Procurement, processing, storage and distribution of cutaneous tissue: the experience of the Emilia-Romagna Regional Skin Bank

Running head Skin banking activities

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SUMMARY

Aims

In this study we described the activities of the Emilia-Romagna Regional Skin Bank involved in the procurement, processing, storage and distribution of cutaneous tissues in the year 2015. In particular, we describe the validation tests required to ensure the suitability of different cutaneous tissues for clinical use. The distribution of cutaneous tissues is also reported, taking into account the different medical fields in which they were required.

Materials and Methods

In order to guarantee the suitability of cutaneous tissue for clinical distribution, microbiological as well as endotoxin tests, histological analysis and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) tests were performed in order to guarantee the sterility, structural integrity and viability of the tissues distributed by our Regional Skin Bank, respectively. The removal of cellular component was only evaluated by viability test on human dermis after the decellularization process. The distribution of cutaneous tissue in the year 2015 and its clinical use was also evaluated using quantitative analysis.

Results

Cutaneous tissue distributed from the Emilia-Romagna Regional Skin Bank is used for several clinical applications in different fields of Medicine. Our results show that, thanks to thorough testing, the Emilia-Romagna Regional Skin Bank is able to ensure the distribution of sterile

cutaneous tissue and to maintain the structural integrity of tissues, as well as the viability of cellular components. The removal of the cellular component, required for the clinical distribution of acellular human dermis, was verified in all samples.

Conclusions

Thanks to their rigorous protocols, the Emilia-Romagna Regional Skin Bank is able to ensure the distribution of safe cutaneous tissue suitable for various clinical applications.

Keywords: acellular dermis, cutaneous tissue, skin bank, tissue bank

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide); CNT (National Transplant Center); ISS (National Health Institute); OT (multi-organ donors); MT (multi-tissue donors); GMP (Good Manufacturing Practice); DMSO (Dimethyl sulphoxide); DED De-epidermized dermis.

INTRODUCTION

Continuous scientific innovation in the field of Medicine has led in recent decades to the development of tissue banks dedicated to the harvesting, processing, storage and distribution of tissues for different clinical applications. Indeed, since the 1960s, several authors have described the effectiveness of homologous skin in the treatment of wounds such as burns or ulcers1-3. As a consequence, several tissue banks have been set up in Europe since the 1980s4.

Today, homologous cutaneous tissue is widely distributed by skin banks for various clinical applications. In order to obviate the risk of infections, donors are carefully selected, and the suitability of tissue has to be assessed. Then, the tissue is transported to the skin bank for processing and storage. According to current regulations5, different techniques can be used by skin banks for tissue preservation; glycerolization is frequently used as the method of choice for skin storage when tissue viability is not required6-8, while cryopreservation is also widely used in order to maintain cell viability in cutaneous tissue9-11.

In Italy, tissue banks began to be established in the mid-nineties, and there are currently five Italian skin banks accredited by the National Transplant Center (CNT) and the National Health Institute (ISS). In particular, the Emilia-Romagna Regional Skin Bank, part of the Burns Center at "M. Bufalini" Hospital - AUSL Romagna, Cesena, is a laboratory accredited for the processing, conservation, storage and distribution of cutaneous tissue derived from multi-organ (OT) and/or multi-tissue (MT) donors. It serves many patients distributed across the nation, and carries out activities in the field of Regenerative Medicine, having established a section dedicated to Clinical Research. It relies on the clean-room environments of the Cell Factory, a leading biotechnology platform built according to European guidelines of Good Manufacturing Practice (GMP), which is able to accommodate the processing, preservation, validation and distribution of cutaneous tissue for transplantation and tissue/cell bioengineering.

In this study, we describe the procurement, processing, storage and distribution of cutaneous tissue performed by the Emilia-Romagna Regional Skin Bank in the year 2015, with particular focus on the validation tests required to ensure the suitability of cutaneous tissue for distribution and clinical applications in different medical fields.

