STANDARDIZATION OF THE CULTURE OF KERATINOCYTES WITH AUTOLOGOUS SERUM FOR COVERING WOUND AREAS.

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Description:

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Abstract

Second and third degree burns, as well as other tissue injuries, cause lesions to the cutaneous epithelial tissue that lead to a loss of keratinocytes, being replaced by a fibrous matrix produced by fibroblasts that act as a feeder layer in *in vivo* conditions. In the world, there are several reports about keratinocyte culture in the construction of regenerative skin patches for burns but not in Colombia. So it raises the need, in our country, for the standardization of a method for the culture of keratinocytes in autologous serum on fibroblasts as feeding layer and with autologous keratinocytes, thus avoiding immunological reactions to cover wound areas improving cutaneous cell viability in invasive processes such as burns and other lesions. We present the cases of patients admitted to the Plastic Surgery service with wound areas managed with autologous keratinocyte culture, which present complete epithelialization in a very short time and their follow-up.

Key Words

Keratinocytes, culture, fibroblasts, autologous, burns, skin culture, dressings.

Abstract

Second and third degrees burns like other soft tissue injuries cause damage to the epithelial layer resulting in a keratinocyte loss, being replaced by a fibrous matrix produced by fibroblasts, which act feed layer in vivo conditions. There are several reports of cultured keratinocytes as apposites of skin for the treatment of burns, but these have not been performed in Colombia, which raises the standardization of a method for cultured keratinocytes in our country with autologous serum on a fibroblasts feeder layer and with autologous keratinocytes, thus avoiding immune responses to improve coverage areas bloody skin cell viability in invasive procedures such as burns and other injuries. We present the cases of patients admitted to the Plastic Surgery Department with soft tissue injuries treated with autologous cultured keratinocytes which present complete epithelialization in a very short time and its follow-up.

Introduction

Second- and third-degree burns, as well as tissue avulsions, lead to lesions of the cutaneous epithelial tissue causing a loss of keratinocytes, these being replaced by a fibrous matrix produced by fibroblasts that act as a feeder layer in *in vivo* conditions. In general, these lesions are managed with healing by secondary intention or with skin grafts, taking between 15 to 20 days in their complete closure and leaving unstable scars and in the case of partial skin grafts, additional scars from the donor area and graft problems such as secondary contraction and pigmentation differences.

In the world, numerous studies have been conducted with keratinocyte culture in the construction of regenerative skin dressings for burns with the development in skin cell cultures, but it had not been done yet in Colombia. So we proposed the standardization of a method for the culture of keratinocytes in our country in autologous serum on fibroblasts as feeder layer and autologous keratinocytes. Because it is serum and skin from the same patient, we avoid the immunological or rejection reactions that could occur and in three days we have a dressing of considerable size to cover these areas with subsequent complete epithelialization on the 5th day of treatment.

Materials and methods

Skin samples of full and partial thickness were taken from different patients after signing informed consent. Samples were washed with PBS and cut into similar sizes to be digested. Three different differentiation protocols(1,2,3) were carried out until it was possible to determine which one was the one with the greater cell viability achieved, the fastest growth and on which dressing they had the highest survival rate.

The sample is placed in 0.25% Trypsin at 4° C. Then the skin is separated into two layers: an upper layer of epidermis and a lower layer (Fig. 1). The explants are repeatedly washed with DMEM-HG medium. The washed sample is collected in a 15 mL Falcon tube and centrifuged. The cells of the supernatant are seeded at a density of 90,000 cells/cm 2 in 6-petri dishes of 3.5 cm in diameter. (Fig. 2)

CELLULAR FEASIBILITY

For all the protocols, cells were counted in Neubauer chamber with trypan blue.

CELL CULTURE

A plate containing 90% confluent fibroblasts (FB) and another plate containing 60-70% confluent mesenchymal cells (MSCs) were used. On the other hand keratinocytes were detached from the plate using PBS, since these cells are not very adhesive. They were taken to a 15mL falcon and centrifuged at 1200rpm for 10 min.

