

HUMAN SKIN FIBROBLAST CULTURE TO TEST GLYCOLIC AND LACTIC ACID SOLUTIONS.

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Received: May 21, 1997.

Key words: human skin fibroblasts, glycolic acid, lactic acid, experimental study.

Synopsis

Alphahydroxy acids (AHAs) have become quite popular as skin rejuvenating agents. The action of AHAs on the skin is affected by the contact time, the concentration and the pH of the solution used. In a previous in vitro study we showed that by using salified derivatives of glycolic and lactic acid one may prolong the contact time for longer than with the pure form. In the present work we test the effects of different concentration of natural glycolic and lactic acid at a pH between 4.5 and 5.5, combined with a special protective mixture formed of gelatine-glycine-aminoacids at the same pH. The investigations performed brought to light no significant differences among the various solution employed. Further study are necessary in order to obtain in vitro results matching the in vivo clinical experience.

Riassunto

Negli ultimi anni gli AHA hanno assunto un ruolo sempre più importante tra i trattamenti anti-ageing. L'effetto dell'applicazione degli AHA sulla cute dipende dal tempo di contatto, dalla concentrazione e dal pH delle soluzioni utilizzate. In un precedente studio eseguito in vitro, abbiamo dimostrato che utilizzando soluzioni salificate di acido glicolico e di acido lattico, anziché le soluzioni pure, il tempo di contatto può essere prolungato. Nel presente lavoro abbiamo testato gli effetti di differenti concentrazioni di acido lattico e acido glicolico ad un pH compreso tra 4.5 e 5.5, combinando questi acidi con una particolare miscela di aminoacidi (gelatina-glicina). Lo studio condotto non ha evidenziato particolari differenze tra le varie soluzioni utilizzate.

INTRODUCTION

Alphahydroxy acids (AHAs) have become quite popular as skin rejuvenating agents. They are a class of compounds that are derived from food sources (e.g., glycolic acid is found in sugar cane while lactic acid is present in sour milk) reported to be effective in the treatment of a condition predisposing to dry, rough skin (1). Although a large number of these compounds are available, glycolic acid has been the most widely used. Therapeutic benefits have been reported with not only dry skin but also in the treatment of acne, keratosis, both seborrhic and actinic, warts and problems related to ageing such as dyschromia and wrinkling (2). Among the AHAs glycolic acid has a low molecular weight and size and hence a high penetrative capacity (3). Alphahydroxyacid compounds cause disadhesion of keratinocytes, and at high concentration epidermolysis (4) as well as increasing water absorption by varying the kind of lipids and enzyme systems produced by Odland's lamellar bodies (3).

The action of AHAs on the skin is affected not only by the contact time but by the concentration and the pH of the solution used (5). There is always some stinging and burning sensation for the patients.

In a previous *in vitro* study we showed that by using salified derivatives of glycolic and lactic acid one may prolong the contact time for longer than with the pure form, since the pH is more physiological (6). That study extended the investigation to lactic acid since, despite having a higher molecular weight than glycolic, this acid has the biological property of converting into chetonic form (pyruvic acid), and this proves far more active in reducing the cohesion of corneocytes (7).

In vivo studies have recently shown that the use of partially salified AHAs does not detract from their clinical effectiveness, and may even improve the penetration (8,9).

The present work thus sets out to test the effects

of various different concentration of natural glycolic and lactic acid at a pH between 4.5 and 5.5, combined with a special protective mixture formed of gelatine-glycine-aminoacids at the same pH. Cutaneous fibroblast cultures were used since such cells are sensitive to treatment with glycolic and lactic acid (10). Investigations consisted in analysis of cell proliferation and assessment of cell morphology by phase contrast light microscopy and scanning electron microscopy.

MATERIALS AND METHODS

Materials

The glycolic acid and lactic acid solutions used in this study are reported in table I. Concentration tested for each solution were 1.2 mM, 4.6 mM e 9.2 mM for 100 gr. of culture medium.

TABLE I.
DETAILS OF AHA SOLUTIONS.

