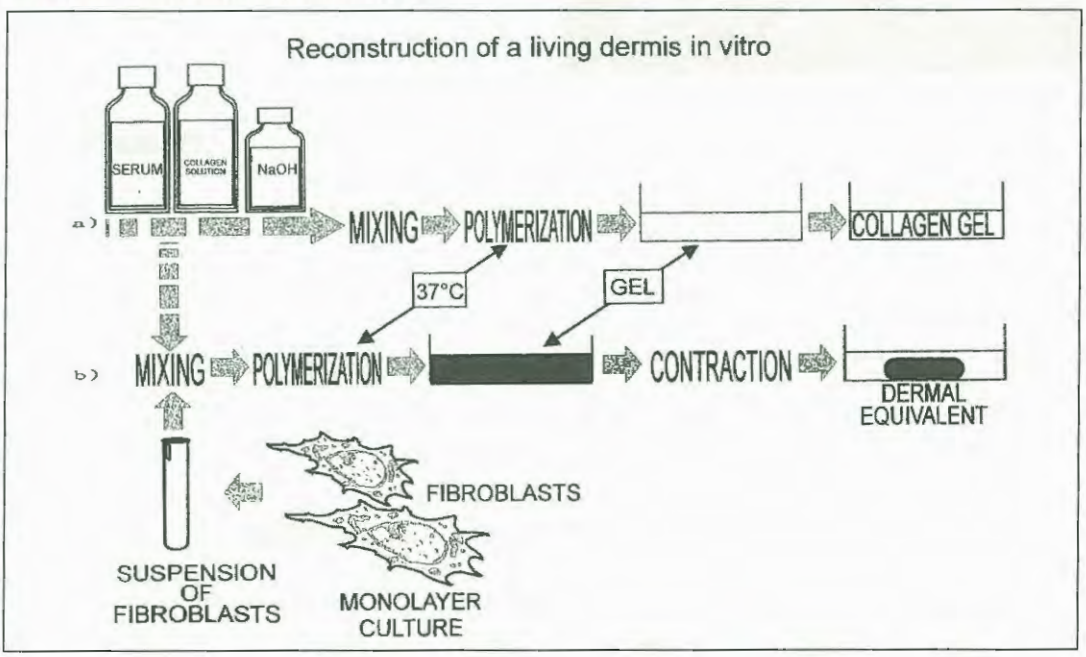
Figure 2:
a) reconstruction of collagen gel in vitro;
b) reconstruction of dermal equivalent in vitro.

- in co-cultures, cells of different types are grown in the same culture dish, for instance fibroblasts and keratinocytes; this allows to reproduce in vitro the complex interactions that occur **in vivo** between different tissues.

a) Monolayer cultures of fibroblasts

In cosmetological research fibroblasts are the cells used more frequently. To reconstruct the dermis in vitro, fibroblasts need to be seeded on a collagen suspension gel that is close to polymerization (dermal equivalent)(6). The fibroblasts attach to the polymerised collagen, contract their fibrils and form the dermal tissue: dermal equivalent (Fig. 2). The three components of this three-dimensional culture model (culture medium, extracellular matrix and fibroblasts) can then be studied separately (6).

Standardization is of central importance in these experiments: donors' age must not exceed a precise range, and fibroblasts must be taken from the same source (skin of the forearm). There are dramatic differences between sites, for instance in enzyme activity (in fibroblasts obtained from



