Clinical application and viability of cryopreserved cadaveric skin allografts in severe burn: A retrospective analysis

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ABSTRACT

Introduction: Cadaveric cutaneous allografts are used in burns surgery both as a temporary bio-dressing and occasionally as definitive management of partial thickness burns. Nonetheless, limitations in the understanding of the biology of these grafts have meant that their role in burns surgery continues to be controversial.

Methods: A review of all patients suffering 20% or greater total body surface area (TBSA) burns over an eight year period that received cadaveric allografts were identified. To investigate whether tissue viability plays a role in engraftment success, five samples of cryopreserved cadaveric cutaneous allograft processed at the Donor Tissue Bank of Victoria (DTBV) were submitted to our laboratory for viability analysis using two methods of Trypan Blue Exclusion and tetrazolium salt (MTT) assays.

Results: During the study period, 36 patients received cadaveric allograft at our institution. The average total burn surface area (TBSA) for this group of patients was 40% and all patients received cadaveric skin as a temporizing measure prior to definitive grafting. Cadaveric allograft was used in complicated cases such as wound contamination, where synthetic dressings had failed. Viability tests showed fewer than 30% viability in processed allografts when compared to fresh skin following the thawing process. However, the skin structure in the frozen allografts was histologically well preserved.

Conclusion: Cryopreserved cutaneous cadaveric allograft has a positive and definite role as an adjunct to conventional dressing and grafting where available, particularly in patients with large TBSA burns. The low viability of cryopreserved specimens processed at DTBV suggests that cell viability in cadaveric allograft may not be essential for its clinical function as a wound dressing or even as permanent dermal substitute.

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1.   Introduction

Human cadaveric cutaneous allograft has been used in the management of burns for over 50 years [1]. During this time, clinical, technological, medico-legal and tissue banking developments have changed the context in which clinicians manage severely injured burn patients. Burns units have evolved their own treatment algorithms which are largely dependent on local resources and clinician preference. This is especially true with respect to wound management practices in those with massive burns.

Despite the availability of various kinds of skin substitutes for clinical and research use, autologous skin grafting remains the primary treatment of choice for deep burns, and indeed if a patient with extensive deep burns is to survive, burns must be grafted eventually with autologous skin. When this is not initially feasible, due to limited donor sites or host wound bed factors, there is a requirement for alternative methods of wound closure. Tissue engineered skin substitutes are currently available for use in uncontaminated wounds, and can adhere and provide wound closure pending availability of autologous grafts; however, they can be highly demanding of technical expertise for production and in the requirement for meticulous wound bed preparation and after application management practices for successful engraftment.

Cadaveric allograft may also be applied to the burn wound as a temporizing measure [2–4]. In addition, cadaveric allografts have been advocated by some authors as a definitive dressing for partial thickness burns and as wound bed preparation after excision of full thickness burns [3]. In contrast to available synthetic skin substitutes, allograft possesses many of the desirable properties of autologous skin. In particular, it has the ability to adhere to and engraft a suboptimal host wound bed, taking a blood supply and providing wound closure until host rejection of the cellular elements. This results in wound closure which promotes retention of moisture and electrolytes and improved thermo-regulation [5,6]. In addition, allografts decrease wound pain, lower bacterial loads in contaminated wounds, and may provide dermal matrix elements which can persist [7,8] and improve final graft properties and scarring after definitive autografting.

Two common methods of preserving cadaveric allografts are in use by tissue banks: cryopreservation and 85% glycerol preservation, and there is ongoing debate regarding the relative clinical merits of glycerol preserved and cryopreserved allograft [9,10]. In comparison to cryopreserved skin, 85% glycerol preservation has antibacterial and antiviral effect [11,12], and allows for more cost efficient long term storage and ease of distribution. However it results in essentially unviable skin, which may be associated with decreased clinical utility [13,14]. The current role of allograft skin in the management of burn varies between burn units, many of which do not have access to or experience with use of this product. In addition, developments in the medico-legal environments in which clinicians and tissue banks operate have increased resource requirements for compliance with various standards. The value of and indications for allograft skin for management of burns patients, and the cost–benefit ratio of different methods of skin tissue banking are not issues on which there is universal agreement. The DTBV is the only fully operational multi tissue bank (skin, musculoskeletal and cardiac tissue) facility in Australia. It developed a skin banking program in 1994 [14]. The Victorian Adult Burns Service (VABS) at the Alfred Hospital is the state-wide provider of burns care for all adults with complex or major burns, serving a population of 5.5 million in south-eastern Australia.