MATERIALS AND METHODS

Procurement of cutaneous tissue

Cutaneous tissue is taken from multi-organ and/or multi-tissue donors under sterile conditions, using a certified electric dermatome and following national rules governing the harvesting, processing, storage and distribution of tissues for transplantation5. Cutaneous tissue is then rinsed in 0.9% NaCl and stored in this solution for transport (at 2-4 °C) to the Emilia-Romagna Regional Skin Bank (< 12 h), where it is aseptically processed under laminar flow hood in GMP-class laboratories. Briefly, cutaneous tissue is dipped for at least 20-30 minutes in RPMI medium containing antibiotics (penicillin, streptomycin and amphotericin B) and a cryo-protector, in order

to maintain cell viability during cryo-freezing. Alternatively, human dermis from the trunk area of multi-organ and/or multi-tissue donors is submitted to the decellularization method patented at the Emilia-Romagna Regional Skin Bank in collaboration with the Rizzoli Orthopaedic Institute, in order to obtain a tissue engineering product. After decellularization, the human derived dermal matrix is dipped in RPMI medium containing antibiotics but no cryoprotector. Cutaneous tissue is then packaged and sealed inside sterile bags within the Cell Factory, and then subjected to cryo-freezing and storage in tanks of liquid nitrogen (180-190°C).

MTT test

Six small pieces of cutaneous tissue are collected using a biopsy punch. Two of them are soaked in liquid nitrogen for 10 minutes in order to eliminate all viable cells, and used as negative controls. Then, cell viability is tested via the MTT assay for cutaneous tissue. Briefly, samples are weighed and incubated in MTT solution (0.5mg/ml; Roche Diagnostic GmbH, Penzberg, Germany) for 3 h at 37 °C in 5% CO2/air. Then, each sample is placed in dimethyl sulphoxide (DMSO) for 10 minutes. The solution is read on a spectrophotometer at 570 nm, and DMSO is assayed as the background. For each sample, the viability rate is calculated as the ratio between the optical density (OD) at 570 nm and the weight (gr). The viability index Iv+ is defined as the mean of the viability rates of samples, and is compared to the viability index Iv- of two negative controls.

Microbiological analysis and LaL test

Microbiological and endotoxin analyses are performed to ensure the sterility of cutaneous tissue for its clinical distribution. After the arrival at the skin bank, cutaneous tissue is immediately incubated on plates selective for the growth of bacteria or fungi (COS Columbia agar + 5% sheep blood, BioMerieux and Sabouraud dextrose agar + CAF, Biolife) for 3 and 14 days, respectively, at 37°C. In order to assess the sterility of cutaneous tissue at all steps performed at the Emilia-Romagna Regional Skin Bank, microbiological testing is also performed on cutaneous tissue after further processing and cryo-freezing. Only sterility testing after processing, decellularization and cryo-freezing is performed on decellularized dermis. For the detection and quantitation of Gram-negative bacterial endotoxins, Lal testing is performed on cutaneous tissue before its clinical distribution. In brief, 200µl of medium in which thawed cutaneous tissue is dipped is mixed thoroughly with Limulus amebocyte lysate (Associates of CAPE COD incorporated, Falmouth, MA, USA) and placed in a dry block incubator at 37°C for 1h. After incubation, the presence of endotoxins in the tissue is evaluated by gelation, inverting the vial containing the solution in one smooth motion.

Histological analysis

Histological analysis is performed on cutaneous tissues in order to ensure the structural integrity of the cutaneous tissue for its clinical distribution. Briefly, for histological analysis, samples are fixed with 10% formalin solution and paraffin embedded. After processing, histological sections (5 µm thick) are stained with haematoxilin and eosin (H&E). The continued structural integrity of cutaneous tissue is evaluated according to the numerical values shown in Table 1.

Clinical distribution

A quantitative analysis of cutaneous tissue distributed from Emilia-Romagna Regional Skin Bank in the year 2015 is here described. An evaluation of the different fields of application was also taken into account.

