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Wound healing after cultured epithelial autografting in patients with massive burn injury: A cohort study

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Summary *Background/aim:* Last century, our laboratory produced Cultured Epithelial Autograft (CEA) for clinical use by the affiliated adult burn service and other burn units across the country. Production of CEA for clinical use was discontinued after several years because of a low success rate and subsequent low demand. Recently, at our burns unit, a cell culture program was reintroduced as a direct response to the need for improvement in ongoing deficiencies and clinical requirements in burn wound closure. The aim of this study was to validate the laboratory processes and clinical algorithms established and share our recent clinical experiences involving CEA.
Methods: This observational cohort study recruited patients with burns exceeding 35% TBSA admitted to the Victorian Adult Burns Service at The Alfred (December 2013–December 2016). Autologous keratinocytes were expanded and delivered through sheets of fibrin carrier.
Results: Twelve patients were recruited to participate in the study. Thirty-two sites were treated with CEA. CEA applied in combination with widely meshed SSG led to the highest take rate (90.1%) at 7–10 days. Further, debridement and grafting were necessary in sixteen of

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thirty-two sites treated, all involving wound beds prepared with Cuono method or sites treated with CEA only.
Conclusion: It is important to address the problem of wound bed contamination, either through increased resistance on the part of the construct or wound bed sterilization. Improved understanding of the relative importance of vascularization, control of cell behavior, the extracellular matrix, immune function, and intrinsic antimicrobial capacity for graft take would then inform a more targeted approach to skin tissue engineering for wound closure in severe burns.

Introduction

Last century, our laboratory produced Cultured Epithelial Autograft (CEA) for clinical use by the affiliated adult burn service and other burn units across the country using the original Rheinwald and Green method.[1,2] Production of CEA for clinical use was discontinued after several years because of a low success rate and subsequent low demand. Early recognition of the importance of dermis and the dermo-epidermal junction in skin substitutes for the treatment of full thickness skin loss, and the development of composites did not lead to the widespread adoption of this technology, despite improved methods of production and sporadic successful case reports and series.[3] Today, standard of care for healing of extensive deep burns in adults does not include cultured skin substitutes as the primary method of wound closure. Nonetheless, it is claimed that CEA application in this cohort can save lives and expedite discharge from hospital. In addition, the problem of inadequate donor sites in massive burn patients and poor cosmesis after split skin grafting persists.[4-6]

At our burns unit, a cell culture program was reintroduced using a modification of the original technique, in which manufacturing and delivery are based on a fibrin carrier.[7] Although the ideal delivery system remains the subject of ongoing investigations, we chose fibrin due to its ease of handling, availability, and cost-efficiency.[7] In addition, fibrin provides good biological support and protection for cultured keratinocytes, promotes epidermal regeneration in vitro, and keratinocyte migration.[7,8] The decision to re-establish our research laboratory and cell culture program was a direct response to the need for improvement in ongoing deficiencies and clinical requirements in burn wound closure.

The aim of this study was to validate the laboratory processes and clinical algorithms established and share our recent clinical experiences involving CEA. Following advances in burn surgery, such as early burn wound excision and wound temporizing measures, we hypothesize that CEA can contribute to burn wound closure and is relevant in the management of patients with burn injuries.

Methods

This is an observational cohort study from December 2013 to December 2016. Eligibility criteria included all adult patients (> 18 years of age) with burns greater than or equal to 35% total body surface area (TBSA) admitted to the Victorian Adult Burns Service at The Alfred. Victorian Adult Burns Service provides the state-wide adult burns service, and treats approximately 250 patients with acute burns annually. This study was approved by The Alfred Ethics Committee (project number 380/13, approved 10 December 2013). The study was registered in the Australian New Zealand Clinical Trials Registry (ANZCTR) (ACTRN12613001342707).

Patient enrolment in this study took place as early as possible following admission. All patients were resuscitated and received nutritional support as per protocol of the Victorian Adult Burns Service prior to surgery. Resuscitation was ongoing during surgical procedures. Excisional debridement of all deep dermal and full thickness burns was performed early.[9] Excised wounds were initially temporized with Biobrane® (synthetic biocomposite semi-permeable bilayer dressing of outer silicone membrane and inner nylon/porcine collagen fabric), while awaiting further clinical decisions to be made.

Within days of admission, skin samples (approximately 2×5 cm) were preferentially harvested from a hair-bearing area, such as groin or axilla.[10] CEA production was based on previous publications by Rheinwald and Green[1] and Ronfard et al.[11] Briefly, skin samples were digested with dispase II and epidermal sheets were minced and digested with trypsin. Isolated keratinocytes were then cultured and expanded with the support of γ irradiated 3T3-J2 (mouse) feeder fibroblast cells. In the second stage of expansion (passage 2), keratinocytes were seeded onto γ-irradiated 3T3-J2 feeder fibroblast cells prepared on a fibrin matrix, which was prepared according to Pellegrini et al with some modifications.[12] Fibrinogen (Tisseel®, Baxter) was diluted 1.6 fold to a final concentration of 45-50 mg/ml (with 13,500 KIU/ml aprotinin) in saline, 1.1% NaCl and 1 mM $CaCl_2$. Diluted fibrinogen was mixed in equal volumes with 1 IU thrombin and immediately poured onto 10×10 cm dishes that were not treated for cell culture (Sterlin) to obtain a 100 μm fibrin gel layer. The fibrin gel layer was left at room temperature for 15 min or longer to fully polymerize before adding media and seeding the cells. Gentamicin 50 μg/ml used during the culturing process was well tolerated by keratinocytes.[13] CEA sheets were ready to be applied approximately 14 days after initial graft harvest.