	COMPOSITION	pH
C	Glycolic acid 70%	4-5.5
C1	Glycolic acid 70% with an 8% (w/w) protective admixture *	4-5.5
D	Lactic acid at 70%	4-5.5
D1	Lactic acid 70% with an 8% (w/w) protective admixture *	4-5.5

* protective admixture = Natural gelatin-glycin-aminoacids.

Cell culture

Human skin fibroblasts, obtained from a lower forearm biopsy of a 20 years old healthy donor, were used to test the above mentioned solutions. Cells were subcultured at the first passage, in a logarithmic growth phase, were used for these experiments.

The fibroblasts were grown at 37° C, in humidified air with 5% CO₂, in 25 cm² flask containing Iscove's modified Dulbecco's medium (Gibco, Grand Island, NY, USA), supplemented with 0.2% sodium bicarbonate (Sigma, St. Louis, Mo, USA), 10% foetal bo-

vine serum (Bio Whittaker, Verviers, Belgium), 200 U/ml penicillin, 200 µg/ml streptomycin (Gibco). Fibroblasts were checked for mycoplasma contamination (PBI International, Milano, Italy). Twenty-four hours after plating cell vitality (90-95%) was measured by Trypan Blue exclusion (Gibco).

Cell proliferation

Fibroblasts were plated on wells (Nunc, Roskilde, Denmark) in a complete medium. After 24 hours the medium was exchanged for serum free IMDM plus 0,1% BSA (Sigma). After 12 hours the fibroblasts were treated with different glycolic and lactic acid solutions diluted in the same serum free culture medium plus 0,1% BSA. Evaluations were performed after 24, 48 and 72 hours.

Haemocytometric Chamber Counts: Fibroblasts were plated in 24 well plates at number/concentration of 20,000 cells/well. Fibroblasts were removed from the substrate by incubation in 200 ml of 0.25% w/v trypsin (Gibco) for 5 min at 37° C and then counted using a haemocytometric chamber.

Tritiated thymidine Incorporation: Fibroblasts were plated, in sextuplet, in flat bottomed 96 well tissue culture plates at a density of 10,000 cells per well. At each time (24, 48 and 72 hours), the cells were exposed to 3H-thymidine (Amersham, Buckinghamshire, U.K.) (0.5 mCi per well) for 6 hours and then stored at - 20° C. Afterwards, the cutaneous fibroblasts were collected on glass microfibre filters using a multiple automated sample harvester, the wells and the filters were washed with distilled water to release all cells from the plates and remove unbound nucleotides. Filters were placed into vials and 0.1 ml Soluene 350 (Cammerra Packard, Meriden, Ct, USA) was added, followed after 40 minutes by 4.5 ml of scintillation liquid (Beckman, Fullerton, Ca, USA). Six hours later, the fiberglass filters were counted using a liquid scintillation counter (LKB WALLAC).

Cell morphology

Phase contrast microscopy: Plated fibroblasts were examined by phase contrast microscopy (Leitz-Labovet F8) and photographed 24, 48 and 72 hours after contact with solutions. In order to study fibroblasts behaviour on first contact with the substances, before and after the cells had time to adapt and organize themselves in terms of culture conditions, we decided to examine the samples over the first 72 hours of contact with solutions. Semiquantitative evaluations were performed by the Kiellstrand method (11), taking 4 images for each samples at 800 magnification phase contrast light microscopy and seeking zone representative of the whole well. Values from 0 to 10 were set for each image, the parameters assessed being: cell morphology, number and distribution of cell present. Totals for these evaluations are plotted in graph against observation times.

Scanning electron microscopy: After 72 hours of contact with solutions, the cells were fixed with 2.5% v/v glutaraldehyde in a 0.1 M cacodylate buffer, post-fixed with 2% w/v osmium tetroxide in 0.1 M cacodylate buffer, dehydrated in an ascending ethanol gradient and critical point dried (CPD). Specimens were then mounted on aluminium holders, gold film metalized and observed with a SEM Philips XL20 at 25 kV.

Statistical analysis

The proliferation data are reported as a mean ± standard deviation. Analysis of variance (ANOVA) was used to evaluate the influence of the compounds, their concentration and their incubation time on cell proliferation.

