EVALUATION OF A COMPOSITE SKIN SUBSTITUTE TO TREAT VENOUS STASIS ULCERS: A Pilot Study

Avaliação do uso de substituto de pele composto no tratamento de úlceras de estase venosa: um estudo piloto

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ABSTRACT

Introduction: Venous leg ulcers represent a therapeutic challenge, and they are associated to significant morbidity and cost. Many advances have been made to develop and apply biological skin substitutes, showing they can be beneficial for patients with burns, leg ulcers, and other skin lesions. Autologous cultivated keratinocytes have been demonstrated to be useful in leg ulcer treatment, but the lack of a dermal component may compromise their effects and the quality of the scar. Purpose: To evaluate a composite skin graft of human acellular dermis and autologous keratinocyte culture to treat leg ulcer. Methods: Two patients with four long-term chronic ulcers were treated with a composite skin graft of human acellular dermis and autologous keratinocyte culture for a month in this pilot study. Results: The four ulcers had a surface reduction of 72.21%, 36.36%, 60.93%, and 15.90% after one month of treatment. No adverse tissue reactions were observed. Histology and immunohistochemistry showed that human acellular dermis was free of donor’s cells, and the composite skin substitute preserved the architecture of the normal skin, including the basement membrane components and stratified epidermis. Conclusions: These preliminary observations suggest that such composite skin substitute could be an alternative to recalcitrant leg ulcers.

Keywords: Varicose Ulcer; Extracellular Matrix; Skin, Artificial; Tissue Culture Techniques; Cell Culture Techniques

INTRODUCTION

Leg ulcers are quite frequent and associated to significant morbidity, high medical costs and major impact on the quality of life and work productivity. Venous stasis ulcers represent 70-90% of cases1, and their management represents a hard medical challenge. It is a consensus that compression is the main step to heal venous ulcers2,3, and, therefore, it is considered the gold standard therapy for such condition4. However, the healing rates can be as low as 22% after 12 week therapy and the 12-month recurrence rate, as high as 69%5. Over the last years, the development of biological skin substitutes brought new strategies to treat difficult-to-heal ulcers6. Keratinocyte sheet autografts have proved to be life saving to treat large third degree burns and have been successfully employed in managing chronic ulcers7. Epidermal equivalents prepared from autologous hair follicle keratinocytes applied on recurrent leg ulcers led to reepithelialization of 70% of the total wound surface after 8 weeks with a healing rate of 32%.8 Although their advantages are permanent and with rapid wound coverage, application onto large areas with material obtained from a small skin biopsy, and faster pain relief9, they present some inconveniences. They are fragile to manipulate, resulting in an unstable epithelium, giving rise to spontaneous blistering many months after grafting. Additionally, they have an increased susceptibility to infections and contractures10. Histologically, the fragility of the grafted cultured epithelial autograft sheets may be related to the incomplete dermal-

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Evaluation of a composite skin substitute to treat venous stasis ulcers: a Pilot Study

METHODS

Patients - Two patients enrolled for the study were inpatients at the Clementino Fraga Filho University Hospital, who signed the informed consent as outlined for the project approved by the Hospital's Ethical Committee.

Case 1 - A 55 year old black man with two five-year old ulcers located at the maleolar external and internal regions of the left leg, measuring 10.15 cm² and 11.00 cm², respectively. Clinically, there were signs of venous insufficiency, with preservation of the arterial pulses. Dopplerfluorometry was compatible with venous insufficiency.

Case 2 - A 65 year old black man with three ten-year old ulcers. Patient had a history of traumatic ulcer (burn) and he had been submitted to a skin graft with no clinical improvement in the previous years. The ulcers were located on the anterior tibial area of the right leg, and they measured 10.65 cm², 0.70 cm², and 6.35 cm², respectively. The skin was covered by an atrophic scar, and there were signs of venous insufficiency. Aerial pulses were preserved and dopplerfluorometry was compatible with venous insufficiency.

In both patients, the autologous keratinocytes were obtained from a skin biopsy on the dorsum. Five and seven weeks later for case 1 and 2, respectively, the composite skin graft was applied over the ulcers. Before applying the skin substitute, the ulcers were cleaned with saline solution and superficially debrided after local anesthesia. The dressing used was A daptic M (Johnson & Johnson).

The dressing was changed 5 days after the application and every 3 days for 8 weeks afterwards.