Debrided burn wounds were closed with autologous split skin grafting wherever possible. Selected sites were chosen for CEA application, most commonly in the upper and lower limbs in nonpressure areas to avoid shear forces and CEA dislodgement. For deep partial tangentially excised wounds, CEA alone was applied. For full thickness wounds, CEA was applied in conjunction with expanded widely meshed autograft (meshing ratio 1:3), or to wounds temporized

Table 1 Summary of patient characteristics, CEA production and application.

Parameter		Results	
Patients	Recruited	12 (10 received CEA)	
	Gender	11 male, 1 female	
	Age: years	49 (22-67)	
	ASA status	3.2 (2-4)	
Injury	Mechanism of injury	Flame related	12
	TBSA (%)	55 (35-68)	
CEA production	Number of grafts harvested	14 (11 FTSG, 3 SSG)	
	Donor site	Axilla	6
		Groin	3
		Thigh	2
		Trunk	3
	Time: injury to graft harvest (days)	5.2 (3-8)	
	Time: graft harvest to CEA application (days)	17 (14-20)	
	Time: injury to CEA application (days)	22.2 (18-27)	
	Sheets of CEA manufactured per patient	8.6 (6-12)	
CEA application	Total number of sites treated with CEA	32	
	Strategy of CEA application	CEA only	7
		Cuono method	13
		CEA + widely meshed SSG	12
	Recipient sites	Upper limb	25
		Lower limb	7

Results are expressed as mean (range).
ASA: American Society of Anesthesiologists.

with cryopreserved cadaver allograft as described by Cuono et al.[14,15] In these cases, Biobrane® dressings were replaced with meshed cadaver allograft, usually at the time of skin sample harvesting for culture within the first week. At the time of grafting with CEA, allograft was de-epithelialized using Versajet® on low setting. Twenty-three sites were treated with the fibrin in direct contact with the wound bed (keratinocytes lying superficially) and in nine sites cultured keratinocytes were in direct contact with the wound bed. The timing of CEA application was determined by CEA availability, wound bed preparation, patient stability, and access to the operating theater. Each patient received one application of CEA only. Serial autologous split skin grafting was carried out whenever donor sites became available to achieve total wound closure.

CEA sheets were dressed with Surfasoft®, an inert monofilament polyamide woven primary wound dressing. To support keratinocyte viability during the early stages of vascularization, this is covered with secondary gauze soaked in Dulbecco's Modified Eagle Medium (DMEM, ThermoFisher®) and crepe bandages.[16] The outer layers of the bandages (leaving Surfasoft® intact) were routinely changed at 3-5 days after CEA application and all dressings were replaced at 7-10 days. Following, dressings were changed according to our protocol at our Burns Unit. Regular microbiological surveillance of all burn and donor site wounds was performed. In the absence of a consensus in the literature, topical antiseptics or antimicrobials were not used due to concerns regarding keratinocyte cytotoxicity.[13,16] Instead, prophylactic perioperative intravenous cephalosporin was administered, and based on clinical status, antimicrobial regimes were then individualized for each patient according to pathology results.

Data were prospectively collected and included baseline patient characteristics, and details of burn injury, CEA manufacturing and application. Each site closed with CEA was defined by the anatomical region. Primary outcome measures take rates assessed clinically at 7-10 days, 4 weeks, 6 months, and 12 months after CEA application, and the requirement for further surgery (repeat debridement and grafting) to achieve wound closure. Wound assessments were supplemented by clinical photography and punch biopsy of wound beds were performed. Scar quality was recorded using Vancouver Scar Scale and patients were followed up for a period of twelve months.[17,18]

Data were collected in Microsoft Excel® for Mac 2011 (Version 14.6.6) and statistical analyses performed using Stata version 14 (StataCorp, Texas, USA). Means and proportions were estimated using the cluster option available in Stata with differences assessed using postestimation commands. To account for multiple observations per patient robust standard errors were estimated. Correlation between variables was assessed by calculating weighted correlation coefficients. All reported P values are two-tailed with $P < 0.05$ indicating statistical significance. Data reporting adheres to STROBE guidelines.[19]

Results

Twelve patients with deep dermal and full thickness burns were recruited to participate in the study and ten patients proceeded to receive CEA (Table 1). Fourteen skin grafts were harvested for CEA manufacturing. Keratinocyte culture from three grafts (harvested from two patients) was aborted due to the bacterial growth detected during