The supply of cadaveric skin allograft is extremely limited in Australia. This paper presents our unit's current algorithm for management of patients with severe burn. The results of analysis of the properties of cryopreserved skin produced by the DTBV are presented, and the indications for use of this skin are discussed in the light of our findings and current logistical realities.

2.   Materials and methods

2.1.   Clinical material

Allograft is used as a temporary method of sealing and stabilizing deep excised burn wounds prior to definitive grafting with autologous split skin graft. Our current management algorithm reserves allograft for use in patients with large burns in whom synthetic or composite skin substitutes have failed (Fig. 1). This is in part due to limited availability of allograft. If stocks allow, allograft is also used for wound closure over widely meshed autograft [15]. Allograft routinely adheres to excised and contaminated burn wounds. The dermis can persist for at least some weeks in a wound bed (Fig. 2), and allograft dermis is not routinely removed prior to autografting. If allograft epidermal elements are present at the time of autografting, these are removed using hydrosurgical excision (Versajet™) prior to grafting. In partial thickness wounds, allograft supports re-epithelialization (Fig. 3). After institutional ethics approval, patients with greater than 20% TBSA burns admitted to the hospital during an 8 year period (January 2002–January 2010) were identified using the Alfred Hospital's VABS database. A chart review of these patients was undertaken and patients receiving cadaveric allograft were identified.

2.2.   Cryopreservation

Skin tissue is retrieved within 24 h of death and exposed to antibiotics for a minimum of 12 h. The skin is exposed to a cryopreservation bath (cell culture media + DMSO), and packaged in double, freeze resistant, plastic and aluminum pouches. Samples for microbiological monitoring are removed at different stages during this process. The skin is frozen to −40 °C at a rate of 1°/min, and stored in quarantine in liquid nitrogen. Tissues are released for clinical use only after the final quality review, which includes all processing data and information contained in the donor file.

2.3.   Viability assays

Cryopreserved skin tissues (I–V) processed at the DTBV, donated from five individuals with an age range of 42–63

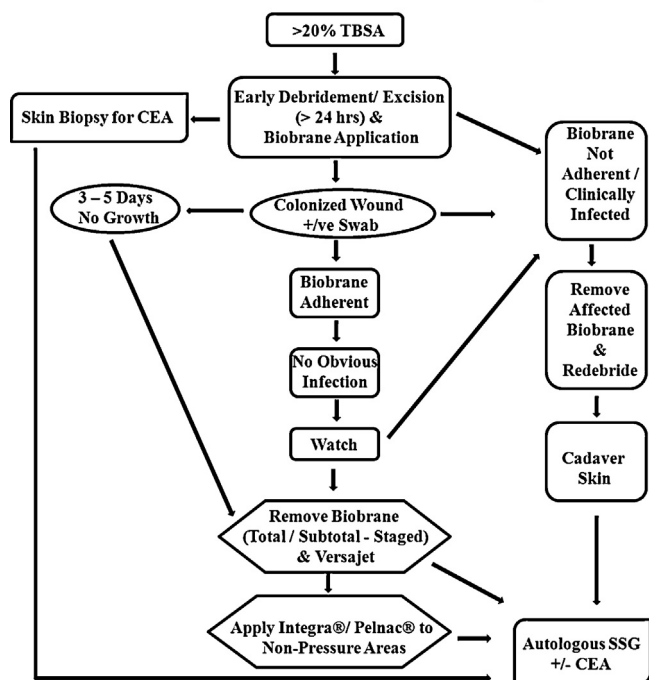## Victorian Adult Burns Service Skin Substitute Algorithm

Fig. 1 – Victorian Adult Burns Service skin substitute algorithm.

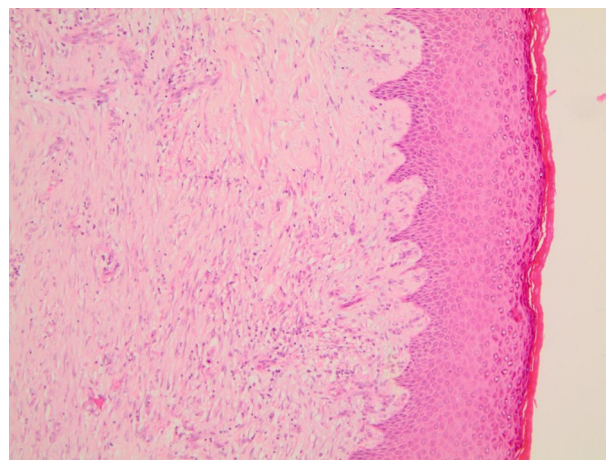**Fig. 2 – Dermis at day 21 after application after failure of autograft.**

