Autologous Graft of Cultivated Epidermis

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ABSTRACT

With the modern techniques of tissue culture, it is possible to obtain from a small fragment of skin, laminae of cultivated keratinocytes to be used for covering raw areas. This cellular population can be rapidly expanded, reaching, at the end of 30 days, a dimension hundreds of times bigger than the original biopsy. The authors analyze the method used for the keratinocytes culture, the difficulties found, the present applications and future possibilities.

INTRODUCTION

Loss of skin is a problem that torments the everyday of Plastic Surgery. We are constantly planning and executing procedures aiming at covering raw areas, not only those of traumatic origin, such as those produced by us after the removal of lesions, but also the donor areas of grafts and flaps. The ideal substitute for skin, which must be good, inexpensive and highly available, has not yet been found. The culture of cells seems to be a promising route since the technological development of this area highlights it as an efficient, relatively fast and economically viable procedure.

Obtaining human keratinocytes, in the form of a

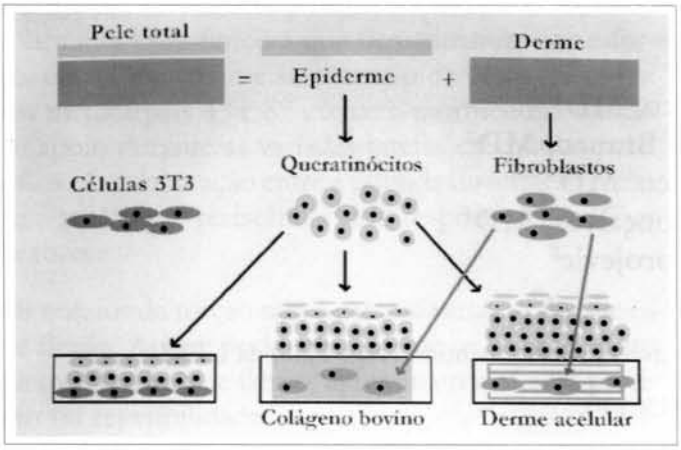

Fig. 1 – Scheme of the 3 types of procedures used: using the dispase enzyme, the epidermis and the corium are dissociated, resulting in keratinocytes, from the first, and fibroblasts, from the second. On the left, the scheme shows keratinocytes sowed over a 3T3 cells feeding layer. In the center, fibroblasts are sowed over collagen gel, forming a new matrix over which keratinocytes are sowed. On the right, acellular corium is populated by fibroblasts and supports the culture of keratinocytes.

Fig. 1 - Esquema dos 3 tipos de procedimento empregados: utilizando a enzima dispase, a epiderme e a derme são dissociadas, obtendo-se queratinócitos, da primeira, e fibroblastos, da segunda. À esquerda, o esquema mostra queratinócitos semeados sobre uma camada alimentadora de células 3T3, evoluindo para multiplicação e discreta diferenciação. No centro, fibroblastos são semeados sobre gel de colágeno, formando nova matriz sobre a qual são semeados queratinócitos. À direita, derme acelular é povoada de fibroblastos e suporta o cultivo de queratinócitos.

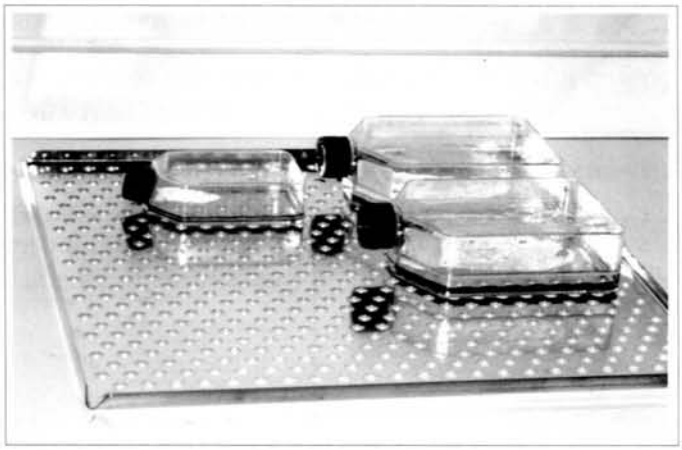

Fig. 2 – Epidermal cells are plated in bottles containing embryonal cells from mice (3T3) as the feeding layer. The culture's evolution is daily monitored by means of a microscope, until a confluence of the keratinocytes is observed, which occurs after 20 to 40 days.

Fig. 2 - As células epidérmicas são plaqueadas em garrafas contendo células embrionárias de camundongo (3T3) como camada alimentadora. O controle da evolução da cultura é feito, diariamente, pelo microscópio, até se observar confluência dos queratinócitos, o que ocorre após 20 a 40 dias.