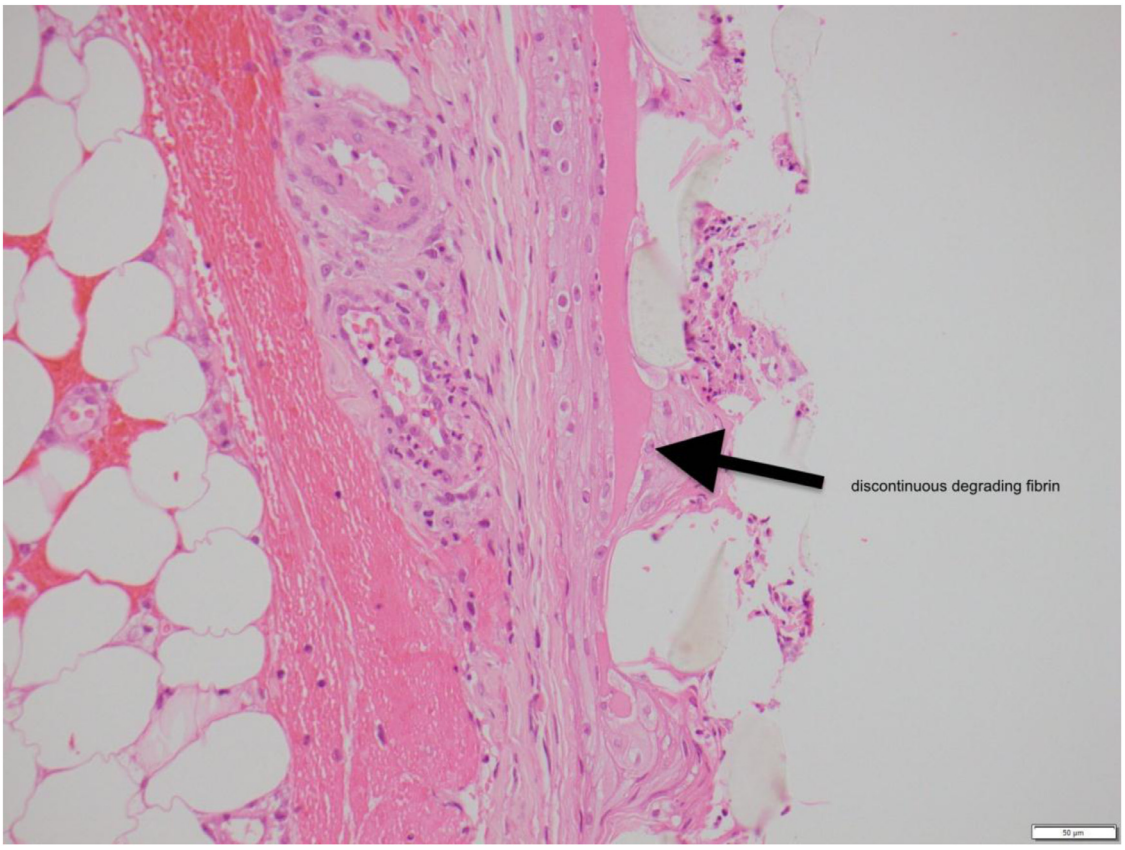

Figure 1 Left forearm wound treated with CEA and widely meshed SSG. Biopsy taken from SSG meshing interstices on day 4 demonstrated degradation of fibrin with keratinocytes deep and superficial to fibrin. Hematoxylin & eosin × 200 magnifications.

manufacturing; these two patients did not receive CEA. In a third patient, a second skin graft harvest was required due to poor yield from the initial graft. Three of 11 FTSG donor sites dehisced and were resutured.

A 59-year-old male (65% TBSA burn) died 21 days following the application of CEA (7 sheets) and widely meshed SSG to his left forearm. By day 10, the treated area had achieved 75% wound closure. The patient died from unrelated complications of sepsis, pulmonary embolism, and multi-organ failure.

A direct comparison in cell recovery between full thickness and split thickness skin on two patients suggested full thickness skin resulted in greater cell recovery compared to split skin. For full thickness biopsies, age significantly affected keratinocyte recovery ($P = 0.024$). There was a trend toward a correlation between cell recovery and cell viability ($P = 0.077$); and a negative correlation between patient age and keratinocyte initial clonogenicity ($P = 0.09$) and subsequent clonogenicity ($P = 0.01$). Initial keratinocyte clonogenicity was improved by at least 10-fold in the subsequent expansion period.

To be conservative in this pilot study, an average of only 8 sheets of CEA (range 6–12) were applied per patient and a meshing ratio of 1:3 of autologous SSG were used (instead of a higher ratio) when CEA and autologous SSG were used in combination. Thirty-two sites were treated with CEA, 12 of which were in combination with autologous split skin graft (SSG) (Table 1. At 7–10 days, epithelialization

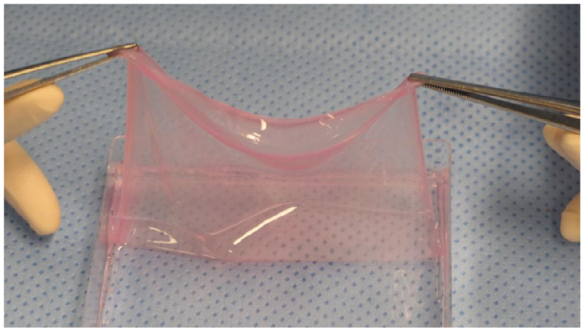

Figure 2 Keratinocytes cultured on semi-translucent 10 × 10 cm sheets of fibrin prior to grafting.

was highest (90.1%) when CEA was applied in combination with meshed SSG (Figures 1–3, Table 2). Qualitative assessment of the sites in which CEA was applied to meshed SSG showed improved epithelialization at 7–10 days when compared to meshed SSG without CEA; however, this was not possible to document objectively. Twenty-three sites were treated with the fibrin in direct contact with the wound bed (keratinocytes lying superficially) and in nine sites cultured keratinocytes were in direct contact with the wound bed. There was no difference in take rates or requirement for further surgery between these two groups. Nine of 32

Table 2 Summary of take rates and other outcomes of CEA application.