RESULTS

Cell proliferation

Counts in Haemocytometric Chamber: Investigations of cell proliferation revealed no significant differences between controls and fibroblasts cultured in the various solutions at the diffe-

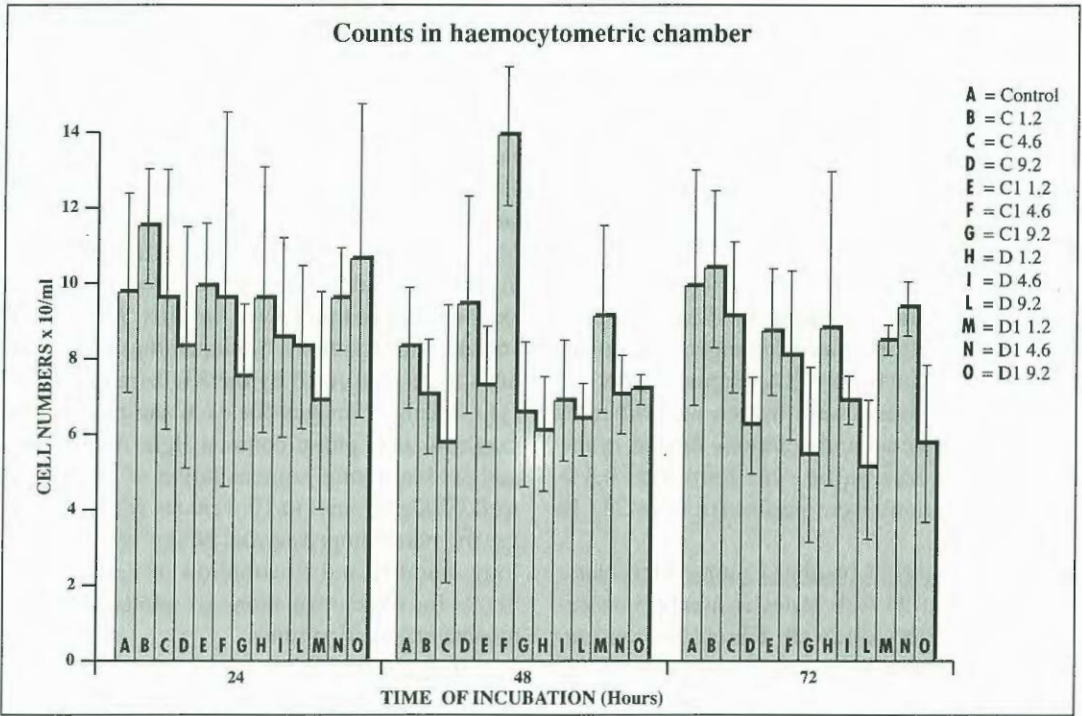


Fig. 1 Evaluation of cell proliferation using a haemocytometric chamber. Mean Values \pm standard deviation are reported.