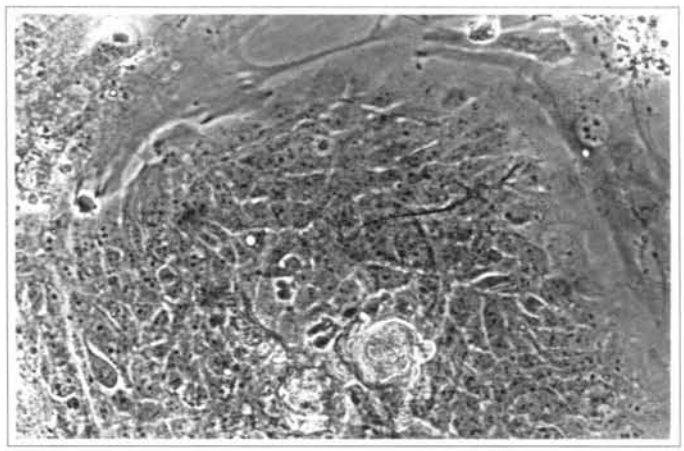

Fig. 3 – Epidermal culture (9th day) - colonies of epidermal cells can be observed; they are surrounded by fibroblasts from the feeding layer. 200 x inverted microscope.

Fig. 3 - Cultura epidérmica (9° dia) - observam-se colônias de células epidérmicas tendo, ao redor, fibroblastos da camada alimentadora. Microscópio invertido 200x.

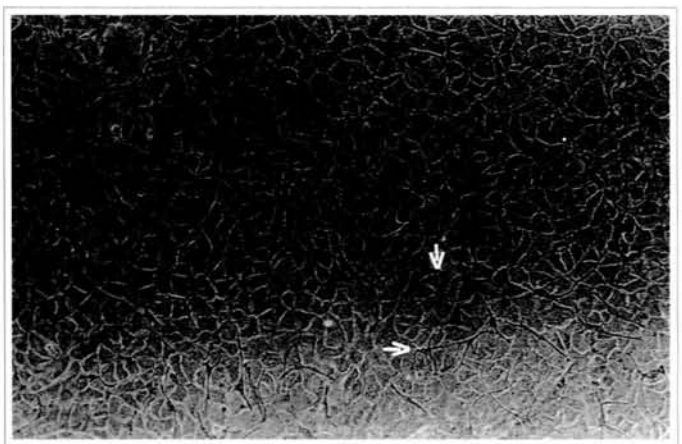

Fig. 4 – Epidermal culture (21st day) - cellular confluence and the presence of melanocytes can be observed. Culture ready to be removed. 100x inverted microscope.

Fig. 4 - Cultura epidérmica (21° dia) - observa-se confluência celular e presença de melanócitos. Cultura pronta para descolamento. Microscópio invertido 100x.

confluent layer, capable of being used as a graft, has been first described by Green[1], in 1979. Based on this pioneer work, improvements on the technique have been introduced, as well as its several applications[2, 3 and 4].

Together with the Cell Bank of Rio de Janeiro - PABCAM, located at the Hospital Universitário Clementino Fraga Filho, a clinical-laboratory study was initiated in 1997, in order to systemize the culture, *in vitro*, of human epidermis and make its practical applications possible. This technique is used in important medical centers worldwide, but is not common yet in Brazil.

From cutaneous biopsy, we can obtain fragments of cultivated autologous epidermis, which allow the covering of raw areas of several extensions and etiologies, such as: burns, chronic ulcers, epidermolysis bullosa, donor areas of cutaneous grafts or areas resulting from recession of giant nevi, etc. Another possible indication is the treatment of some cases of vitiligo, due to the presence of melanocytes in the culture.

MATERIAL AND METHOD

CLINICAL PHASE

In the period between April, 1998 to October, 1999, 19 patients with giant nevi (4), tattoos (5), vitiligo (5), or who would be submitted to the removal of partial skin grafts for the covering of complex wounds (5) were selected. Ten patients were females and 9 were males. The ages ranged from 8 months to 72 years.

At the first visit, the need for immediate cutaneous coverage or for the coverage after the treatment of the basic lesion was evaluated. In accordance with the *Norms for Research in Human Beings*, the procedures were explained to the patients and they were asked whether they accepted them. Once they had agreed, a medical file was created at the Institution, followed by anamnesis and physical examination, for the beginning of treatment.

The biopsy was performed under local anesthesia, in an outpatient basis (15 patients), or during the surgery of another procedure, under general anesthesia (4 patients), obtaining fragments of about 2 cm^2.

This material was taken to the Cell Bank for cultivation, for an average period of 30 days, during which the complementary examinations and the clinical preparation of the patients were performed.