Strategy	Number of sites	Wound closure (%)[a]				Sites requiring further surgery (%)	VSS (12 months)[a,b]
		7–10 days	4 weeks	6 months	12 months		
Cuono method	13	29.2	41.4	99.7	100	10 (76.9)	4.3
CEA only	7	32.3	35	100	100	6 (85.7)	8
CEA + widely meshed SSG	12	90.1	94.4	100	100	0 (0)	5.5

[a] wound closure and Vancouver Scar Scale (VSS) results are expressed as mean.
[b] assessment of Vancouver Scar Scale excluded sites which required further grafting.

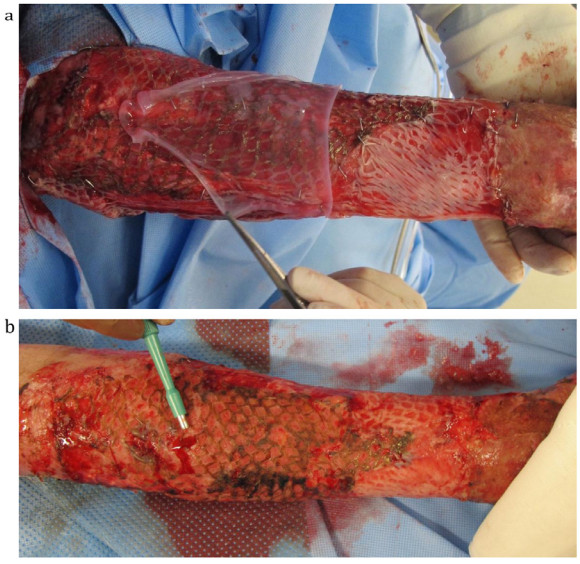

Figure 3 A 53-year-old male with 54% TBSA flame burn injury (a) CEA sheets were applied over widely meshed autograft over proximal aspect of right dorsal forearm. Interstices of meshed autograft (meshing ratio 1:3) are clearly larger than the distal forearm treated with SSG only (meshing ratio 1:2) (b) seven days after CEA application the take rate was approximately 85%. Punch biopsy of wound bed was taken for analysis.

sites were colonized with pathological microorganisms at the time of grafting.

Requirement for further debridement and grafting occurred in sixteen of thirty-two sites treated overall (50%), all involving wound beds prepared with Cuono method (Figure 4) or sites treated with CEA only (Table 3, Figure 5). No successful CEA graft using either the Cuono method or CEA alone had positive wound swabs on grafting; in contrast, several wounds treated with SSG and CEA healed uneventfully despite pathogens isolated on wound swabs. Clinically successful wound temporization with Biobrane® was not necessarily reflected in negative wound swabs.

Thirteen of 20 sites treated with either Cuono method or CEA only had partial initial graft take, but sites with initial take rates $< 60\%$ did not heal (Table 3). Ultimately, 4/20 of these sites healed without the need for further grafting. Statistical analysis did not reveal any difference between CEA alone and Cuono method with respect to healing efficacy. Ten sites underwent further debridement and grafting within 4 weeks and 6 sites required further surgery more than 4 weeks after CEA application.

A total of 53 punch biopsy samples were collected for histological assessments immediately prior to CEA application and at 7–10 days after CEA application. Biopsy findings were highly variable, and overall difficult to interpret in this acute setting; in part due to heterogeneity of wounds. Sampling errors and false negatives cannot be excluded due to the nature of punch biopsies. However, these histological assessments confirmed clinical impressions of wound depth, fragmentation of fibrin matrix within days, and viable interspersed cytokeratin-positive keratinocytes. Many samples revealed inflammatory changes consistent with an acute burn injury. Poorly vascularized or unintegrated cadaver allograft and other necrotic debris were seen in wounds treated with the Cuono method (Figure 6). Residual Biobrane® was found in four sites treated with CEA, surrounded by inflammatory cells including macrophages; Biobrane® adherence and retention in wound beds (Figure 7) has been previously reported.[20]

Discussion

We recently recommissioned our skin culture laboratory with the intention of treating severe burn patients with cultured keratinocyte products and developing improved products and protocols for doing so. Here, we present our initial recent clinical experience with CEA produced in accordance with Good Manufacturing Practice (GMP) principles, in an era when active surgical protocols and skin substitutes have become standard of care for patients with severe burns in our unit. In this study, a multi-layer confluence of keratinocytes were successfully cultured and delivered on fibrin by our laboratory. However despite the absence of granulating wounds, and significant improvements in sepsis rates, delivery of adequate nutrition, wound management, and antibiotic stewardship since 1980s, we essentially observed no improvement in efficacy of a modified version of CEA.