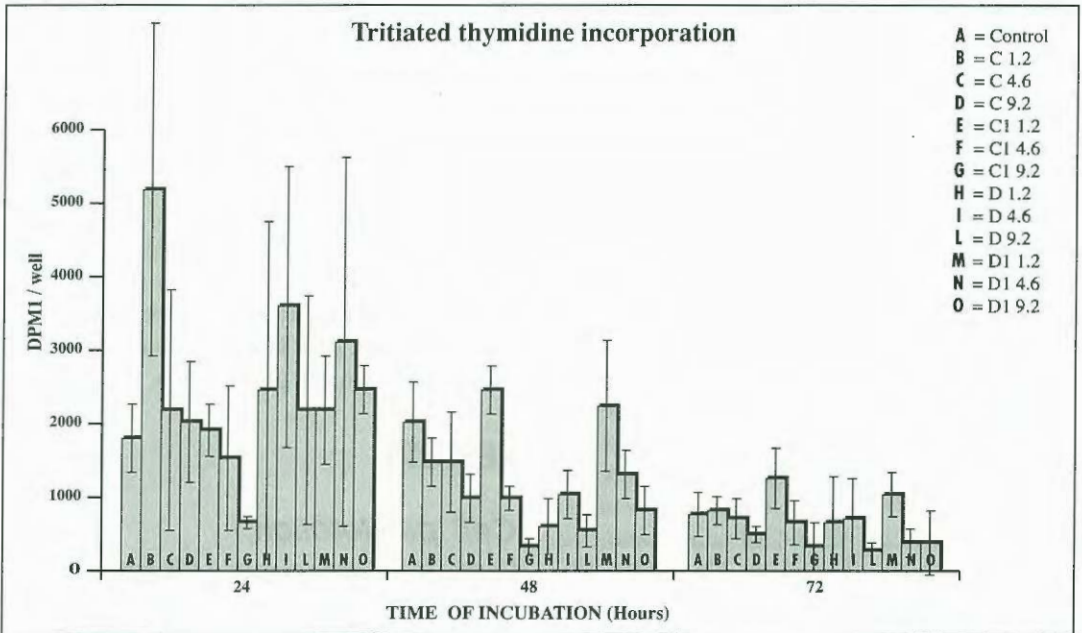


Fig. 2 Evaluation of cell proliferation by incorporation of ^3H -thymidine. Mean Values \pm standard deviation are reported. DPM = disintegration per minute

rent contact times. A slight reduction in cell number may nonetheless be detected as the contact time increases (Fig.1).

Comparison of glycolic with lactic acid as culture solutions shows a lower cell count with the latter, though not significantly so (Fig 1). In general, all solution and contact times confirm that the use of high concentration (9.2mM) involves a grater reduction in cell count. The only exception to this was when lactic and glycolic acid contained a protective admixture, at 24 and 48 hours respectively (Fig.1) .

Tritiated thymidine Incorporation: A more sensitive test than the foregoing, it confirms that there are no significant differences between controls and fibroblasts cultured with the various solutions where DNA duplication is concerned. The only exception was low concentration (1.2 mM) glycolic acid at 24 hours. A marked decrease in DNA duplication is seen at the contact time increases (Fig.2). Comparison between glycolic and lactic acid shows there to be less DNA duplication with

the latter, though not significantly (Fig.2). A similar picture emerges between the solutions with and without the protective admixture. High concentration (9.2 mM) had consistently lower scores than the other concentrations (Fig.2).

Cell morphology

Phase contrast microscopy: As the graph shows (Fig.3), there were no significant differences between the various samples and controls, whether at the same time intervals or comparing across intervals. Fig.4 reports the morphology of a) control cells against low concentration (1.2 mM) of b) glycolic and c) lactic acid. Non confluent cells shows numerous cell prominences in both controls and the glycolic acid samples, unlike that with lactic acid where the cell shape itself is more elongated. Fig. 5 shows the normal cell morphology of samples treated with a) glycolic and b) lactic acid at a medium (4.6 mM) concentration. The morphologic appearance of cells cultured with a high (9.2 mM) con-