LABORATORY PHASE

The technique described by O'Connor[16] in 1981 was used with a few changes. The cutaneous fragments were transported to the laboratory in Eagle's culture medium, modified by Dulbecco (DMEM) (Sigma Chemical Co., St. Louis, MO, USA), with fetal bovine serum at 10% (FBS) (Cultilab,

Table I

Name	Collection	Surgery	Diagnosis	Graft	Result
A.F.D.	02/02/98	01/04/98	Giant nevus	Over dermis Over fascia	100% integration 20% integration
A.M.S.	03/06/98	20/07/98	Donor area partial skin	Over dermis	100% integration
A.B.L.	06/10/98	-	Tattoo	Culture didn't progress	-
T.C.S.	12/08/98	31/08/98	Giant nevus	Culture didn't progress	-
R.S.P.	14/07/98	10/08/98	Donor area partial skin	Over dermis	100% integration
M.C.L.	06/04/98	15/09/98	Giant nevus	Over dermis	60% integration
V.O.S.	19/10/98	15/10/98	Raw area	Scarring 2nd intention	-
M.A.D.	20/07/98	17/08/98	Giant nevus	Over dermis Over fascia	90% integration Total loss
A.N.P.	29/09/98	23/10/98	Donor area partial skin	Over dermis	100% integration
A.S.	01/02/99	08/03/99	Donor area partial skin	Over dermis	100% integration
C.M.M.	17/03/99	05/04/99	Tattoo	Over dermis	Total loss
R.C.S	12/05/99	02/06/99	Vitiligo	Over dermis (abrasion)	100% integration - Hyperchromia
A.E.S.	19/05/99	16/06/99	Vitiligo	Over dermis (abrasion)	100% integration - Achromia
S.A.R.	16/06/99	06/07/99	Vitiligo	Over dermis (abrasion)	100% integration - Hyperchromia
A.S.	07/07/99	30/07/99	Tattoo	Over dermis (dermatome)	Total loss
C.S.Q.	20/07/99	13/08/99	Tattoo	Over dermis (dermatome)	100% integration
J.F.F.	03/08/99	06/09/99	Vitiligo	Over dermis (laser)	100% integration - Achromia
A.C.S.	01/08/99	06/09/99	Vitiligo	Over dermis (laser)	100% integration - Eritema
L.C.A.	30/06/99	27/09/99	Tattoo	Over colagen + fibroblasts	No follow-up

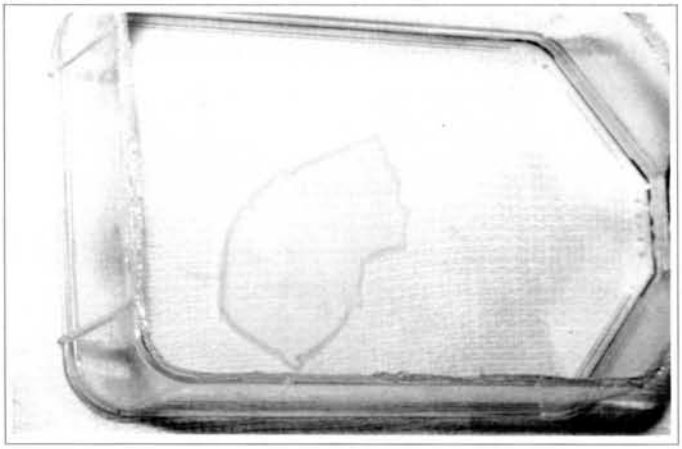

Fig. 5 – After 4 to 7 days in confluence, the cultivated epidermis is enzymatically removed from the bottle, using dispase.

Fig. 5 - Após 4 a 7 dias em confluência, a epiderme cultivada é descolada, enzimaticamente, da garrafa, com o uso de dispase.

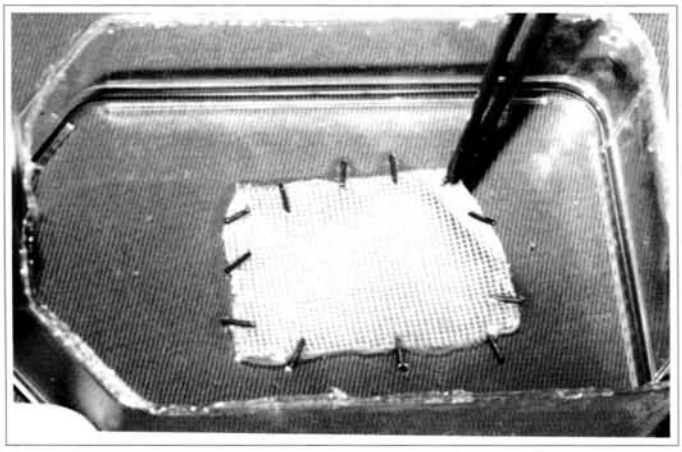

Fig. 6 – The cultivated epidermis lamina is framed over a gauze soaked with petrolatum, using surgical clips.

Fig. 6 - A lâmina de epiderme cultivada é fixada sobre gaze embebida em petrolatum, utilizando-se clipes cirúrgicos.

Fig. 7 – The gauze+epidermis sets are taken to the surgery center, observing the proper asepsis and refrigeration. In this figure, the epidermis has been elevated from its gauze bed to show its thickness.

Fig. 7 - Os conjuntos de gaze+epiderme são levados ao centro cirúrgico sob cuidados de assepsia e refrigeração. Nesta figura, a epiderme foi elevada de seu leito de gaze, para mostrar sua espessura.