RESULTS

The Emilia-Romagna Regional Skin Bank is certified for the clinical distribution of three different bio-products of cutaneous tissue: homologous skin, de-epidermized dermis and decellularized dermis. Homologous skin is composed of human epidermal layers and a thin underlying dermal layer. It is considered life-saving in extensive burns, and a valid therapeutic option for chronic ulcers; vascular, traumatic and non-healing wounds; full-thickness loss of substance; and osteo-articular injuries of the tendon. It decreases the pain threshold and bacterial contamination in addition to promoting the formation of granulation tissue and re-epithelialization of the injured area. De-epidermized dermis (DED) consists of human dermis alone, without the overlying epidermal layers. DED is widely used as a skin substitute and in composite skin grafting techniques. The use of cryo-preserved DED is particularly effective in the treatment of relapsing chronic ulcers and traumatic ulcers, but also in vascular and diabetic ulcers of different aetiologies, and in those with full-thickness loss of substance that do not respond to standard medical treatment. Decellularized dermis is a tissue engineering product consisting of human dermis subjected to a decellularization process patented at the Emilia-Romagna Regional Skin Bank in collaboration with the Rizzoli Orthopaedic Institute; this is able to remove the cellular component while maintaining the structural integrity of the collagen and fibrous matrix of dermal tissue. This provides a cell-free scaffold widely used in different fields of Regenerative Medicine, being suitable as a permanent dressing that prevents rejection by the receiving patients.

Cutaneous tissue is harvested by dedicated personnel and transported to the Emilia-Romagna Regional Skin Bank, where specialized personnel receive it and assign it a unique identification code. After acceptance, cutaneous tissue is processed within the Cell Factory. Here, the following activities are carried out: 1) quantitative analysis of the collected cutaneous tissue, assessing its suitability in compliance with the standards of tissue harvesting defined by the skin bank; 2) decontamination treatment in order to eliminate any previous contamination of cutaneous tissue, and 3) the packaging of cutaneous tissue inside sterile bags.

The human dermis processed within the Cell Factory is also subjected to a decellularization process before packaging in order to obtain a tissue-engineering product usable for clinical purposes. All cutaneous tissue packaged and sealed inside sterile bags within the Cell Factory is then subjected to cryo-freezing by means of a programmed, gradual drop in temperature, and is then stored in tanks of liquid nitrogen. Subsequently, validation tests are performed in order to ensure the suitability of tissue for clinical distribution. The results here described were obtained from validation tests performed on cutaneous tissue in the year 2015 in order to guarantee the distribution of safe tissues, suitable for clinical use. In particular, a total of 71 cutaneous tissue donors were identified that year. Among them, 5 donors showed contamination over the accepted values, and their cutaneous tissue was disposed of in line with current regulations5. Hence, the results here described are based on validation tests performed on 66 donors of cutaneous tissue. In particular, validation was performed on both homologous skin collected from 66 explants and on DED or decellularized dermis, collected respectively from 42 and 44 explants.

Sterility of cutaneous tissue

The results of our microbiological analysis, performed on homologous skin, DED and decellularized dermis, show that the majority of cutaneous tissue collected and transported at the Emilia-Romagna Regional Skin Bank is already sterile before processing (Fig. 1 A-B-C, left panel). However, a small percentage of tissue, corresponding to 6%, 12% and 15%, respectively, of homologous skin, DED and dermis before decellularization, showed contamination by Staphylococcus epidermidis (=102); no fungal contamination was identified in any cutaneous tissue tested (Fig. 1 A-B-C, left panel).

After processing in clean-room environments in GMP-class laboratories, all bio-products were found to be sterile (Fig. 1 A-B-C, central panel). In addition, the decellularization method applied to the human dermis was found able to maintain the sterility of processed human dermis. Sterility

testing was also performed on processed and thawed cutaneous tissue previously cryo-frozen and stored in liquid nitrogen, as described above. Also in this case, we determined that sterility had been maintained in all cutaneous tissue analysed (Fig. 1 A-B-C, right panel). Regarding endotoxin analysis, this was performed only before the clinical distribution of cutaneous tissue. In this case too, all samples were found to be sterile (data not shown).

Maintenance of structural integrity of cutaneous tissue

Our results, deriving from the histological analysis performed on homologous skin, DED and decellularized dermis show that good levels of structural integrity were maintained in the three bio-products of cutaneous tissue processed and cryo-frozen at Emilia-Romagna Regional Skin Bank. In fact, qualitative analysis performed according to parameters described in Table 1 shows that the average values of structural integrity obtained for all cutaneous tissue analysed in the year 2015 were between 6 and 7, corresponding, respectively, to good and very good structural integrity (Fig. 2A). As shown in Figure 2B, the histological characteristics of each bio-product analysed are well maintained. In particular, the presence of cellular and vascular inclusions is evident in homologous skin and DED, while they are removed from the dermis by the process of decellularization, without compromising the structural integrity of collagen and the fibrous matrix (Fig. 2B).