Fig. 3 – Dermis healed wound at day 21. A biopsy punch of the healed wound at day 21 was processed and stained with H&E.

were analyzed for cell (keratinocyte) viability. Skin cell viability was compared to fresh tissues donated after informed consent by seven individuals with a similar age range (between 39 and 62 + a 19 year old), after approval by the relevant institutional Ethics Committee. These tissues were processed within the first 18 h of retrieval. For each experiment at least one fresh sample was tested as positive control.

2.3.1. Whole tissue MTT viability assay

All DTBV processed human skin samples (I–V) were thawed in pre-warmed phosphate buffer saline. 4 mm biopsy disks, in triplicate, were incubated with 2 mL 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT, Sigma) at 0.5 mg/mL for 2–3 h at 37 °C, shaking at 100 rpm. MTT is converted to a blue/purple salt (Formazan) by mitochondrial dehydrogenase in live cells. The salt was eluted overnight by incubating the skin disks in 1 mL 2-methoxyethanol (Sigma) and its optical density was measured at 570 nm with a reference wavelength of 690 nm. The absorbance values were normalized against absorbance values of fresh skin that was tested within the first 18 h of retrieval.

2.3.2. Cell viability – extraction and Trypan Blue Exclusion assay

A 5×2 cm^2 piece of each DTBV processed human skin (I–V) was digested with Dispase II (4 mg/mL, Roche Diagnostics) for 1 h at 37 °C: the epidermis separated easily and did not require the 2 h conventional incubation time. The fresh control skin was digested either for 2 h at 37 °C or overnight at 4 °C. Following the digest, the epidermal sheets were separated from the dermis, minced and digested in 0.25% trypsin (Invitrogen) for 5–10 min at 37 °C to retrieve epithelial cells. Trypsin was deactivated by adding 5-fold the volume of 10% serum containing growth medium, and isolated keratinocytes were tested for viability based on 0.2% Trypan Blue (Sigma) exclusion. Dead or membrane damaged keratinocytes that take up 0.2% Trypan Blue were counted against viable cells using a haemocytometer.

Table 1 – Comparison of allograft and no allograft patients.			
	No allograft (n = 244)	Allograft (n = 36)	p-Value
Age (years)	41.25 ± 18.02	44.8 ± 16.96	0.27
Male (%)	173 (70.9)	25 (69.4)	0.82
TBSA% burn	32.59 ± 13.53	56.94 ± 20.6	<0.001
TBSA% full thickness burn	10 (3–20)	40 (16–55)	<0.001
Deceased (%)	15 (6.1)	10 (27.8)	<0.001
LOS (days)	31 (18.5–47)	77.5 (49–131.5)	<0.001
Number of operations	2 (1–4)	10 (5–11)	<0.001
Total operating time (h)	4.5 (1.5–9)	19.5 (14–30.5)	<0.001
Days to first debridement (days)	2 (0–5.5)	0 (0–1)	<0.001
Data are presented as mean $\pm$ standard deviation, median (inter-quartile range) or number (percentage) where appropriate.			

2.3.3. Histology

All fresh and DTBV cryopreserved human skin tissues were fixed in 10% formalin and processed for histological analysis according to standard protocols. Sections were stained with H&E.

2.4. Statistical analysis

All data were analyzed using the SAS software version 9.2 (SAS Institute, Cary, NC, USA). Comparisons between groups (allograft versus no allograft) were made using the Student's t test for normally distributed data and Wilcoxon rank sum test for nonparametric data. Differences in proportions between groups were compared using the chi-square test for equal proportion or Fisher's exact test where numbers were small. Statistical significance was set at a two-sided p-value of 0.05.