Subsequently, cell count was made using Neubauer chamber. Keratinocytes (K) (250000 cells per dish) were seeded using collagen dressings as support.

GROWTH OF CELLS ON FB WITHOUT SUPPORT4

The extraction of the medium and the seeding of keratinocytes were performed on a plate containing 90% confluent fibroblasts (FB) in the manner described above, by seeding the keratinocytes directly over the FB (250000 cells per dish). (FIG 3)

In an initial stage, 50% fetal bovine serum was used and 4 portions of 2.5 x 2.5 cm collagen mesh were immersed in it. They were divided into two groups: in group 1 two immersion portions were left for 24 hours and later, fibroblasts were seeded at a density of 100,000 cell/cm2. In the other group fibroblasts were immediately seeded at a density of 100,000 cell/cm2 and the adhesion and confluence of the cells were observed without finding differences in growth, adhesion and confluence of the cells during 24, 36 and 48 hrs.

Then, the 4 dishes were shaken on an oscillating table for 4 hours and left to stand for 48 hours to achieve greater adhesion of the fibroblasts to the collagen meshes. The keratinocytes were seeded in the meshes with fibroblasts; adequate cell growth, confluence and adhesion of the keratinocytes were obtained with impregnation of the whole mesh in both groups. So it was concluded that the fibroblasts can be seeded immediately over the collagen meshes and 50% fetal bovine serum or autologous serum and subsequently the keratinocytes are seeded. (Fig. 4)

In this way, the culture of keratinocytes on collagen meshes with fibroblasts was standardized. The next step was to take a skin sample of partial thickness of 2 mm in length and 20 cc of blood from a patient admitted to the Plastic Surgery Unit with a deep second degree burn in the right upper limb, after signing an informed consent. Autologous serum was made and skin digestion was performed at the same time that the fibroblasts were seeded in the collagen meshes impregnated with the serum obtained from the patient's blood. The meshes were subjected to the aforementioned procedure for 48 hours, during which time an adequate population of keratinocytes was obtained. Subsequently, keratinocytes were seeded on the meshes with fibroblasts and allowed to stand for 24 hours, at which time an adequate confluence and cell adhesion was observed on the dressings (Fig. 5).

The dressings were placed with an aseptic technique over the patient's residual wound area and covered with a transparent dressing and bandages to ensure their non-mobilization. The uncovering of the wound area was performed 5 days later, finding a complete epithelialization of the treated area.

Results

The protocol for the management of patients admitted to the Plastic Surgery Department of San Ignacio Hospital with deep second degree burns is established.
They were handled in the following way:

* Day 0 (Fig. 6):
- The patient is admitted.
- Washing and debridement of the burn
- Take skin sample (3mm) and 20 cc of blood.
- Digestion of the skin
- Obtaining of autologous serum from the patient's blood.

*Day 1:
- Separation of the two layers of the skin

- Culture and replication of keratinocytes in autologous serum (72 hours) obtained from the epidermis
- Culture and replication of FB obtained from the dermis

*Day 3:
- Seeding of the FB grown on a collagen mesh
- Seeding of the keratinocytes grown on the fibroblasts and the collagen mesh (24hours) (Fig. 7).

*Day 4:
- Wash the burn area
- Application of the obtained dressing on the burn and covering of it.

* Day 9:
- Uncover the burn

In this way, 10 patients admitted to the Plastic Surgery Service with second degree burns were managed and they were followed up for 12 months.

Three days after taking the sample, we already have a dressing with fibroblasts and keratinocytes with a suitable cell density ready to be placed (Fig. 7). The burn is washed again and the dressing is covered with gauze and a transparent dressing.

Complete epithelialization of the deep second degree burn was obtained in 1 week with the implants (Fig. 8 and 9). None of the patients had rejection reactions of the implant.

Photographic monitoring of the patients was carried out, finding that the areas managed with keratinocytes presented a better quality of the scar compared with the areas healed by secondary intention in the same patient (Fig. 8).

The costs were calculated at 15,000 pesos to manufacture a 10 x 10 cm dressing.