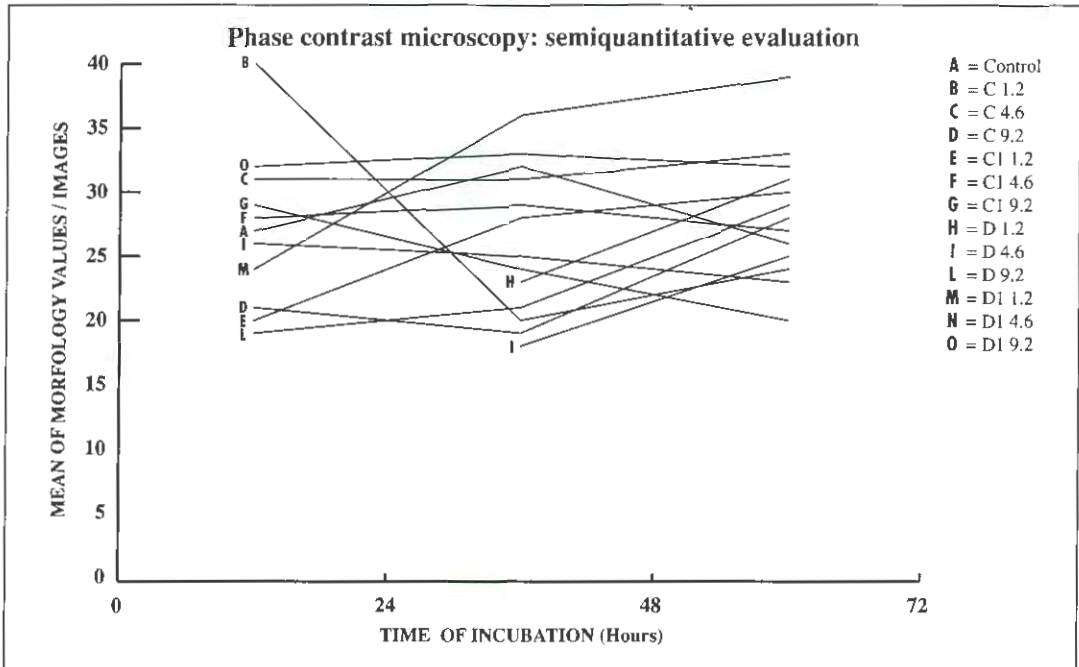


Fig. 3 Graph of the semiquantitative evaluation of cell growth by phase contrast microscopy.

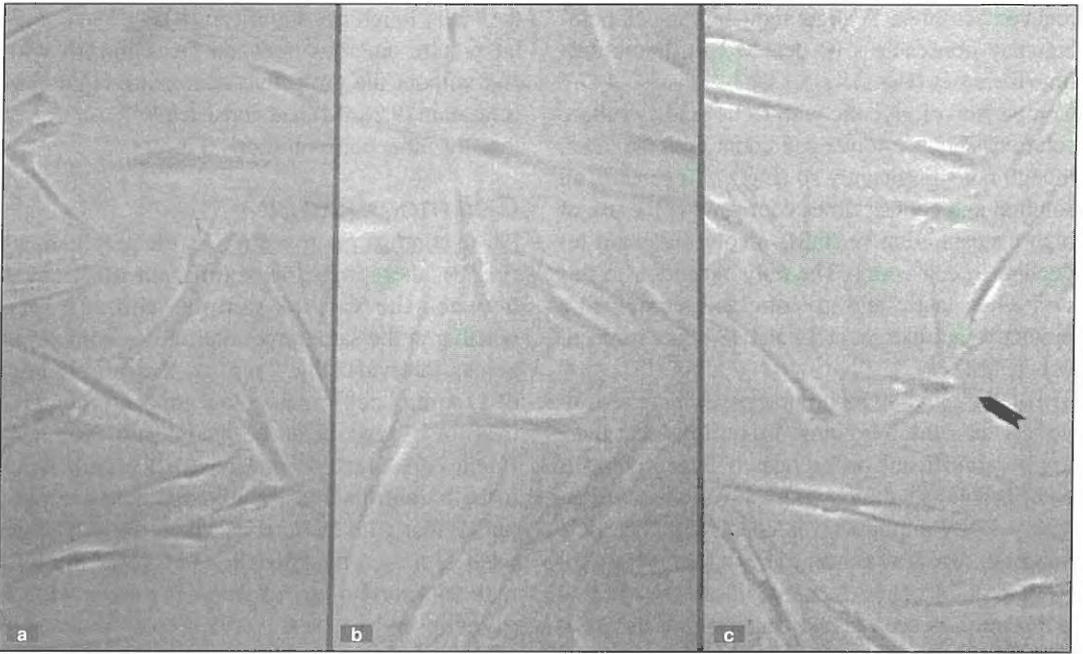


Fig.4 Fibroblast cultures of a) controls vs cultures treated with a low concentration (1.2 mM) of b) glycolic acid and c) lactic acid, observed by phase contrast light microscopy (x250). N.B. The elongated appearance connected with lactic acid (➡).