3. Results

3.1. Demography

In the years 2002–2010, 36 patients were treated with cadaver skin supplied by the DTBV. During this time, 244 admitted patients with burns >20% TBSA who were actively treated did not receive allograft. The characteristics of these patients were
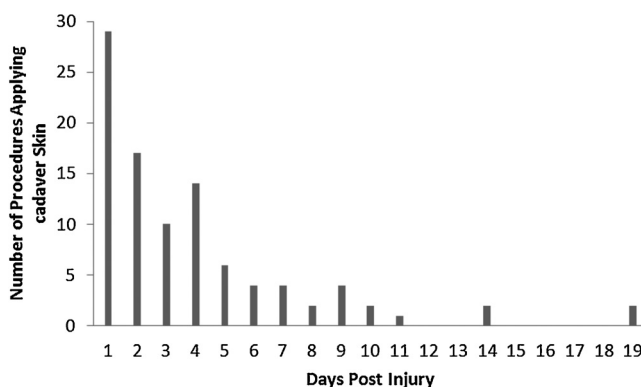

Fig. 4 – Time to allograft procedure. A total of 32 out of 36 patients who received allograft were reviewed further. Allograft was applied in 98 procedures; mostly in days immediately after injury.

compared with actively treated patients not receiving allograft (Table 1). A total of 32 charts were available for more detailed review. The average number of allografting procedures per patient was 2.83 for survivors (n = 24) and 3.75 for non-survivors (n = 8). Overall, allografts were used in 98 episodes. The indicators for use of allograft were: skin closure after primary burn wound excision (n = 33); infected burn wound/failed split skin graft (n = 16); failed skin substitute (Biobrane or Integra) (n = 9); cover widely meshed autologous skin graft (n = 40). Fig. 4 shows the time to allograft procedure in days after burn.

3.2. Viability assays

Two methods of MTT assay and Trypan Blue Exclusion were used to estimate whole tissue and isolated keratinocytes viability, respectively. The MTT assay on the whole tissue showed between 18 and 26% viability compared to freshly isolated skin (Fig. 5A). The viability was slightly lower in isolated keratinocytes, possibly due to further damage to the cells during enzymatic digestion and single cell suspension preparation (i.e. 4–12%, Fig. 5B). However, histological analysis showed that the structural integrity of the skin allografts was maintained during cryo preservation and thawing out process compared to the fresh skin (Fig. 6). The epidermal cell layers appeared normal with no sign of gross damage compared to fresh skin. Stratum corneum was not fragmented and dermal–epidermal junction had remained intact. Fibroblasts were still present in the dermis.

4. Discussion

Cryopreserved cadaveric allograft can be used to achieve a stable wound in the context of complicated burn wound management. Allografts are successful where other treatments such as Biobrane™ have failed due to wound contamination. This type of ''salvage'' application now represents the most common scenario in our practice, as the commercially available synthetic skin substitutes (Biobrane™ and Integra™) are now our method of choice for initial wound closure after acute burn wound excision in patients with extensive burns. We now no longer remove cadaver skin prior to definitive autografting: wound bed preparation with Versajet™ (Smith and Nephew) hydrodissection is aimed at removing residual allograft epidermis and
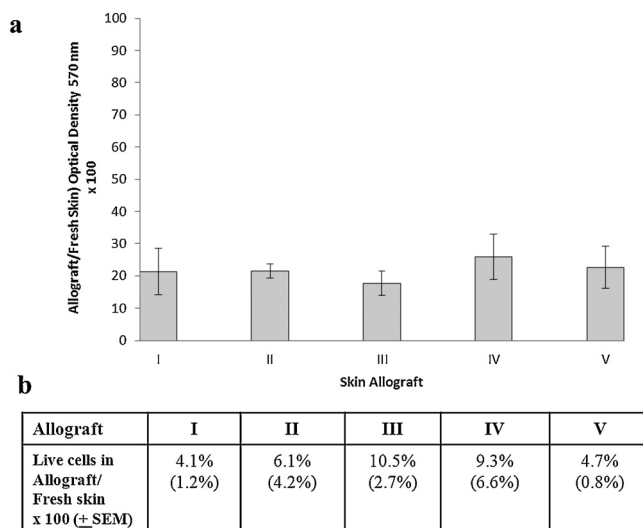
Fig. 5 – Allografts have low viability compared to fresh skin. (a) Biopsy punches of allografts (I–V) and fresh skin samples were incubated with MTT reagent for 2–3 h at 37 °C while shaking. The blue/purple precipitated salt in live cells was eluted using 2-methoxyethanol overnight and its absorbance measured at 570 nm with a reference at 690 nm wavelength. The chart represents three independent experiments with the error bars showing SEM. (b) A 2 cm × 5 cm of each cadaver skin (I–V) or fresh sample were digested with Dispase II (4 mg/mL) to separate the epidermis and the epithelial sheets were digested with 0.25% trypsin-EDTA for 5–10 min to retrieve epithelial cells. The viability of isolated epithelial cells was tested by Trypan Blue Exclusion according to standard protocols. This table shows the proportion (%) of viable cells isolated from allografts compared to the cells isolated from fresh skin.

any granulation tissue while preserving allograft dermis. Patients receiving allograft skin have more extensive burns, require more procedures and have a higher mortality rate than those not receiving allograft, which is to be expected given that as a result of a shortage of allograft, and also of the efficacy of Biobrane™ in the temporary closure of primarily excised burn wounds, use of allograft is now confined to patients with complications. Our mortality rate in this group of patients is however comparable with reports from other burn units [9].