Campinas, SP, Brazil) and 1μg/mL amphotericin B (Eurofarma Laboratórios Ltda., São Paulo, SP, Brazil), under refrigeration. The largest possible amount of skin was removed, and the fragments containing the epidermis were fragmented with a scalpel. This latter portion was then submitted to enzymatic dissociation, with 0,3% trypsin (Sigma) in a balanced salt solution calcium- and magnesium-free (BSS-CMF), during 18 to 20 hours, at 4° C, followed by incubation in a new solution, from 30 to 60 minutes at 37°C, with orbital jar. The epidermal cells, now apart from each other, were plated in culture bottles (Nunc, Roskilde, Denmark), $6x10^6$ cells/ 75 cm^2, containing 3T3 (embryonal cells from mice) as a feeding layer. The 3T3, obtained from the Cell Bank of Rio de Janeiro, was previously cultivated until it reached confluence, and then it was treated with 10μg/mL Mitomicyn C (Sigma), so it would not proliferate anymore (Figs. 1 and 2).

The cultures were kept for 20 to 40 days in medium for keratinocytes: minimum essential medium, D-valine modification, supplemented with 5 mg/L insulin, 4mM L-glutamine, 0,4 mg/mL hydrocortisone, 10^{-10} M cholera toxin, 5 mg/L transferrin, $2x10^{-9}$M T3, 10ng/mL epidermal growth factor (all from Sigma) and fetal bovine serum at 20% (Cultilab), at 37°C and CO_2 at 5%. Cellular growth was monitored by means of an inverted microscope with phase contrast. The culture medium was changed every 2 or 3 days. Cellular confluence was reached in a period of 10 to 20 days, depending on the patient's age (Figs. 3 and 4). After 4 to 7 days in confluence, the epidermal layer was removed from the bottle, using 1.2U/mL dispase in DMEM (Sigma), for 60 minutes at 37°C. This layer was then washed with a balanced salt solution (BSS), fixed on a gauze soaked with petrolatum (Adapatic®), using surgical clips (Ligaclip – both from Johnson & Johnson Medical INC., São Paulo, Brazil), transported to the surgery center on Petri dishes, which were refrigerated, and used for grafting (Figs. 5 and 6). The period between cell removal and grafting was of 4 hours (Fig. 7).

SURGICAL PHASE

The grafts were applied directly on the main raw area in 11 cases, or as a complement to the method used for covering this area in 6 cases (Figs. 8 and

9). The receptor bed was prepared according to the basic disease: with scalpel (4 cases), deepithelization with CO_2 laser (2 cases), abrasion with water-resistant sandpaper (3 cases), deepithelization with electric dermatome (8 cases).

Due to their reduced thickness, the cultivated skin fragments were framed to gauze with petrolatum, which served as an anchor to the stitches, 5-0 mononylon. All the set was covered with vaseline gauze and tie-over dressing. The dressing was removed after 5 to 7 days for evaluation, keeping the area covered with soft gauze for at least 14 days.

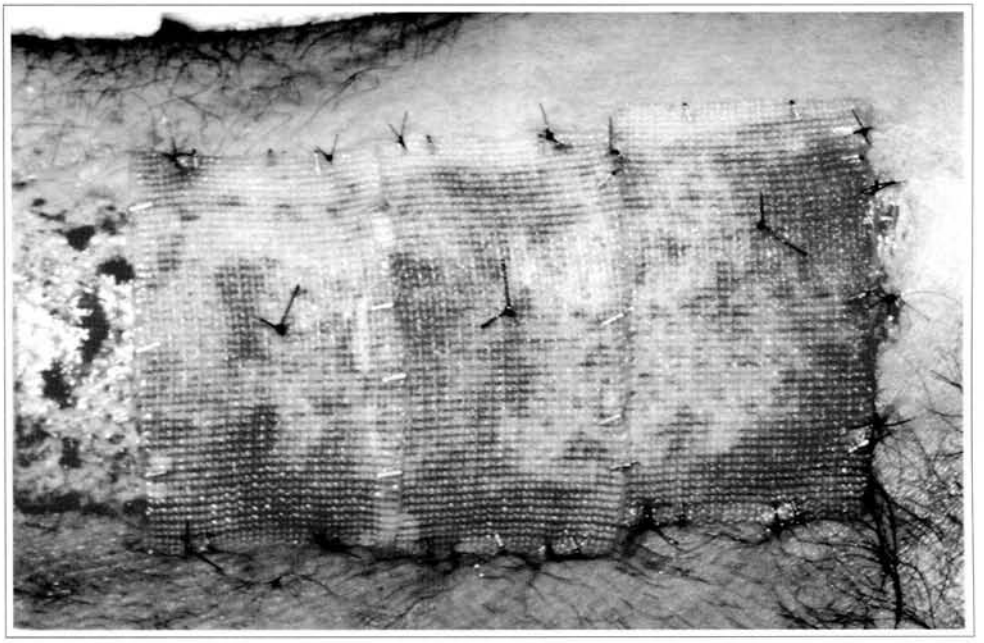

Fig. 8 - The gauze+epidermis sets are fixed over the raw area, and the gauze is used to support the fixation points. In this case, a non-grafted strip was left, so as to allow the comparison with the integration of the cultivated epidermis.