Maintenance/removal of cell viability in cutaneous tissue

The results of MTT testing performed on homologous skin and DED show that cell viability is significantly maintained in the former after its processing and cryo-freezing (Fig. 3A). On the other hand, a drastic reduction of viable cellular component was identified in processed human dermis after decellularization, as required for its clinical use as a permanent dressing, in order to avoid rejection by the recipient patients (Fig. 3B). Only histological analysis on the biological products of DED (fresh and processed/thawed DED) is routinely performed in order to evaluate cell viability.

Clinical distribution of cutaneous tissue

After the validation of the cutaneous tissue processed and stored at the Emilia-Romagna Regional Skin Bank, it is ready for distribution, both regionally and nationally, to public and private structures operating in the healthcare sector. Distribution outside the Emilia-Romagna region is possible only if the structure does not have a regional skin bank of its own, or if the skin bank of reference does not have the amount of tissue required to meet the clinical demands at the time of the request, and therefore authorizes tissue distribution from another skin bank.

In the year 2015, the distribution of cutaneous tissue from the Emilia-Romagna Regional Skin Bank was used to treat different clinical conditions in several fields of Medicine. Homologous skin was mainly used for the temporary coverage of burns, as expected. In addition, it was also used in vascular surgery, dermatological applications, and the treatment of diabetic foot, taking advantage of its ability to act as a temporary coverage for different damaged areas (Fig. 4A). Regarding DED, this was frequently used in the field of vascular surgery, mainly in the treatment of ulcers of different aetiologies, but also found clinical applications in the field of dermatology (Fig. 4B). Finally, the DED produced and distributed by the Emilia-Romagna Regional Skin Bank was used as a permanent dressing in several clinical fields. In particular, our cell-free human dermal matrix was widely used in the field of breast surgery, for post-mastectomy surgical reconstruction, and in orthopaedics, for the repair of damaged tissue, including tendons, the rotator cuff and cartilage. It was also applied in emergency surgery for the reconstruction of abdominal wall, as well as in other fields, as reported in Figure 4C.

DISCUSSION

The activities of procurement, processing, storage and distribution of cutaneous tissue performed by the Emilia-Romagna Regional Skin Bank, accredited by the National Transplant Center and the

National Health Institute are aimed at the final clinical distribution of cutaneous tissue for different applications in several fields of Medicine.

In this article, we focus on both the validation process that our skin bank uses to ensure the suitability of cutaneous tissue for clinical use, and the clinical areas in which it is currently applied. We took into account the year 2015, which provided the most recent data analysed in terms of validation tests and distribution. As demonstrated by validation tests, the Emilia-Romagna Regional Skin Bank is able to distribute sterile cutaneous tissue. In particular, it is able to maintain the sterility of cutaneous tissue during its processing under sterile conditions, as well as the ability to remove tissue contaminants. Indeed, current regulation requires that highly contaminated tissue not be sent for clinical use and be disposed of.

Moreover, the processing and cryo-freezing process used by the Emilia-Romagna Regional Skin Bank is able to ensure good maintenance of the structural integrity of cutaneous tissue necessary for its clinical application. In particular, it is noteworthy that human dermis maintains its structural integrity after the application of the decellularization method, required to remove its cellular component, and thereby provides a cell-free scaffold suitable as a permanent dressing for several fields of Medicine.

Finally, MTT testing shows that the processing and cryo-freezing of homologous skin are both able to maintain its cellular component viability. In particular, the percentage of cell viability maintained after cryopreservation seems not to be affected by donors' age. In fact, it did not tend to decrease progressively with the increasing age of the donors, as previously reported11. However, the restriction of our analysis to 71 donors in only one year (2015) must be taken into account. The decellularization method applied to human dermis also appears to be effective, removing its cellular component, as required for its clinical distribution.