CONCLUSIONS

Patients with burns have been managed through time by using different methods and currently there are different treatment options ranging from closure by secondary intention to cell cultures. In the world, keratinocyte cultures have been developed and the method for culturing them has been standardized; but in our country, these cultures had not been developed because of the cost of the immunological studies, since they are foreign cells to the receiving host.

Given this difficulty, we developed and standardized a method that allows us to obtain dressings from autologous keratinocytes and autologous serum from a very small sample of skin in three days. Complete epithelialization is achieved by the 5th day without allergic reactions or rejection of the implant. This scar is different to the one shown by patients with skin grafts or with closure by secondary intention since there is no contraction or retraction.

We discovered a standardized, reproducible, and low cost method with low comorbidity of donor areas, which is of special importance in patients with large burn areas. These patients do not have donor areas for grafts and with this method you get large dressings from small portions of skin.

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FIGURES

Figure 1. Separation of the skin after digestion

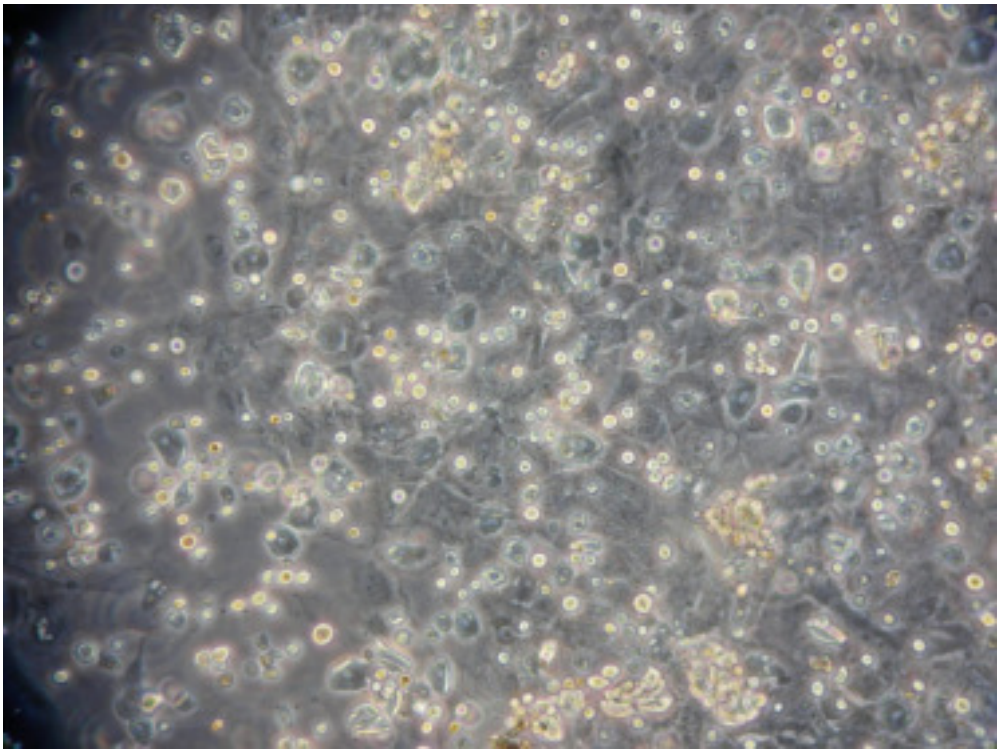

Figure 2. Monolayers of keratinocytes after 96 hours of growth

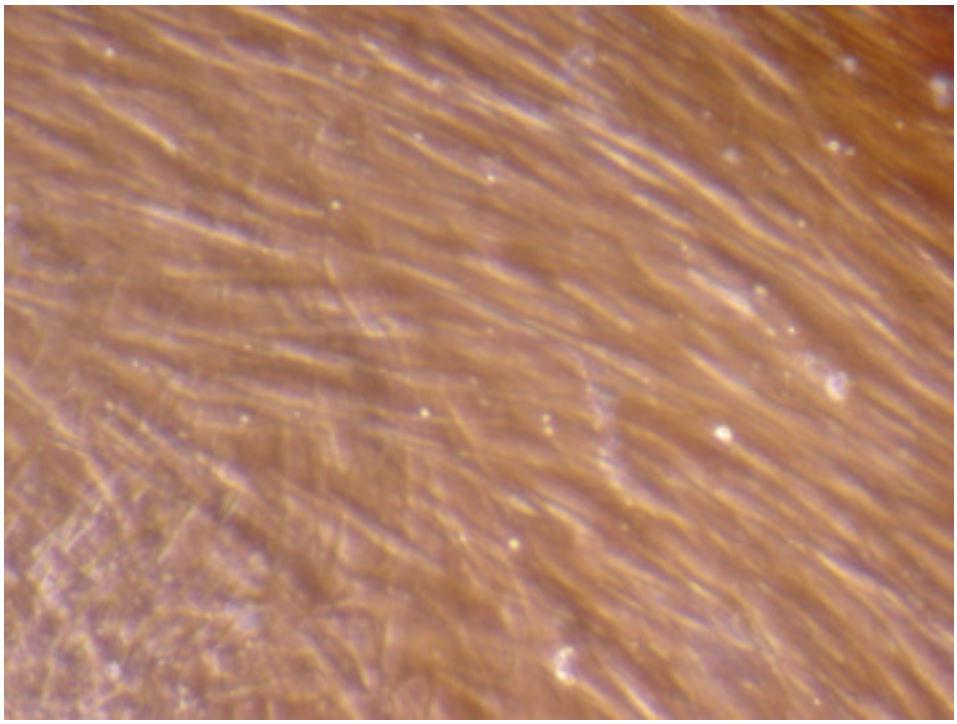

Figure 3. Keratinocytes on fibroblasts

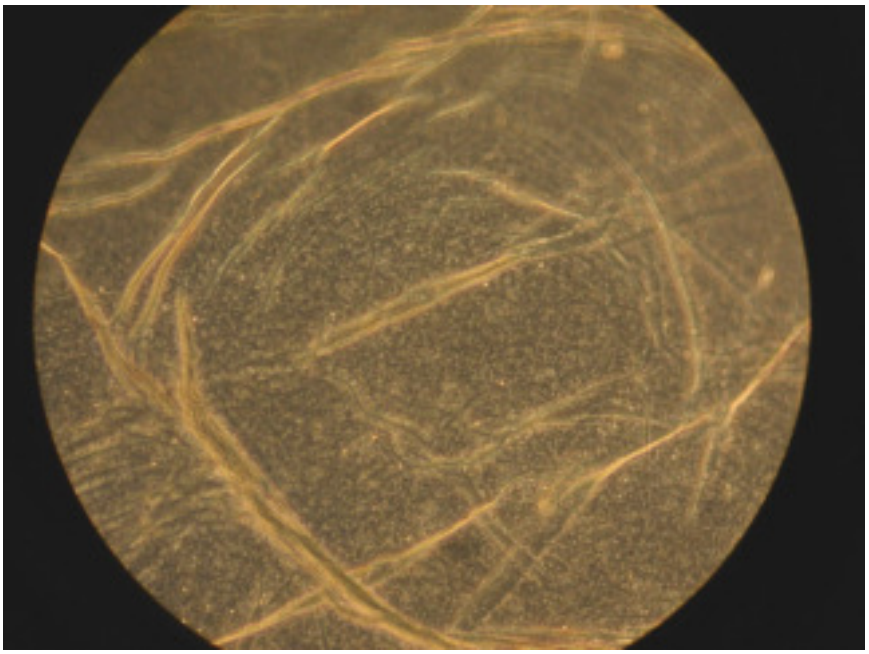

Figure 4. Collagen dressings with fibroblasts and keratinocytes

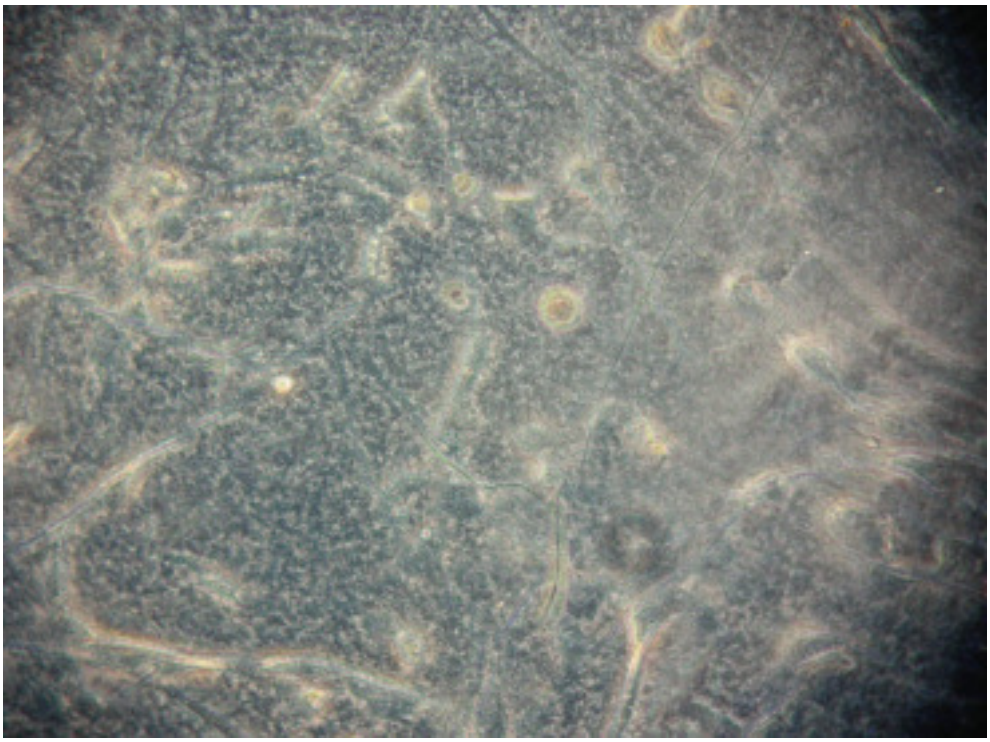

Figure 5. Condition of the culture of keratinocytes and fibroblasts with autologous serum from the patient on collagen mesh. We observe the adequate confluence and cell adhesion.