Fig. 8 - Os conjuntos gaze+epiderme são fixados sobre a área cruenta, sendo a gaze utilizada como apoio dos pontos de fixação. Neste caso, foi deixada uma faixa sem enxertia, para permitir a comparação com a integração da epiderme cultivada.

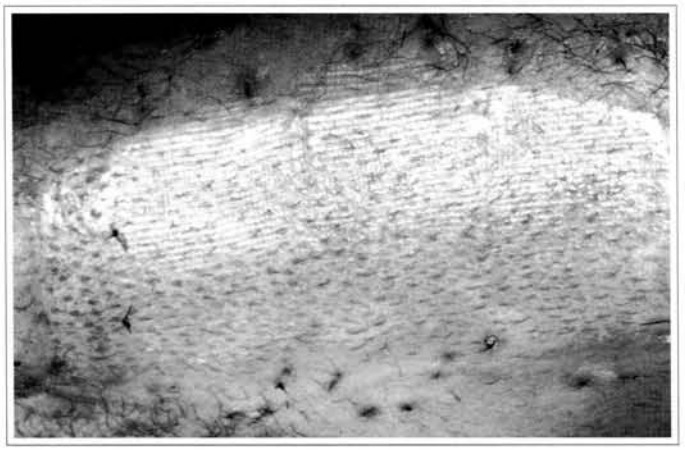

Fig. 9 – After 10 days, complete epithelization of the treated area can be observed, noticing that the grafted part with the cultivated epidermis presents a thicker covering than the strip which epithelized without grafting. The limit between the two parts is marked by two points.

Fig. 9 - Após 10 dias, observa-se epitelização completa da área tratada, percebendo-se que a parte enxertada com a epiderme cultivada apresenta cobertura mais espessa do que a faixa que epitelizou sem enxertia. O limite entre as duas partes está assinalado por dois pontos.

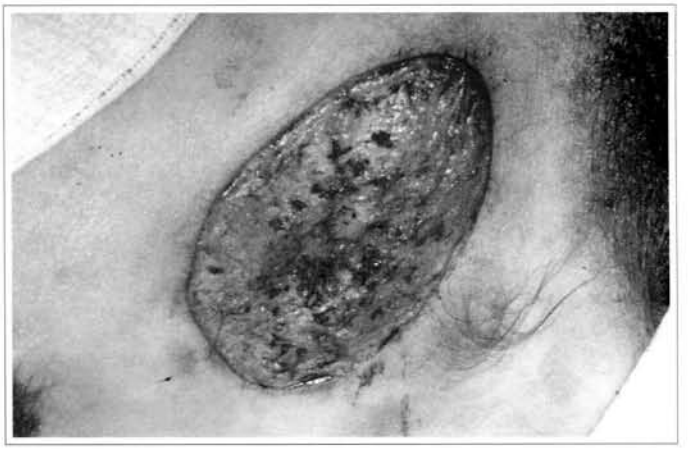

Fig.10 – Ressection of the nevus preserving the dermal bed, over which the cultivated epidermis will be grafted.

Fig.10 - Ressecção de nevo preservando o leito dérmico, sobre o qual será enxertada a epiderme cultivada.

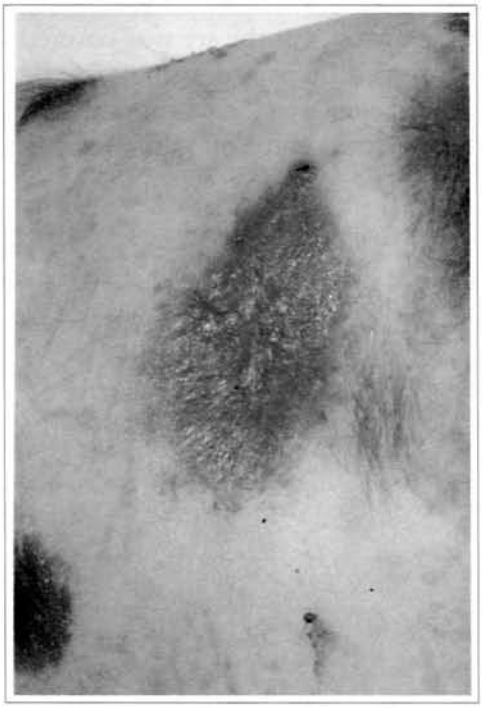

Fig. 11 – Ten days after the cultivated epidermis grafting, complete epithelization can be observed.

Fig. 11 - Dez dias após a enxertia da epiderme cultivada, observa-se epitelização completa.