Our findings of low viability of DTBV-processed allografts suggest that a high degree of cellular viability of the graft is not an essential factor in successful engraftment of the wound bed, and this is confirmed by other authors [3,9,16]. The DTBV-processed samples have a lower viability compared to other skin banks (50–60%) [3,17–19]; however, the viability of

cryopreserved allografts decreases with increasing age, and it is possible that DTBV-processed allografts from younger donors would have higher viability rates [20]. There are concerns regarding transmission of infectious diseases associated with the use of cutaneous allografts; however such concerns need to be weighed against the life threatening condition for which skin allografts are employed in the treatment of patients with severe burns. Using current donor tissue preparation protocols, no documented case of disease transmission has ever been reported in the literature. Indeed, a recent study attempting to demonstrate donor provenance of common wound swab cultures in allografted patients was unable to do so [20].

Cryopreservation of donor skin is labor intensive, and its long term storage and distribution are also costly processes compared with those for glycerol preserved grafts, which may
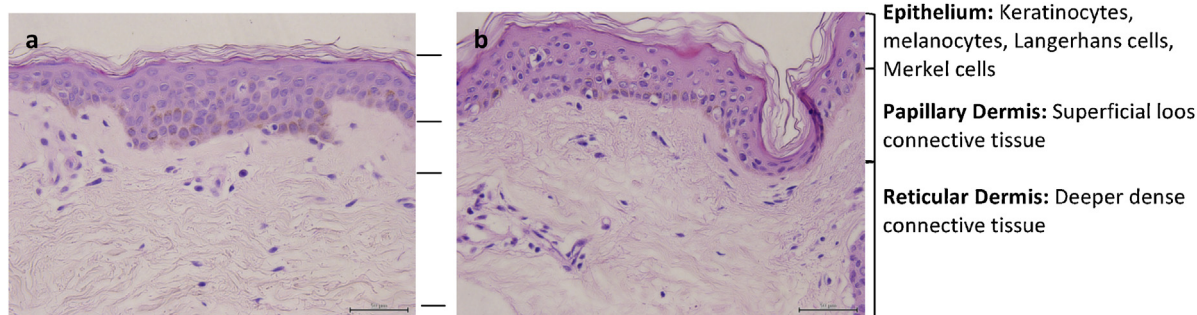

Fig. 6 – Allografts maintain normal skin architecture. All tissues were fixed, sectioned and stained with H&E. (a) Fresh skin (b) allograft sample III All other allografts showed similar structure (data not shown). Scale bar represents 50 μm.

also be associated with decreased disease transmission risk and reduced antigenicity. Kua et al. [9] suggest that improved outcomes using cryopreserved allograft compared with glycerol preserved may be clinically significant; however, conclusive evidence of relative clinical efficacy of the two products is lacking, and many skin banks, for example the Euro Skin Bank, use glycerol preservation exclusively [13]. Allograft skin is a vital component of the treatment of patients with life threatening complicated burn; however its limited availability in Australia restricts use. We are currently pursuing strategies to increase opportunities for skin donation within our own institution: including skin retrieval from organ donors and processes to improve the efficiency and timeliness of identification of potential donors. There is an urgent need for improved processes for donor identification and skin retrieval in Australia and as part of a significant development effort in this area, an opportunity exists to examine possible improved efficiencies in skin banking through the trialing of glycerol preservation techniques in the Australian setting. More studies comparing outcomes of cryopreserved vs. glycerol allografts is also recommended.

In conclusion, cryopreserved human cadaveric cutaneous allograft has a role in the management of extensive deep burns injuries. Our experience has shown that patients with limited autologous skin availability benefit from excision of full thickness burns and treatment with cadaveric allograft. This study provides further evidence that skin tissue viability is not absolutely required for its significance as a dermal substitute or temporary coverage in burns patients' outcome. Other preservation methods based on cost effectiveness and product safety should be investigated further. Effect of preservation method on clinical outcome rate requires further investigation.

Conflict of interest

We as authors do not have a conflict of interest.

Acknowledgments

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