In the year 2015, we distributed our three biological products for use in several fields of Medicine. In addition to burns-the main clinical condition in which homologous skin is applied, it was also used in Vascular Surgery, Dermatology and General Surgery, as well as for the treatment of skin ulcers and diabetic foot ulcers, in which it is used as a temporary dressing for covering the damaged area.

The clinical distribution of DED is growing, since it finds application in several clinical fields. In fact, as an acellular biological scaffold, DED could be potentially applied in all fields of clinical Medicine. Currently, several surgeons have tested the decellularized human dermis produced at the Emilia-Romagna Regional Skin Bank with satisfactory results and, as a consequence, its distribution increased in the year 2016.

CONCLUSIONS

The validation tests currently performed at the Emilia-Romagna Regional Skin Bank are able to ensure distribution of safe and suitable cutaneous tissue that retains all the biological characteristics required for its clinical application. In fact, the clinical distribution here described is clear evidence that the cutaneous tissue provided by our skin bank is effective in the treatment of several clinical conditions.

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FIGURE LEGENDS

Fig. 1 Microbiological analysis of homologous skin, de-epidermized dermis and decellularized dermis (A) Evaluation of homologous skin sterility immediately (left panel), after processing (central panel), and after further processing and cryo-freezing (right panel). (B) Evaluation of de-epidermized dermis sterility immediately (left panel), after processing (central panel), and after further processing and cryo-freezing (right panel). (C) Evaluation of decellularized dermis sterility immediately (left panel), after processing (central panel), and after further processing and cryo-freezing (right panel).

Fig. 2 Structural integrity of cutaneous tissue. (A) Qualitative evaluation of structural integrity of homologous skin, de-epidermized dermis and decellularized dermis, according to the numerical values in Table 1. (B) Morphological analysis of homologous skin, de-epidermized dermis and decellularized dermis.

Fig. 3 Maintenance/removal of cell viability in cutaneous tissue. (A) Quantitative analysis of cell viability in homologous skin immediately and after its processing and cryo-freezing. (B) Quantitative analysis of cell viability in dermis before and after processing, decellularization and cryo-freezing.

Fig. 4 Clinical distribution of cutaneous tissue. Quantitative analysis of transplants of homologous skin (A) de-epidermized dermis (B) and decellularized dermis (C) in different fields of clinical Medicine.