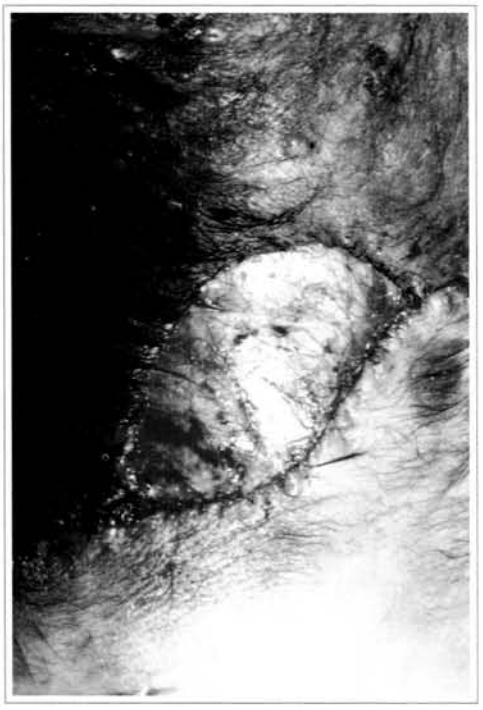

Fig. 12 – Ressection of the nevus with exposition of the deep fascia, over which the epidermis will be grafted.

Fig. 12 - Ressecção de nevo com exposição da fáscia profunda sobre a qual será enxertada a epiderme.

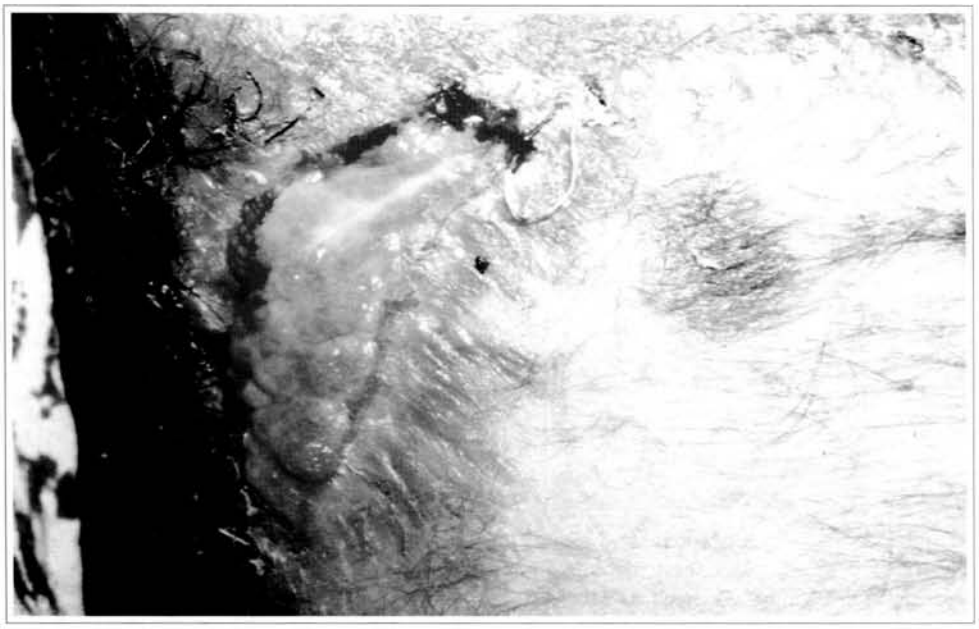

Fig. 13 – After 15 days, a complete loss of the graft can be observed.

Fig. 13 - Após 15 dias, observa-se perda total do enxerto.

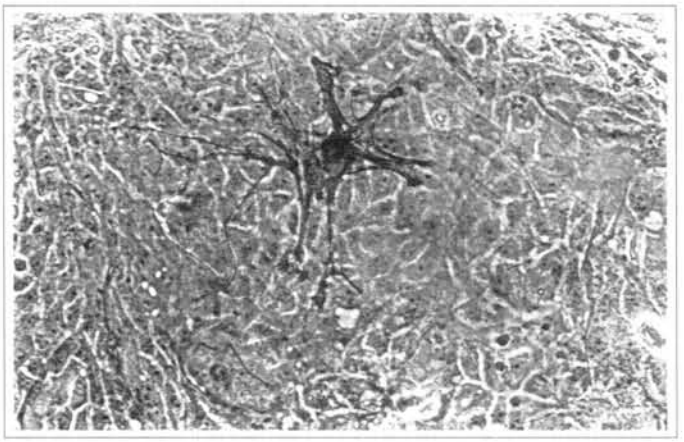

Fig. 14 – Epidermal culture (9th day). The present melanocyte is highlighted. 200x inverted microscope.

Fig. 14 - Cultura epidérmica (9º dia). Observa-se destaque de melanócito presente. Microscópio invertido 200x .

Fig. 15 – Achromic area in patient suffering from vitiligo. An abrasion with water-resistant sandpaper was performed and cultivated epidermis was grafted.

Fig. 15 - Área acrômica em paciente de vitiligo. Sobre esta área foi feita abrasão com lixa d'água e enxertada epiderme cultivada.