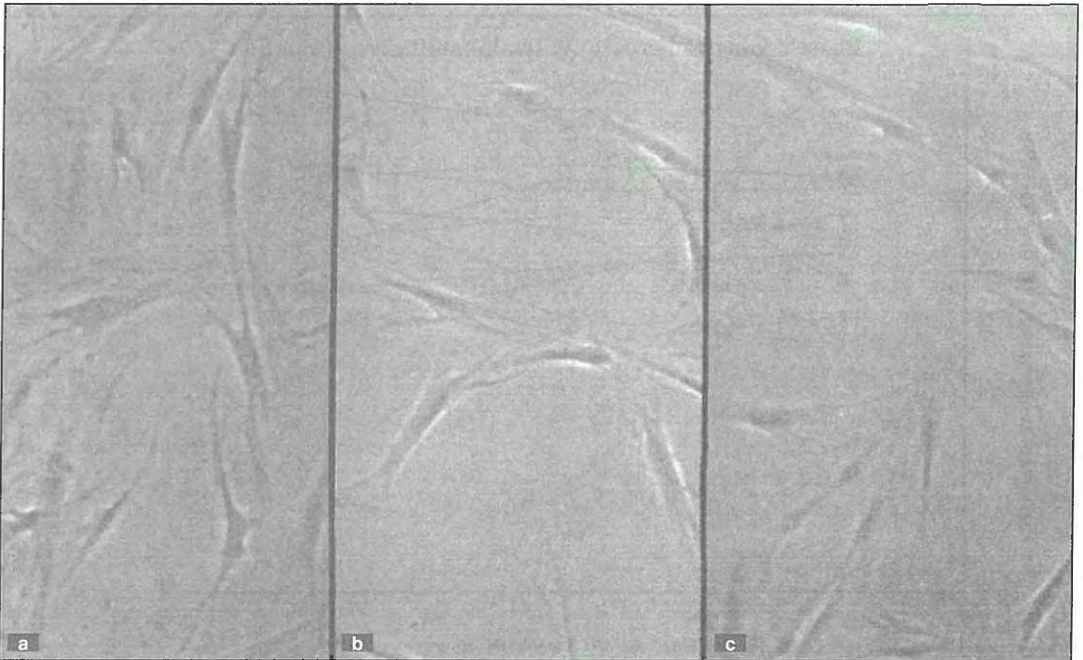


Fig.5 Appearance under phase contrast light microscope of fibroblast cultured a) with glycolic acid at an intermediate concentration (4.6 mM) and b) with intermediate (4.6 mM) and c) high concentration (9.2 mM) lactic acid (x250).