the pubic region the 5 α-reductase enzyme exhibits a five- to six-fold activity as compared to those from the forearm). As for collagen, its origin and extraction must be well characterized and standardized, and working with the same batch for the whole series of experiments is highly recommended. Type I collagen is extracted with acetic acid from rat-tail tendons or from calf skin, while a mixture of types I and III is obtained from human placenta by digestion with pepsin (6). The culture medium is EMEM (Earle's modified Eagles medium), with the addition of 10% foetal calf serum, of pooled AB serum from healthy donors, or serum from patients. Serum greatly influences the life and proliferation of fibroblasts, so the same batch must be used throughout the study. Cultures must be maintained at 37°C in 5% CO_2 /95% air athmosphere. Fibroblasts raised in monolayer cultures divide until they reach confluency, while in dermal equivalents they remain separated (Fig. 3). Their growth rate depends on the density of cells seeded: from $4x10^4$ to 10^5 cells/ml^{-1} proliferation is inversely related to the number of cells seeded. The dermal equivalent can be used as a model of cell growth homeostasis in relation with the external environment. It can also be employed in quantitative studies on collagen fibril condensation by fibroblasts. Rate and extent of contraction are measured by monitoring the diameter decrease of the dermal equivalent. Contraction is directly related to the initial concentration of fibroblasts, and inversely related to collagen concentration. Contraction is very sensitive to pharmacological treatment. The ability of fibroblasts to contract the collagen matrix is inhibited in a linear, dose-dependent manner when dexamethasone or hydrocortisone (2.5 - 1,5 x 10^{-4} M) are added to the culture medium in the presence of an inhibitor of cell division that does not affect the cytoskeleton (1 µl ml^{-1} cytosine arabinoside). Contraction is inbibited by the addition to the culture medium of Acid Fibroblast Growth Factor (a-FGF) in the presence of heparin, which is a factor that stabilises a-FGF (6).

Figure 3: *adherent fibroblasts grown in culture with typical cytoplasmic protrusion and mostly spindle-like features.*

b) Co-cultures of fibroblasts and keratinocytes

To reconstruct in vitro a simplified human skin, an association of dermis and epidermis is necessary as in vivo (5). There are several models, of which we describe the simplest. The dermal equivalent described above is utilized in close

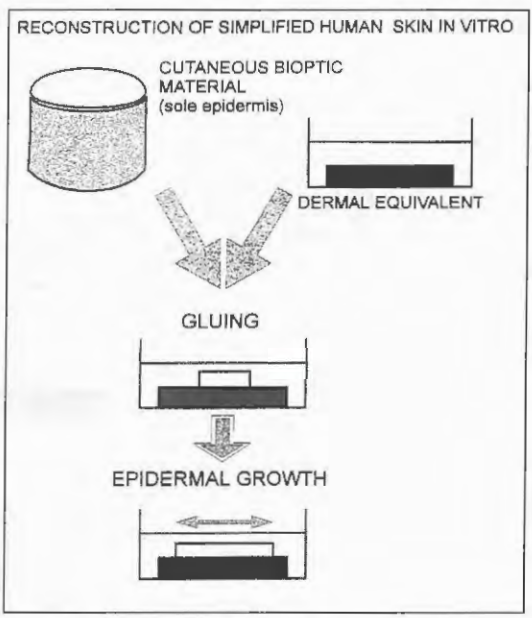


Figure 4: *reconstruction of simplified human skin in vitro.The authors are grateful to Mr G. Forniti of MAVI SUD for providing the drawings*

association with cutaneous bioptic material constituted by the sole epidermis (Fig. 4). It is well known that fibroblasts and collagen affect the growth and differentiation of epithelial cells. A layer of lethally irradiated fibroblasts supports the culture of keratinocytes and increases their lifespan. Collagen provides for their adhesion and for plating efficiency, and is essential in the formation of the basal lamina (6). Keratinocyte sources (donors aged 20 to 40 years) can be superficial cutaneaous biopsies, which are implanted in a dermal layer, or epidermal biopsies, which are glued to the dermis during its fibroblast-mediated formative organization with a drop of soluble collagen. The same source of keratinocytes must be used throughout each study. The donor of keratinocytes differs from that of fibroblasts for practical reasons. No interference is observed between allogenic cells in vitro because there are no cytotoxic cells. Langerhans cells do not appear in the epidermis that is thus formed. Allogenic fibroblasts are not only tolerated when the equivalent epidermis is grafted onto animals, but they are actually integrated structurally. Rejection is not observed when the epidermal equivalent is grafted onto humans, but its tolerance and persistence are yet to be demonstrated (7). Culture conditions are the same as those for the dermal equivalent, except for the addition to the medium of Epidermal Growth Factor (EGF; 10-20 $\mu g\ ml^{-1}$), hydrocortisone (0.4 $\mu g\ ml^{-1}$) and cholera toxin (8.4 $ng\ ml^{-1}$), traditionally used in keratinocyte cultures (6). When there are no specific treatment indications, only EGF is essential, to promote keratinocyte migration and support proliferation as well as the epidermalization of the biopsy. Experiments should however be conducted both with and without EGF (6). The epidermal equivalent can be either completely immersed in the medium, when privileging the study of cell growth, or kept in contact with air when studying prevalently differentiation. The keratinocytes grow and divide, and form an epidermis which spreads throughout the dermal equivalent. This new epidermis can differentiate as in vivo by forming cuboid basal cells, keratohyaline granules, etc (7). This method of epidermalization has several advantages. It allows not only to study differentiation qualitatively in terms of morphology and keratin analysis, but also to study epidermal growth quantitatively in terms of surface area. To study cell proliferation, tritiated thymidine is incorporated and DNA content is evaluated by flow cytometry. DNA content per unit of surface area reflects the subtle balance between proliferating, migrating and differentiating cells. The surface area of the epidermis is visualised by staining the whole culture with Nile blue sulphate before separating the dermis from the epidermis. The surface area can be measured with an image analyser. The mutual influences of the two tissues can be studied by analysing the dermis and epidermis separately. The results obtained from this "organ" reconstructed in vitro permit to identify among the various interactions, those of greater biological significance, and to devise predictive models for the screening of products with pharmacological, toxicological or cosmetic properties.