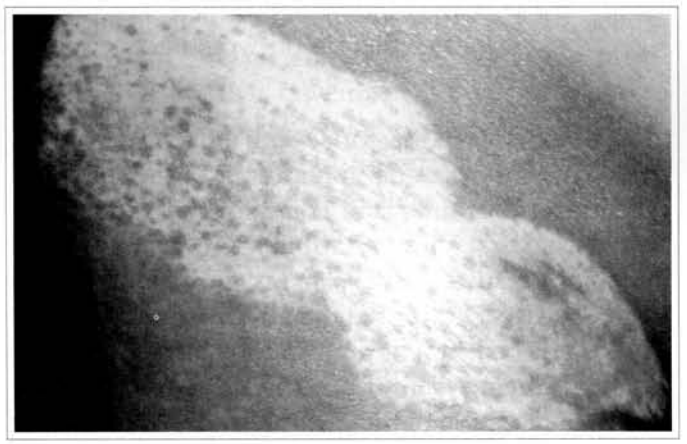

Fig. 16 – Local appearance, 4 months after the grafting, observing a subtle and sparse pigmentation.

Fig. 16 - Aspecto do local, 4 meses após a enxertia, observando-se pigmentação esparsa e tênue.

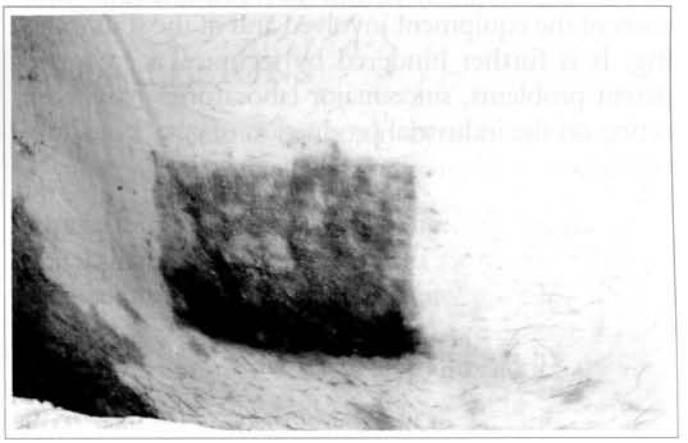

Fig. 17 – Another patient suffering from vitiligo, in whom cultivated epidermis was grafted, close to the pubic hairs, over abraded area. Appearance after 5 months, observing hyperchromia and irregular distribution of the pigments.

Fig. 17 - Outra paciente de vitiligo na qual foi feita enxertia de epiderme cultivada, próximo aos pêlos pubianos, sobre área abrasada. Aspecto após 5 meses, observando-se hipercromia e distribuição irregular do pigmento.

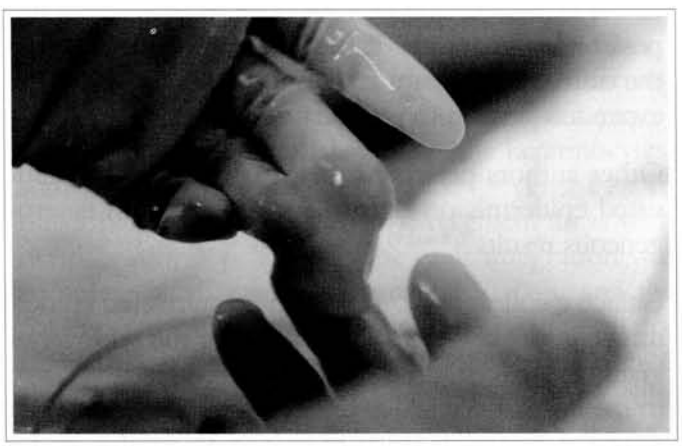

Fig. 18 – Bovine collagen preparation with fibroblasts, to be used as a dermal substitute.

Fig. 18 - Preparado de colágeno bovino com fibroblastos, para ser utilizado como substituto dérmico.

Fig. 19 – Acellular corium preparation.

Fig. 19 - Preparado de derme acelular.

RESULTS

From the 19 patients initially selected, 1 abandoned the treatment, 1 presented healing by second intention in the area to be treated and, in 2 of them, the culture did not evolve, and another repair technique was applied at the laboratorial phase. Thus, a total of 15 patients was reached, with 16 treated areas.

At the postoperative period, it was observed that the 9 patients presented a complete integration of the grafts, 3 had an integration varying from 60 to 90 %, and 2 of them did no present any integration (Table I).

DISCUSSION

The culture of cells is hindered, at first, by the elevated costs of the equipment involved and of the staff training. It is further hindered by technical secrets and patent problems, since major laboratories have been acting on the industrial production of cutaneous substitutes.

Therefore, even though the basic techniques can be found in a number of publications, the small details of the execution, which are vital to the complete performance of the procedures, need to be rediscovered, individually, by different researchers.

The constant lack of funds and the scarcity of specialized technicians available add up to the difficulties. All this, together with the attempts, by other institutions, to take the few skilled professionals we have, by means of attractive financial proposals, makes up the scenario in which university research is developed in our country.

On the other hand, however sophisticated, research on cell culture is perfectly practicable and of current interest. It is also surrounded by a touch of magic, since the ability to multiply, in laboratories, human beings, wholly or in part, is still perceived as a novelty, even after some decades of observation. And, most importantly, these are not routine procedures, which justifies new and varied research.