In this study, the only reliable healing group was the group treated with combination of SSG and CEA (Figure 3). This synergistic combination is believed to help wound closure, improve CEA take rate and provide a dermal matrix for stable wound coverage.[21,22] Several other burn centers consistently achieved similarly high take rates using this technique.[13,21–23] However, it is difficult to quantify the proportion of final epithelium that resulted from CEA application as opposed to the meshed autograft. Epithelialization and wound closure did seem to occur earlier than SSG with no CEA, however healing rates were similar

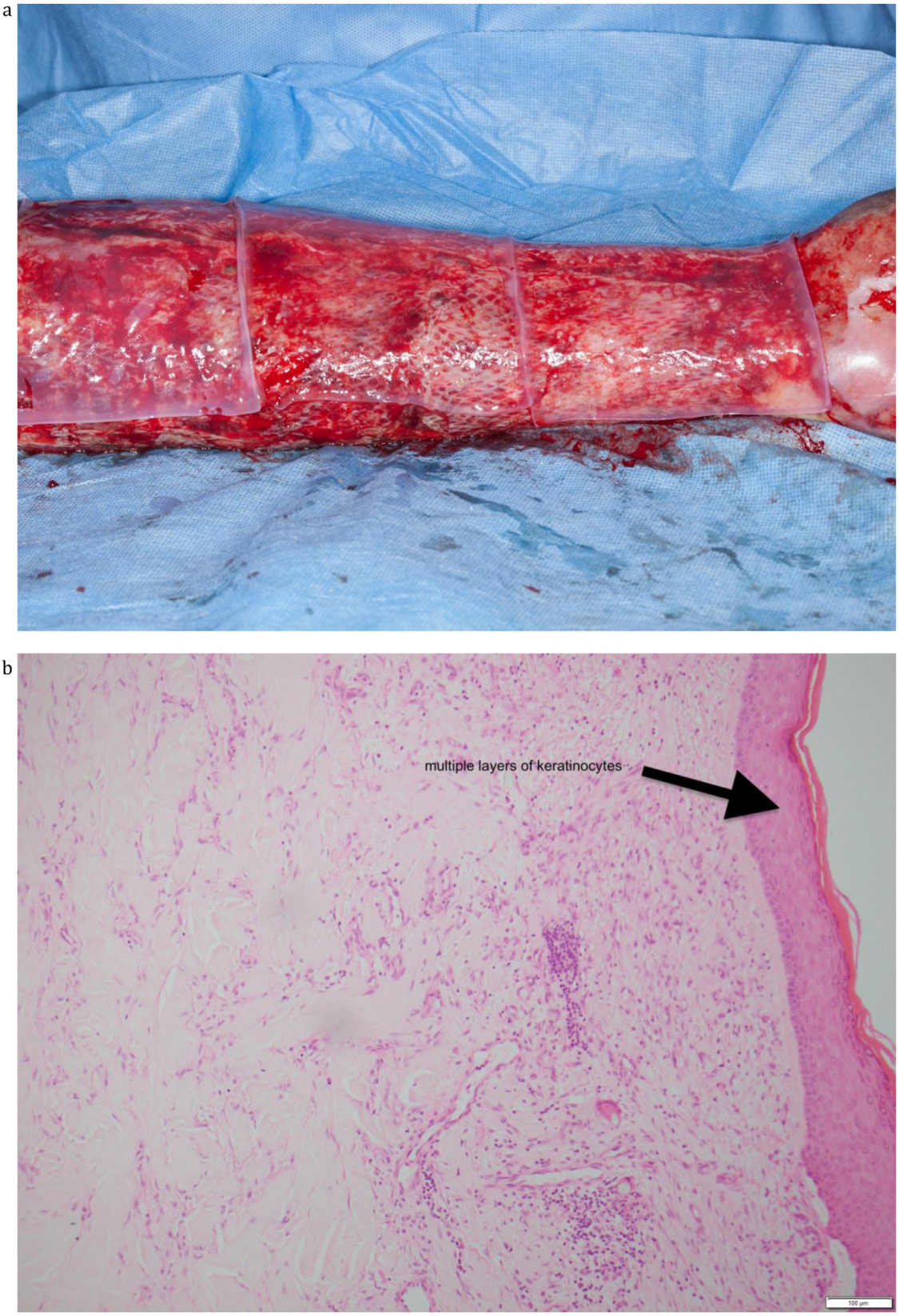

Figure 4 Cuono technique (a) Left volar forearm wound in a 67-year-old male with 60% TBSA flame burn injury treated with Cuono technique: meshed allodermis visible underneath fibrin CEA sheets (b) Biopsy taken from the right lower leg of a 35-year-old male with 44% TBSA flame burn injury treated with Cuono technique 12 days prior: multiple layers of keratinocytes & epithelialization clearly visible in the setting of successful wound closure. Hematoxylin & eosin × 100 magnifications.