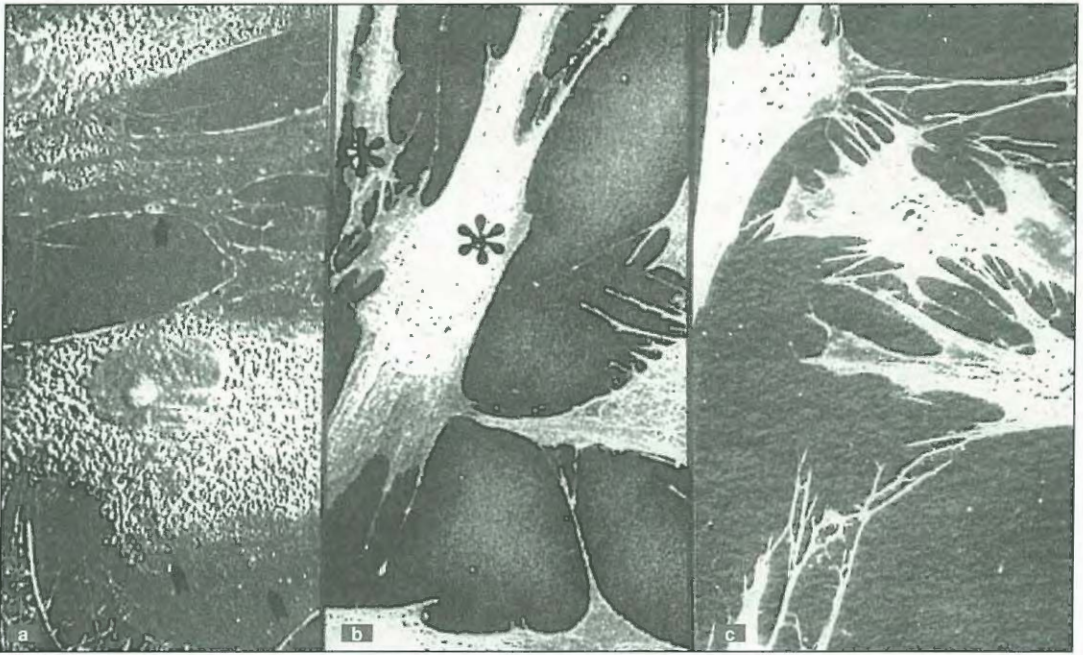


Fig.6 Scanning electron microscope photograph of a) control fibroblast (SEM x700), as well as fibroblasts cultured with b) glycolic acid (SEM x400) and c) lactic acid (SEM x400) at a low concentration (1.2 mM). N.B. The flattened appearance (➔) of control fibroblast and the marked cell spreading (*) with glycolic acid.

centration of lactic acid is somewhat altered, on the other hand, the cytoplasm being very diaphanous and a great number of thin prominences present.

Scanning electron microscopy (SEM): Cell growth among controls seemed good, the cells being mainly arranged round the outer edge of the well. Cells were generally spindle-shaped and no signs of degradation were noted (Fig.6a).

After the various treatment, morphological investigations of cultured fibroblasts revealed the tendency of cells to take an increasingly stellate shape as the glycolic acid concentrations rose (Fig.6b; Fig.7c). At high concentrations there was extremely pronounced cell spreading as well as many signs of cell degeneration (Fig.8a). No significant differences could be seen between those with and without the protective admixture. At both low (Fig.6a) and high (Fig.8b) concentrations, whether "protected" or

not, lactic acid cultures were comparable with glycolic. A contrast to this was observed at the intermediate concentration, where the pure solution yielded a cell appearance similar to controls (Fig.7), while considerable degeneration was observed where the protecting solution had been used.

DISCUSSION

The recent literature on AHAs treatment has been rich in vivo trials (12-15) but undersupplied with in vitro experiments on cells. In vivo studies confirmed that the pH, and hence salification level, of AHAs is a factor of the highest importance. With salified solutions at a pH between 4 and 5.5 one notes a reduction in the side effects of AHA application, such as stinging and burning (8). In a previous study we showed, in vitro, that salified derivatives of AHAs have better biotolerance than pure solu-