Keratinocyte cell culture - Normal skin autologous keratinocytes were obtained according to Green and colleagues. Biopsies were cut into fragments and submitted to mechanical dissociation of the epidermis from dermis followed by enzymatic dissociation with a 0.3% trypsin in calcium and magnesium-free saline solution. Cells were harvested by centrifugation and cocultured with 3-T 3-12 mouse fibroblasts (obtained from the Rio de Janeiro Cell Bank), pretreated with 15 µg/ml mitomycin-C solution (Boehringer-Mannheim, Indianapolis, IN). They were maintained in keratinocyte culture medium consisting of a 1:1 mixture of Dulbecco’s (DMEM, high glucose) and Ham’s F12 media (both from GIBCO/BRL, Gaithersburg, MD, USA), supplemented with 10% fetal bovine serum (JRH Bioscience, Lenexa, KS), adenine, 1.8 x 10⁻⁴ M (Sigma Chemical, St Louis, MO, USA), cholesterin, 10⁻¹⁰ M (Vibrio Cholerae, type Inaba 569 B; Calbiochem, La Jolla, CA, USA), hydrocortisone, 0.4 µg/ml (Novo Nordisk, Princeton, NJ, USA), transferrin, 5µg/ml (Boehringer-Mannheim), triiodo-l-thyronine, 2 x 10⁻⁹ M (Sigma), amphotericin B, 2.5 µg/ml, and penicillin-streptomycin, 100 U/ml to 100 µg/ml (Boehringer-Mannheim), and epidermal growth factor, 10 µg/ml (Collaborative Biomedical Products, Bedford, MA, USA). Cultures were incubated in a humidified 10% CO₂, at 37°C.

Preparation of the acellular dermis - A cellular dermis was prepared from donor cadaver skin obtained from Rio Transplante Skin Bank (hepatitis B- and HIV negative). Acellularization was obtained as per the modified protocol described by Ralston and colleagues.

Fragments of skin were submitted to three rapid freeze-thaw cycles in liquid nitrogen to devitalize cells, washed three times in sterile PBS, and immersed in a series of increasing concentration of glycerol in phosphate saline buffers.

After being dried, acellular dermis was sterilized in ethylene oxide, rehydrated, and extensively washed in an 1 M NaCl solution. The epidermis was gently stripped from the dermis with a forceps, and the dermis was immersed in PBS with antibiotics solution (gentamycin at 100 µg/ml, ciprofloxacin at 10 µg/ml, amphotericin B at 2.5 µg/ml, and penicillin-streptomycin at 100 U/ml to 100 µg/ml) at 40°C for 4 weeks or more. Before the use, the acellular dermis was washed three times in a saline solution to remove residual antibiotics.

Preparation of the composite skin grafts - Pieces of acellular dermis were placed into a 35-mm tissue culture dishes, with the papillary side up. Cultured keratinocytes (0.5 ml of 1.0 x 10⁶ cells/ml) were seeded on top of the papillary surface of the acellular dermis and maintained 24 hours immersed in DM EM supplemented with 10% fetal bovine serum. This “skin composite” was subsequently raised and maintained at the air-liquid interface for 1 week prior to grafting.

Clinical assessment - Two months of following patients up after the surgery each week. Photographs of ulcers were taken with a digital SONY Cybershot 2.1 megapixels camera, and the areas were measured by the software Image Tool for Windows (version 3.00), and a mean of three consecutive measurements was performed.

Immunostaining and histology - To monitor the structure of the composite skin substitute, a fragment was processed by routine histologic techniques.

A n immunohistochemical study on paraffin sections characterized the presence of laminin and collagen type IV (DAKO, Carpenteria, CA, USA).
Antibodies were detected with LSAB+ - HRP (DAKO), followed by diaminobenzidyne (DAB, DAKO), and hematoxylin counterstaining.

RESULTS
Composite skin substitutes' biopsy showed a stratified epidermis with presence of laminin and type IV collagen at the dermo-epidermal junction, as well as a complete acellularized dermis maintaining its original structure (Figure 1). To the application, grafts had approximately the same size of each ulcer. After the graft application, the integration of the composite skin substitute was visually recognizable, in both patients. The graft adhered to the underlying wound within 5 days. Patients referred pain reduction. They did not present any complain and no adverse or side effects related to the application were noticeable upon clinical examination.