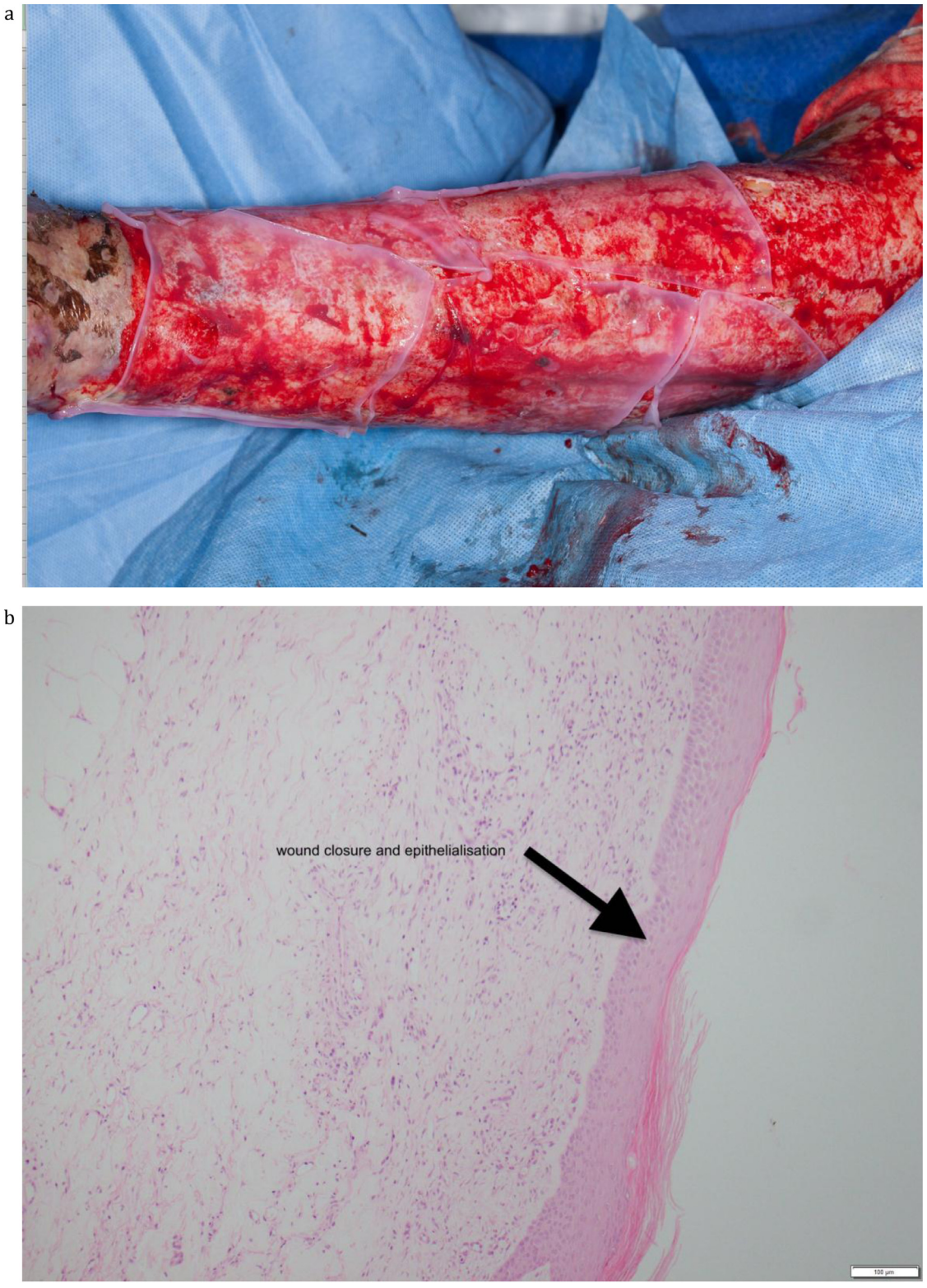

Figure 5 CEA only (a) fibrin CEA sheets applied over tangentially excised wound with deep dermis in the left dorsal forearm (b) biopsy taken from a left dorsal forearm wound 11 days after CEA application showing successful wound closure and epithelialization. Hematoxylin & eosin $\times$ 100 magnifications.

Table 3 Summary of take rates, microbiology and further surgery in sites treated with Cuono method and CEA only.

Site	CEA application	Take rates at 7–10 days (%)	Take rates at 4 weeks (%)	Wound microbiology at time of grafting	Further surgery required
1	Cuono method	Not available	90	None	No
2	Cuono method	40	30	None	Yes
3	Cuono method	20	20	None	Yes
4	Cuono method	80	Not available	None	No
5	Cuono method	30	Not available	None	Yes
6	Cuono method	20	30	None	Yes
7	Cuono method	0	40	None	Yes
8	Cuono method	30	30	None	Yes
9	Cuono method	Not available	Not available	Candida albicans	Yes
10	Cuono method	Not available	Not available	Candida albicans	Yes
11	Cuono method	0	Not available	Pseudomonas/Enterococcus/Serratia	Yes
12	Cuono method	0	Not available	Pseudomonas	Yes
13	Cuono method	60	50	Skin flora	No
14	CEA only	10	Not available	None	Yes
15	CEA only	20	Not available	None	Yes
16	CEA only	0	Not available	None	Yes
17	CEA only	40	0	None	Yes
18	CEA only	1	Not available	Pseudomonas/Enterococcus/Serratia	Yes
19	CEA only	75	Not available	Candida albicans/Pseudomonas	Yes
20	CEA only	80	70	Skin flora	No

Take rates not available as a result of further wound debridement and grafting or data not being collected.