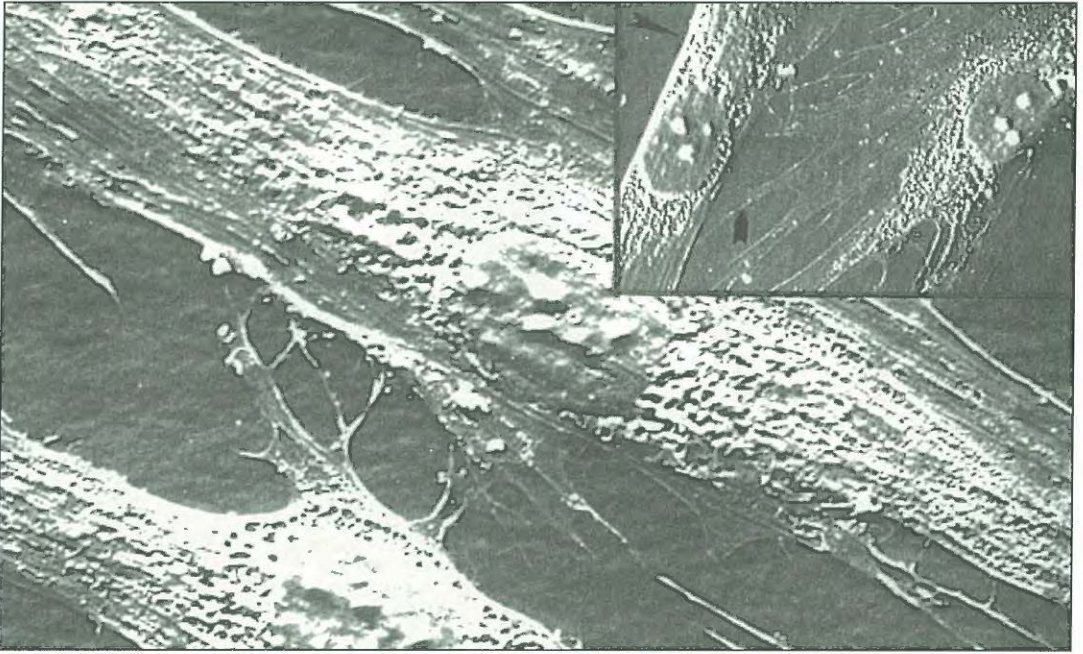


Fig.7 Scanning electron microphotograph of fibroblasts cultured with an intermediate concentration (4.6 mM) of lactic acid (SEM x1300). Insertion : fibroblasts (>) cultured with glycolic acid (SEM x700) at an intermediate concentration (4.6 mM) showing cell spreading (➡).

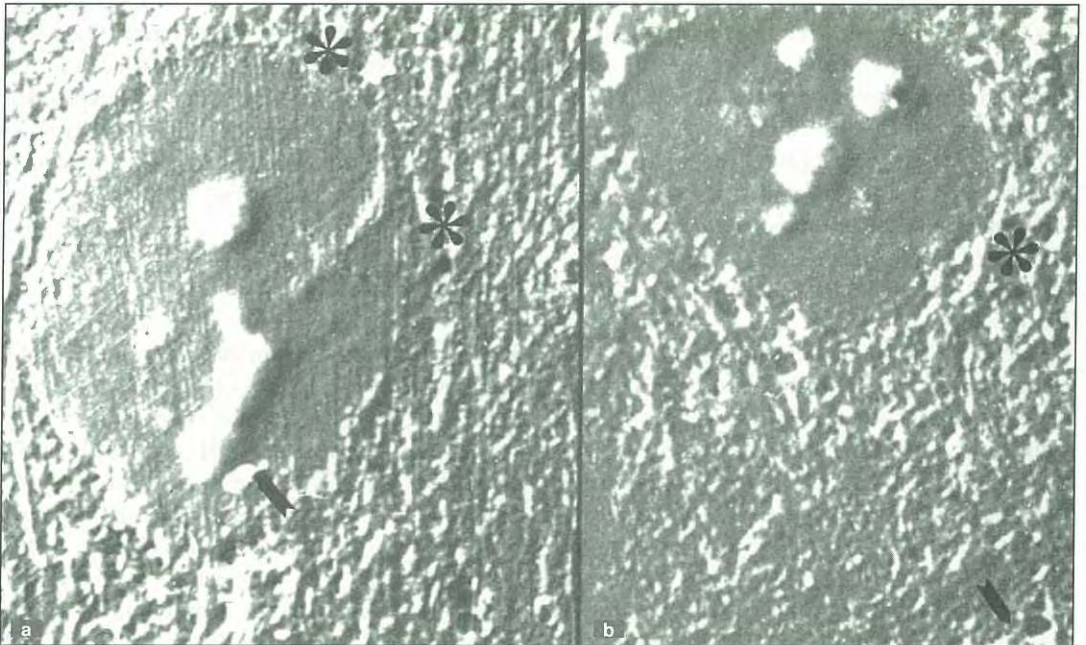


Fig.8 Scanning electron microphotograph of fibroblasts cultured with a) glycolic (SEM x2000) and lactic (SEM x2000) acid at high concentration (9.2 mM). Cell spreading (*:*) is most marked and cell degeneration evident

tions (6). The present study does not show any significant differences among pure glycolic or pure lactic acid treated cultures, or those with protective admixtures, or controls.; there is simply a tendency for lactic acid solution to be less compatible. Evidently the solution brought into contact with cells do not significantly alter the microenvironment, and the fibroblasts preserve a stable state similar to controls throughout the experiment. One other decisive factor proves to be the concentration of AHAs used: as this rises, so does the compatibility to fall.

Since investigations brought to light no differences among the various solution employed, it is reasonable to assume that the buffer mixture was no suitable either in quality or in quantity in order to obtain in vitro results matching the in vivo clinical experience (8). Optimising the buffer mixture will be attempted in subsequent studies designed to test the correlation between in vitro predictive models and in vivo clinical results.

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