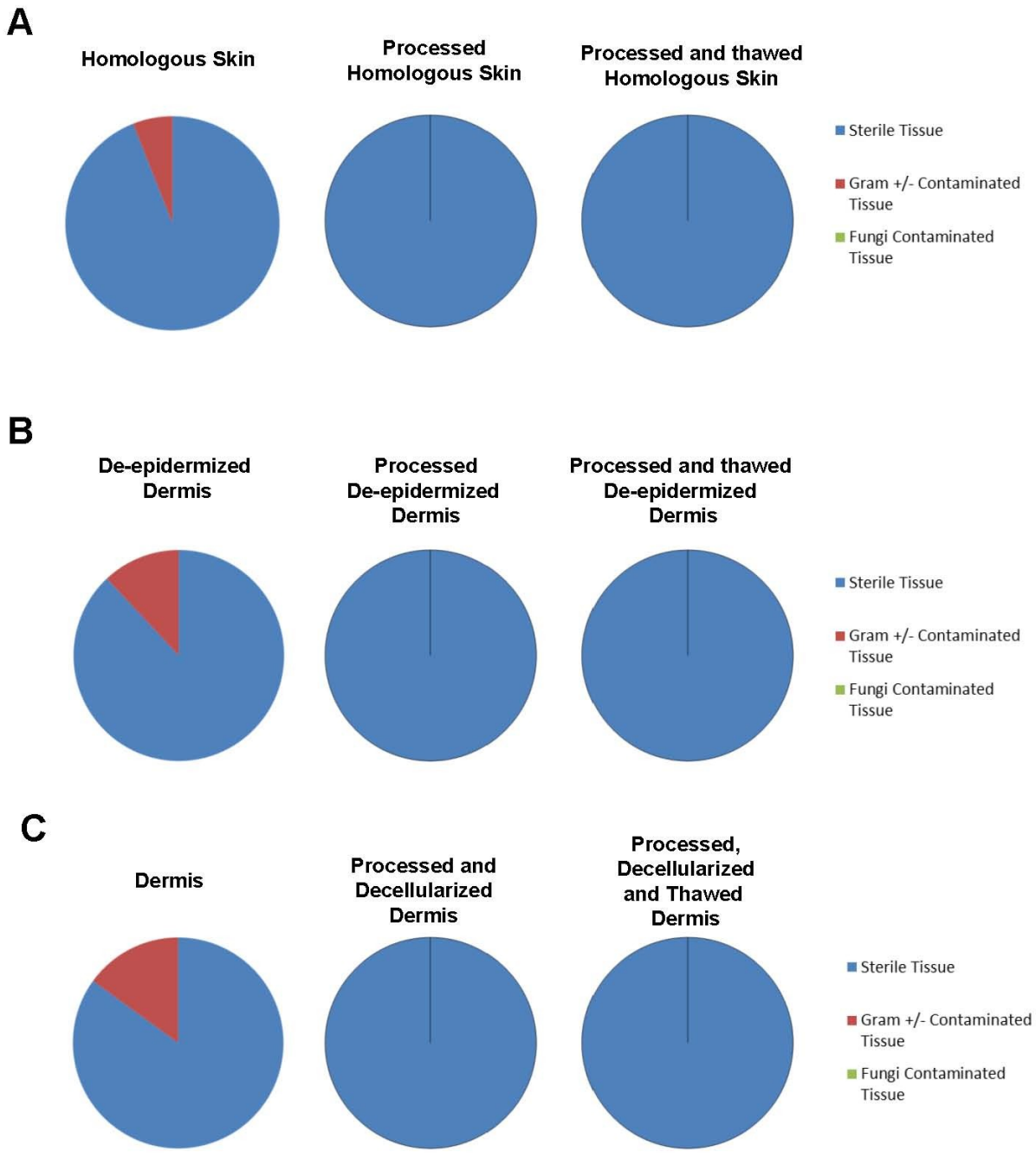
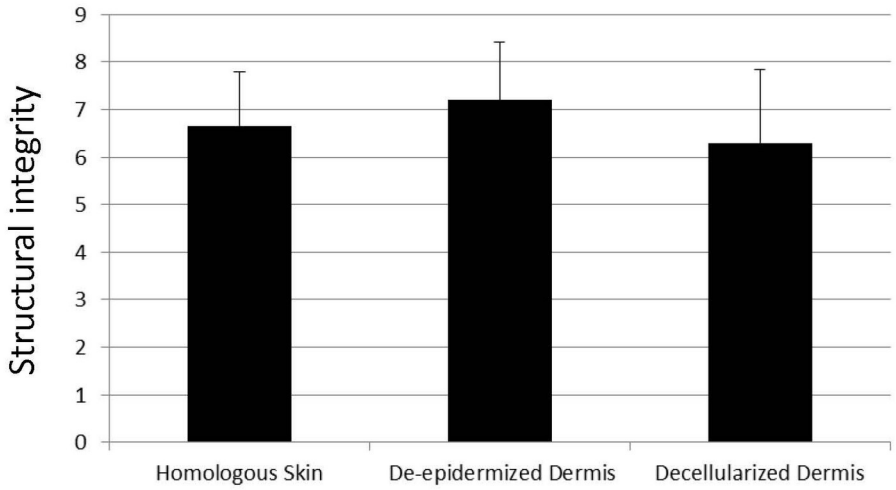


Fig 1

A


Structural integrity
9
8
7
6
5
4
3
2
1
0
Homologous Skin
De-epidermized Dermis
Decellularized Dermis