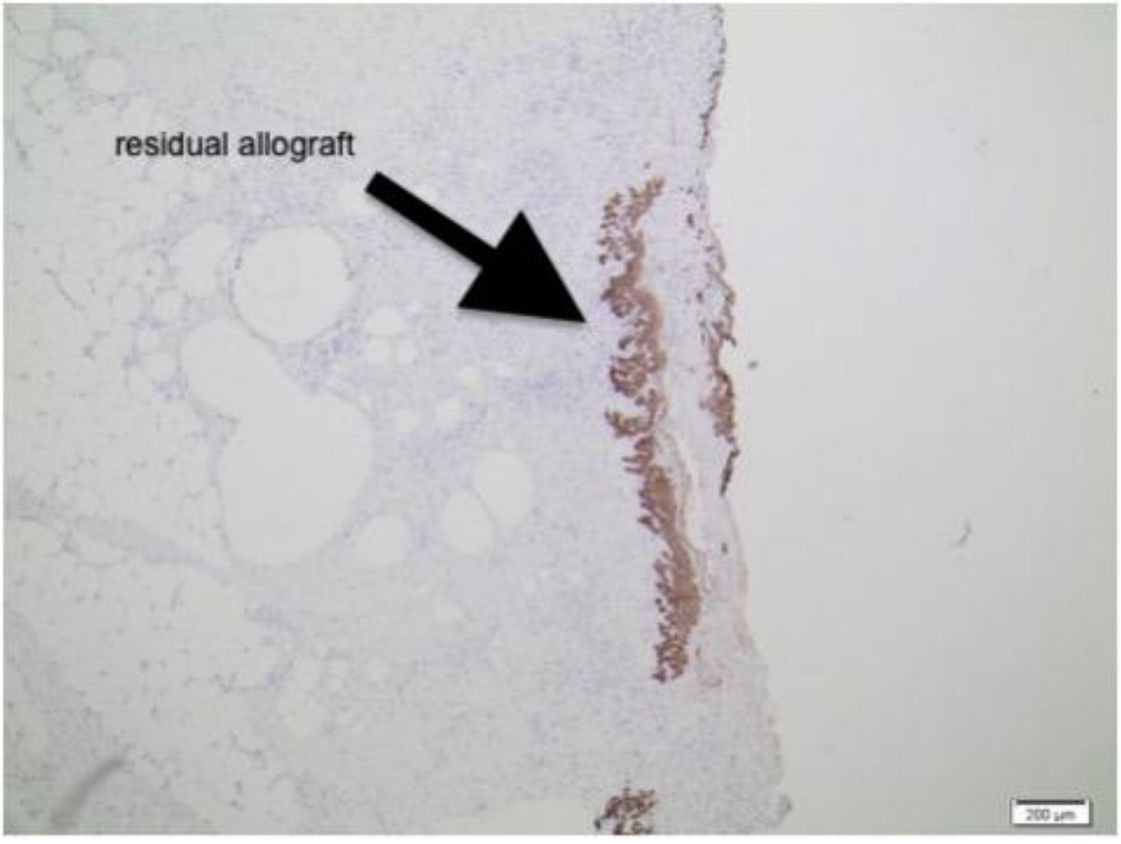

Figure 6 Biopsy taken 9 days after CEA application from a wound bed prepared via Cuono method showing residual necrotic cadaver epidermis deep to overlying fibrin and cultured keratinocytes. AE 1 and 3 cytokeratin immunoperoxidase × 100 actual magnifications.