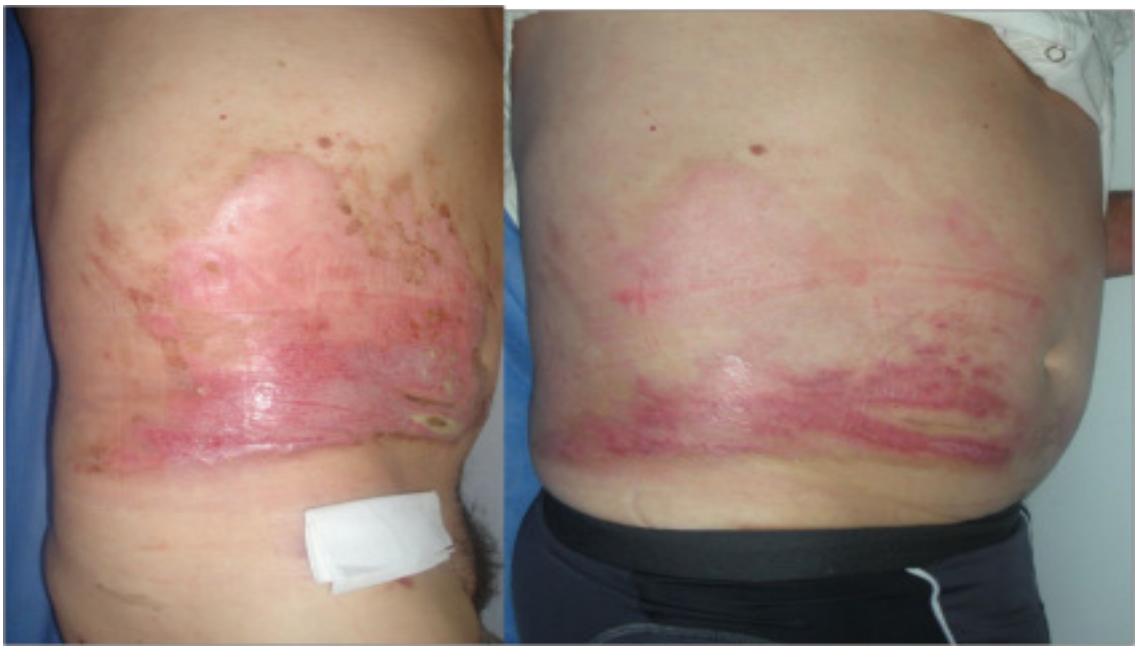

Figure 6. A 56-year-old patient with a second degree burn in the abdomen who was managed with autologous keratinocyte culture, showing complete epithelialization on the 5th day.

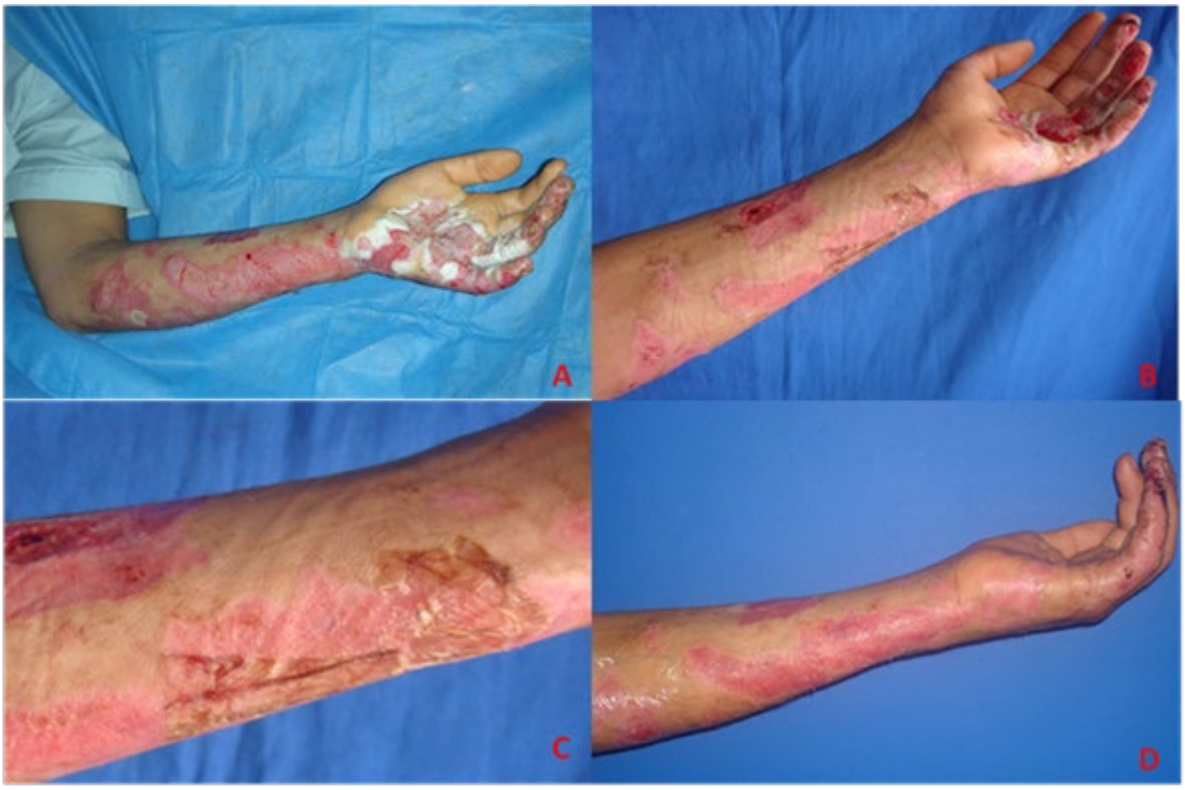

Figure 7. Patient with burned forearm managed with cultured keratinocytes. Note (C) the appearance of the dressing on the 3rd day of adhesion and how it comes off as the epithelialization progresses until it is completely detached with complete closure of the wound (D).