Once we were not familiar with the technique, we chose cases in which the failure of the grafting would not bring about any inconvenience other than the small scar on the biopsy area, that is:

1) Donor areas of partial skin grafts, where the epithelization would normally occur, even without the graft of cultivated skin.

2) Giant nevi, which usual treatment consists of multiple ressections. If the graft did not integrate, we would proceed to the previously planned ressection.

3) Vitiligo - the receptor areas were prepared by means of dermabrasion with sand paper or laser. If there was no integration of the cultivated skin, there would be spontaneous epithelization.

4) Tattoos - the tattooed area could be abraded or deepithelized with dermatome or scalpel. The layer of cultivated skin would accelerate the epithelization, and also contribute to disguise residual pigments. If there was no integration, epithelization would occur spontaneously.

A preliminary reading of several published articles misled us of the possible applications of the cultivated epidermis. O'Connor and colleagues[6] (1981) published the first clinical application of cultivated keratinocytes, used in two patients with extensive third degree burns. They tested the grafting on three types of receptor bed: recent granulation, chronic granulation and recently-debrided fascia. They refer to satisfactory integration of the cultivated epidermis in both patients and in the three types of raw areas. However, they also reported that "both patients were extensively grafted with expanded autologous grafts" and that "after 3 to 4 weeks, the cultivated epidermis reached the surrounding expanded skin", which leads to the conclusion that the grafts were placed on the orifices of the expanded skin or very close to them. It is impossible to guarantee if the resulting epidermis was the cultivated one or was a result of the growth of the expanded skin borders.

Other authors presented their experience with cultivated epidermis on burnt patients, but with heterogeneous results[7, 8 and 9].

Gallico e colleagues[10] (1989) used cultivated epidermis to cover raw areas resulting from the removal of giant nevi in 8 children. The ressections reached down to the fascia, over which the grafts were placed. They refer to an average integration of 68% with a maximum of 84% in one case.

Our experience does not confirm these results. We

tried to place cultivated epidermis segments directly over raw areas of several types; however, we concluded that the epidermis does not integrate with irregular surfaces and where there is no corium. We also considered equally doubtful the possibility of deriving any benefits from the placement of cultivated epidermis over burnt areas. This fact was confirmed when we tried to graft the epidermis over muscular fascias, in the patients who had giant nevi, after the ressection of the impaired tissues. There was no integration and, positively, the surface of the fascia is a better receptor area than the burnt surfaces (Figs. 10, 11, 12 and 13).

We believe that the use of isolated epidermis should be limited to raw areas which have corium.

The question as to what is the advantage of using cultivated epidermis to cover areas that will epithelize spontaneously, such as donor areas of grafts, can be answered by the patients themselves. Every plastic surgeon knows that raw areas resulting from the removal of split-thickness grafts are extremely painful and adherent to dressings, thus making their removal more difficult; besides, such areas produce serum-hematic secretion, which produces local bad smell and is a culture medium for bacteria. The discomfort on the donor area is always worse than that on the area of the main disease which impelled the graft removal. This lasts for at least two weeks, if there are no complications.

The use of cultivated skin totally eliminated the pain and the secretions, producing complete epithelization of the wounds in less than 7 days. A strip of raw area, over which no graft was placed, was left in all patients. The external checking of the dressing always highlighted moistening on that area and the patient explained that the pain was limited to it.

Another attempt was made with patients suffering from vitiligo. Several articles recommend this procedure, due to the fact that, among the keratinocytes, some melanocytes are cultivated [11, 12] (Fig. 14). In our 5 patients, we prepared the recepient areas with dermabrasion[3] or laser[2]. The graft integration was complete in all cases, after around 7 days, and the patients were instructed to follow the usual dermatological therapies for the disease. However, after 6 months of follow-up, we concluded that the results are poor or insufficient, since the pigmentation either does not occur or is irregular, both in tint and distribution. Therefore, we consider that, for such patients, the used techniques still have to be improved (Figs. 15, 16 and 17).

The experience acquired with keratinocytes culture indicates that there is the need to develop artificial or biogenous supports to substitute the skin. At the moment, we are investigating two kinds of dermal substitution: bovine collagen and human acellular corium, both containing human fibroblasts (Figs. 18 and 19). Such substitutes would serve both for temporary and permanent coverage. In the first case, they would prepare the raw area for subsequent grafting, minimizing the pain and hydroelectrolytic losses. The definite coverage of the raw area could also be attained, by means of the association of skin substitutes and epidermal cells cultivated in their surface or subsequently applied. Both possibilities seem to be promising and will motivate further research.

CONCLUSIONS

a) The culture of keratinocytes is a viable technique, capable of producing large tissue segments from small biopsies, in short periods of time.

b) The integration of the cultivated epithelial laminae occurs perfectly on dermal surfaces, promoting a faster epithelization and eliminating the pain and the secretions from the wound.

c) The cultivated epidermis does not integrate over raw areas without corium.

d) The use of cultivated epidermis on vitiligo areas did not result in homogeneous pigmentation.

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