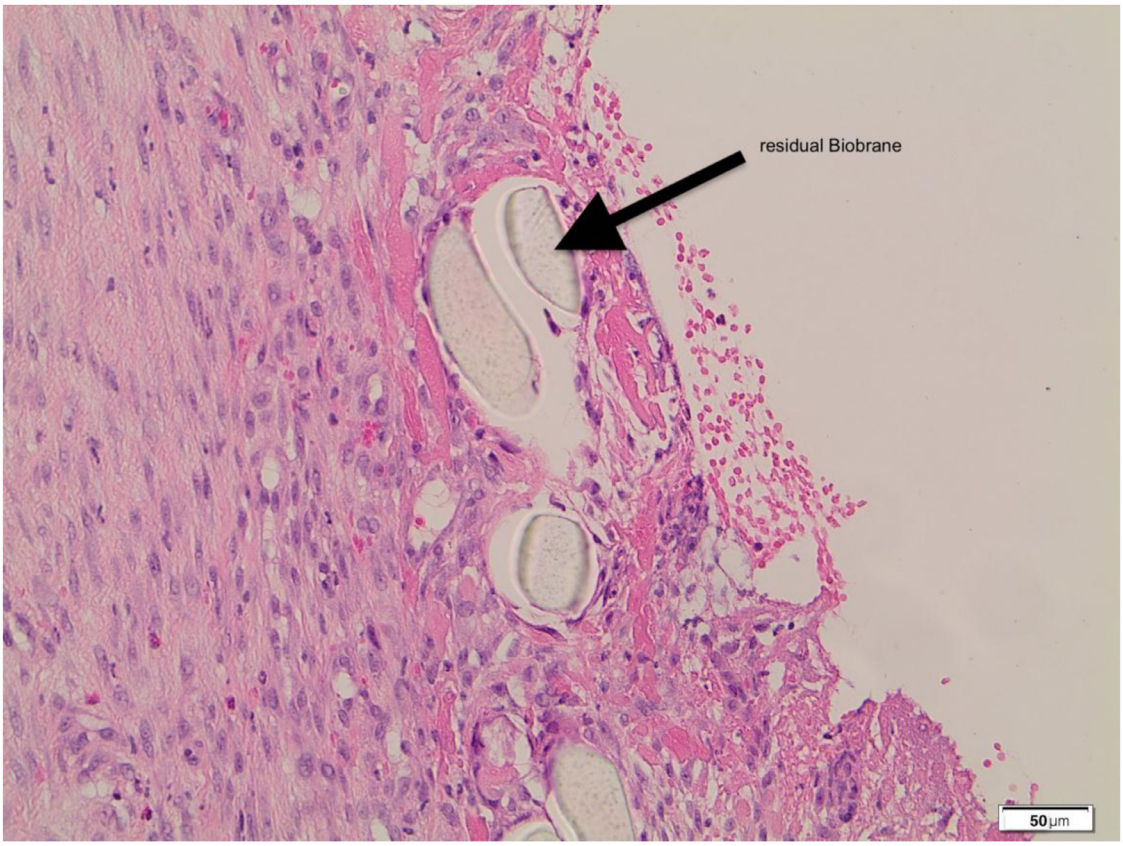

Figure 7 Residual Biobrane® and associated inflammatory and foreign body reactions within wound bed temporized with Biobrane®. Hematoxylin & eosin × 200 actual magnifications.

at 4 weeks and any positive effect of the CEA may not be clinically relevant at our meshing ratio.

Cuono et al treated an adult burn patient involving 55% TBSA with cadaver skin allografts.[14] The allografts were later serially abraded to remove allogeneic epidermis and resurfaced with autologous cultured keratinocytes. The Cuono method were used in many recently published series and considered by some authors to be the best skin substitute and preparation of wound bed for CEA; however reported take rates were highly variable at 0-95%.[10,13,21,22,24-26] In this study, relatively low take rates achieved with this technique may be attributed to several factors. When cadaver allografts were used, they were left in situ only long enough for CEA to be ready, approximately 2-3 weeks, whereas others removed cadaver epidermis after more than 3-4 weeks.[13,14,21,22,27] This period of allodermis vascularization and integration may be inadequate, as indicated by the biopsy findings of residual necrotic nonviable tissue following debridement, immediately prior to CEA application (Figure 6). Removal of cadaver epidermis in this study was challenging, and cadaver graft occasionally dislodged easily from the wound bed. Cuono et al. removed the allodermis by using a cylindrical air-driven carborundum wheel; whereas other authors have applied tangential excision.[14] Dermabrasion may be associated with bleeding and aerosolization of abraded bloody particles, presenting difficulties, such as poor visibility and health risk to health workers in the operating room. Tangential excision may lead to inadvertent but complete removal of allograft. We have used Versajet® in burn debridement for many years and found it effective in the selective and precise removal of nonviable tissue and preservation of healthy dermis.[13] Depth of removal may be controlled via settings on the machine and varying the duration of application of the handpiece over the wound. We applied Versajet® until punctate bleeding suggestive of healthy viable tissue, often leaving behind an embedded meshed pattern of allodermis.

CEA sheets were applied in isolation only to tangentially excise partial thickness wound beds with preserved deep dermis (Figure 5). It is well established that CEA take rate is improved by the presence of dermal elements, however in this study successful grafting occurred in only 1 in 7 sites with residual deep dermis treated with CEA alone.[2,28] Colonization of excised burn wounds occurred in nearly a third of sites, and was not completely prevented by temporization of excised wounds with Biobrane® nor allograft; cultured cell sheets are known to be highly vulnerable to bacterial proteases and cytotoxins.[28-30] Sites successfully treated did not have any microbiological growth, or grew skin flora only. Whereas bacterial colonization may have little or no effect on the take of meshed autograft, a similar level of contamination can cause significant loss of CEA.[30,31]

Residual Biobrane® (likely nylon) was unexpectedly found in four sites treated with CEA, surrounded by inflammatory cells including macrophages. Although, Biobrane® adherence and retention in wound beds has been previously

reported, it is not widely recognized.[20] This unexpected finding in our study highlights the importance of timely removal or replacement of Biobrane® as the foreign tissue load may add to the infection risk (Figure 7).

It is approaching 40 years since the clinical application of cultured autologous keratinocytes in the treatment of burn wounds was first published, and the healing capacity of cultured epithelial cells is well established.[32] Mastery of stem cell technologies and understanding of wound healing mechanisms has increased massively since then.[33] However, regardless of how sophisticated the engineered skin construct, if the problem of wound bed contamination is not addressed, either through increased resistance on the part of the construct, or wound bed sterilization, then even the most complex of bioengineered skin substitutes will remain frustratingly unpredictable. Understanding of the factors necessary for skin grafts to take has not advanced in keeping with advances in knowledge of stem cells, wound healing, inflammation, and tissue engineering. Since the relationship between the quantity of pathogenic bacteria and skin graft survival was described more than 50 years ago, research has been directed to control wound bed infection, but it seems that eradication of harmful bacteria in extensive burn wounds may be unachievable, and the key determinants of skin graft take beyond broad clinical requirements remain unclear.[34,35] Because a wound only has to be "good enough" for successful autologous grafting, the minimum necessary conditions for graft take are not known, and what factors to specifically target in the production of a tissue engineered skin substitute that performs as well as an autologous SSG are as yet undetermined. In the same way that synthetic dermal replacements may be more effective in a clinical setting than those composed of biological materials, mimicking a SSG may be less important than identifying and addressing minimum conditions for cell survival and proliferation in vivo after grafting.[36–38] An understanding of the relative importance of vascularization, control of cell behavior, the extracellular matrix, immune function, and intrinsic antimicrobial capacity for graft take would then inform a more targeted approach to skin tissue engineering for wound closure in severe burns.

Conflict of interest

None.

Acknowledgments

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