c) Three-dimensional culture system

SKIN 2 TM (a three-dimensional culture system) is currently used more and more frequently (7) (Fig. 3). It is constituted by a living human three-dimensional tissue substrate. Fibroblasts taken from neonatal foreskin are seeded on a nylon mesh. They attach to this mesh and grow, secreting their matrix protein. Keratinocytes are successively seeded on this dermis and proliferate, forming a multi-layer differentiated epidermis with a well developed horny layer (8). This model has been successfully experimented in the study of collagen deposition, irritancy responses, cytotoxicity and percutaneous absorption (7). Validation studies have demonstrated its reproducibility, while characterization has evidenced its similarity to the human epidermis (9). This model is widely used in the evaluation of phototoxicity and photoprotection

processes. The advantage of utilizing in phototoxicity studies a model providing both the dermis and epidermis lies in the possibility of obtaining a series of specimens in a relatively short time, and of eliminating those species or individual factors that can mask or modify the potential risk. The process of recognition of the validity of this model for the study of phototoxicity has been conducted and approved by the Commission of the European Communities and by the Comité de Liaison des Associations Europeennes des Industries de la Parfumerie, des Produits Cosmetiques et de Toilette (COLIPA). In the framework of this European study, the most promising in vitro photoirritancy tests were selected and their methods standardized to obtain validation.

Conclusions

In vitro techniques can play an important role in the fast acquisition of information that will allow the selection of ingredients characterized by low toxicity and high biological activity, at least in the field of cosmetic products. Cell cultures are probably the most promising biological models, in that they are simpler than the organism in toto, but are constituted by organized living units, thus opening interesting prospects also in toxicological studies. Their characteristic features and intrinsic limitations must however be taken into consideration when programming experiments or interpreting their results. Some studies have demonstrated the suitability of cell cultures in studying the activity of melanocytes (10), the basal membrane in all its components (11), and skin metabolism and permeability (12). In view of the need to standardize validation techniques and related tests, a series of research bodies have been set up at national level, and subdivided in committees to deal with specific fields. The most important of these, and the main national bodies in this field are: in the USA the American Society for Testing and Materials, the Health Industry Manufacturers Association, the American Dental Association, the Food and Drug Administration; in the UK the British Standard Institute; in France the Association Francaise de Normalisation; in Germany the Deutsches Institut fur Normung, in Italy the Ente Nazionale Italiano di Unificazione. At international level, the main regulatory body is the International Organization for Standardization (ISO), constituted by the research units on standardization of the most industrialized countries, also organized in technical committees. The EEC has issued directives setting the minimum standards for safety, reliability and for every aspect of the medical sector, in part already enacted by the Italian Parliament. The European Committee for Standardization (ECS) on behalf of the EEC is currently dealing with the harmonization of a series of basic technical rules and regulations in line with the requirements of EEC directives. In parallel with harmonization in the EEC, an attempt at "global" harmonization is being made by Japan, the USA and Europe for a wide series of rules, tests and certification systems.

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