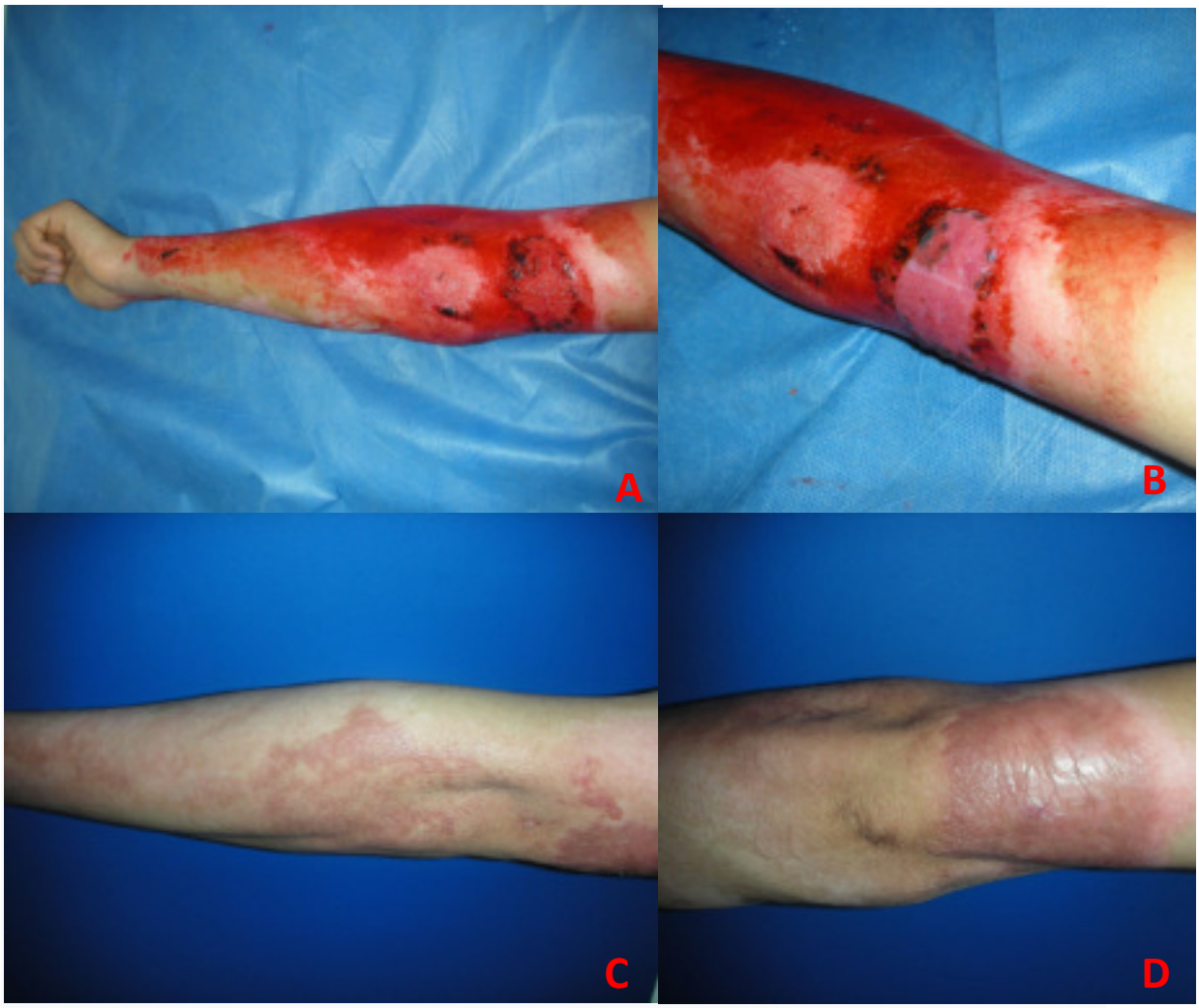

Figure 8. (A) Patient with superficial and deep second degree burn of the right upper limb with residual wound area in the arm (B) Residual wound area covered with the dressings. (C) Complete epithelialization on the 5th day. Note that the zone that healed by secondary intention presents a hypertrophic scar. (D) Area managed with the dressings, which presents a flat scar not indurated, 7 months after treatment.