Case 1 - One month after the application of the skin substitute, the ulcers' diameters reduced from 10.15 cm$^2$ to 2.82 cm$^2$ and from 11.00 cm$^2$ to 7.00 cm$^2$, representing a reduction rate of 72.21% and 36.36% respectively.

Case 2 - A fter 1 month of the composite skin substitute application, the area of the ulcers reduced from 10.65 cm$^2$ to 4.16 cm$^2$, and from 6.35 cm$^2$ to 5.34 cm$^2$. The reduction rate were respectively: 60.93% and 15.90%. The third and smaller ulcer (0.70 cm$^2$) completely healed. (Figure 2).

DISCUSSION
The established treatment protocols to leg ulcers, including debridement, compression therapy, and venous surgery optimized the wound healing environment but did not stimulate healing directly. New advances in wound healing must provide an active stimulus to the wound. Although split-thickness autograft remains an option, the pain at the donor site, healing delays, infection and hypertrophic scarring are a limitation for its current use. The optimum characteristics of a skin substitute are: 1. rapid attachment to the wound allowing the revascularization; 2. provide a barrier against fluid loss and infection; and 3. easy handling. Additionally, it should be nonantigenic, with minimal inflammatory or foreign body response and nontoxic.

Although autologous culture keratinocytes may be useful to treat leg ulcers and to provide a new epidermal surface, they are difficult to handle, generating an epidermis subject to physical stress, resulting in early graft loss, blistering and scarring. The use of composite skin grafts here described disclosed no adverse events, made the manipulation of the graft easier, and increased the graft integration. Preservation of the basement membrane complex at the dermo-epidermal junction granted better mechanical properties of the healing skin. Although treated patients did not completely heal the ulcers in one month, their size had a significant reduction as well as morbidity. It is relevant to emphasize that those were long standing ulcers (5 and 10 years evolution), with larger than 5 cm$^2$ area were considered refractory to traditional treatments. Prognostic indicators of venous ulcers demonstrated that in subjects with a greater than 5 cm$^2$ only 40% healed baseline ulcer area with over 3 years duration, only 24% healed. Additionally, patient 2 had a history of injury (burn) in the affected leg, and the skin bed was not optimum, surrounded by an atrophic skin, which could made the graft taking more difficult.

On the other hand, the encouraging results described with other composite skin substitute on leg ulcers (GraftskinTM) were only attained with more than one application of graft. It is possible that successive applications of the present composite skin substitute may bring better results.

CONCLUSION
In this pilot study there were no side effect upon the application of the graft, and it was demonstrated the feasibility of this skin substitute to treat refractory leg ulcers. We are now engaged in a randomized study with a more expressive number of cases to estimate the accuracy of such composite skin substitute on leg ulcers compared to compression.
RESUMO

Introdução: As úlceras de estase venosa de perna representam um desafio terapêutico associado a significativa morbidade e custo. Muitos avanços têm sido realizados no desenvolvimento e aplicação de substitutos de pele biológicos, mostrando que estes podem ser benéficos para pacientes com queimaduras, úlceras e outras lesões de pele. Os queratinócitos autólogos cultivados têm demonstrado utilidade no tratamento de úlceras de perna, apesar da ausência de componentes dérmicos poder comprometer seus efeitos e a qualidade da cicatriz.

Objetivo: Avaliar o uso de um substituto dermocutâneo e queratinocítico autólogos cultivados no tratamento de úlceras de perna.

Métodos: Neste estudo piloto, dois pacientes com quatro úlceras crônicas de perna foram tratados com substituto dermocutâneo e queratinocítico autólogos cultivados durante um mês.

Resultados: As quatro úlceras tiveram uma redução de tamanho de 72,21%, 36,36%, 60,93% e 15,90% em um mês de tratamento. Nenhuma reação adversa foi observada. A histologia e a imuno-histoquímica mostraram que a derme acelarizada humanizou estava livre de células do doador, e o substituto composto preservou a arquitetura normal de pele, incluindo componentes de membrana basal e epiderme estratificada.

Conclusões: Os resultados preliminares sugerem que o substituto de pele composto pode ser uma alternativa para úlceras refratárias de perna.

Descritores: Úlceras Varicosas; Matriz Extracelular; Pele Artificial; Técnicas de Cultura de Células; Técnicas de Cultura de Células.

REFERENCES