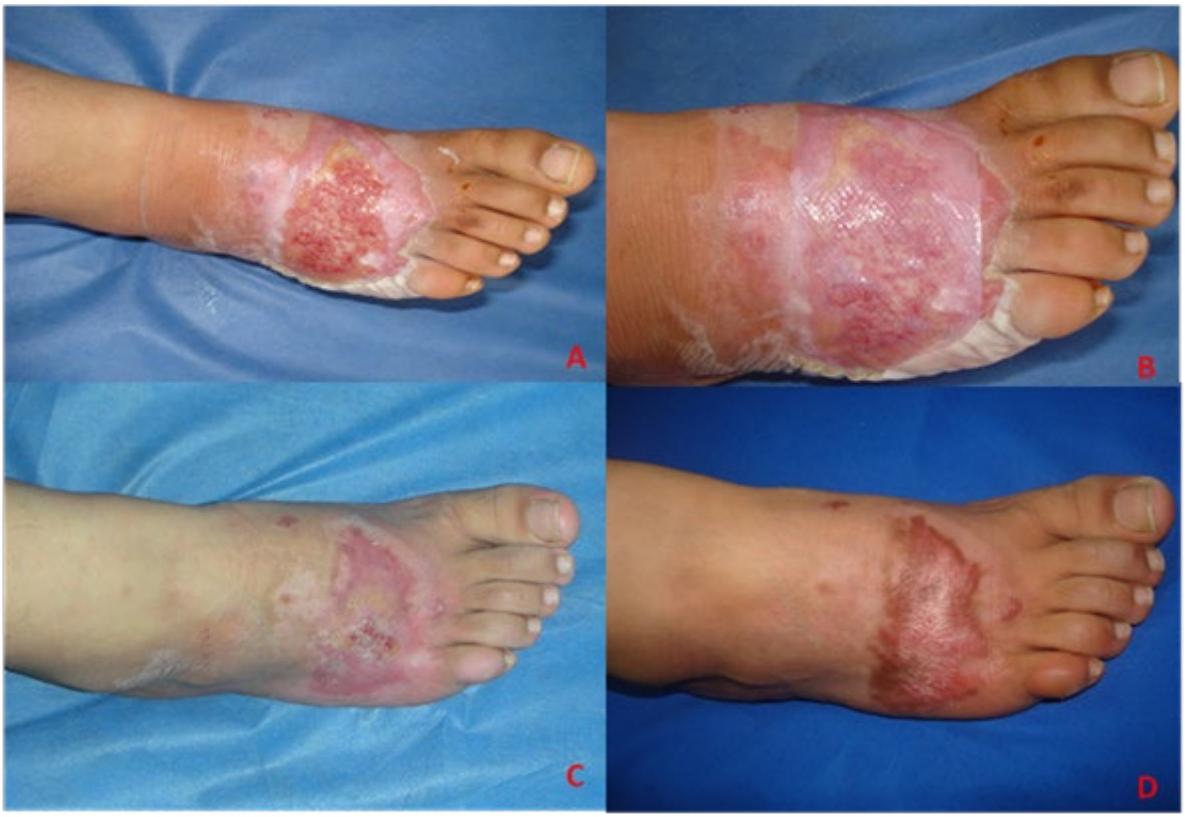

Figure 9. A) A 10-year-old patient with a burn on the back of his right foot. (B) Covered with a dressing with autologous keratinocytes, (C) uncovered the 5th day and showing adequate epithelialization. (D) Follow up for 6 months showing a flat scar, slightly pigmented but without induration.