B

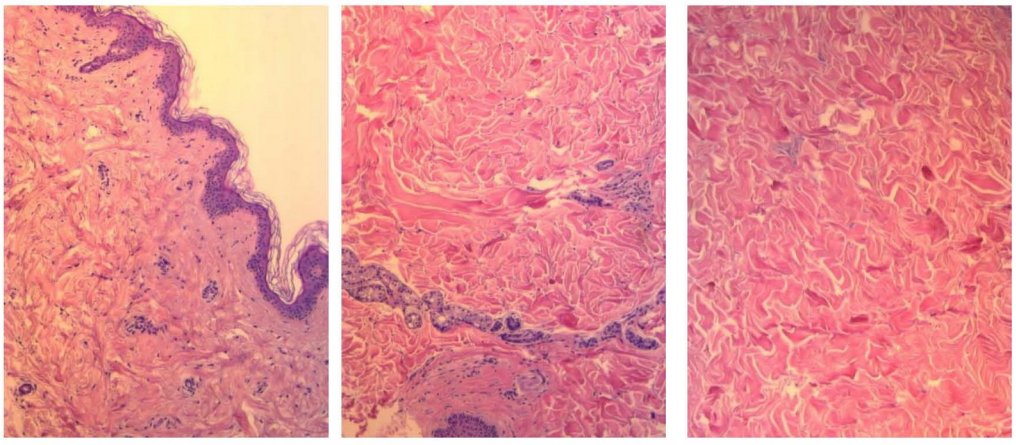
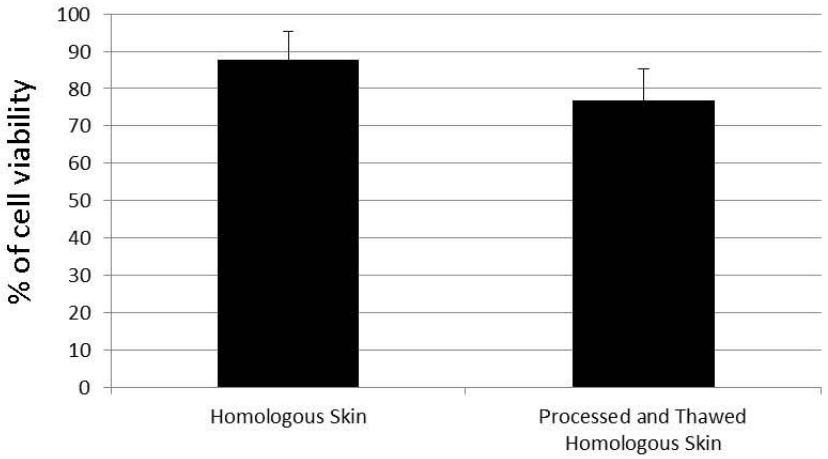

Homologous Skin
De-epidermized Dermis
Decellularized Dermis

Fig 2

A


% of cell viability
100
90
80
70
60
50
40
30
20
10
0
Homologous Skin
Processed and Thawed Homologous Skin

B

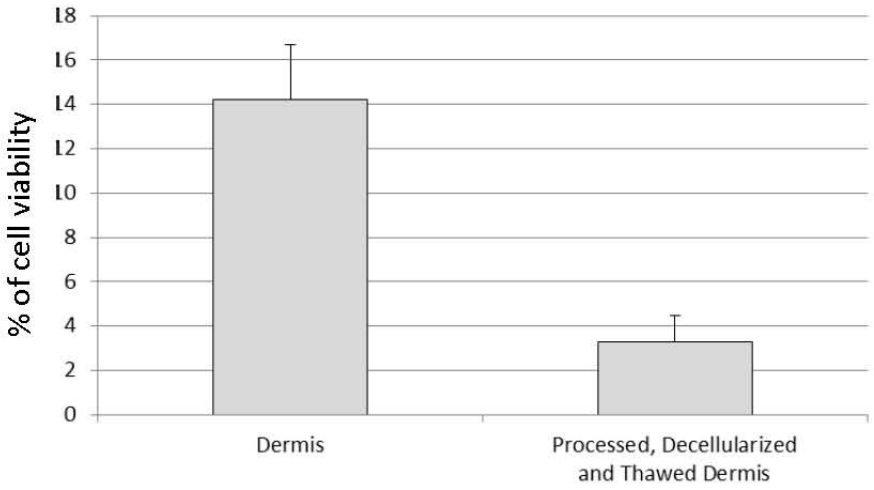

% of cell viability
18
16
14
12
10
8
6
4
2
0
Dermis
Processed, Decellularized and Thawed Dermis

Fig 3

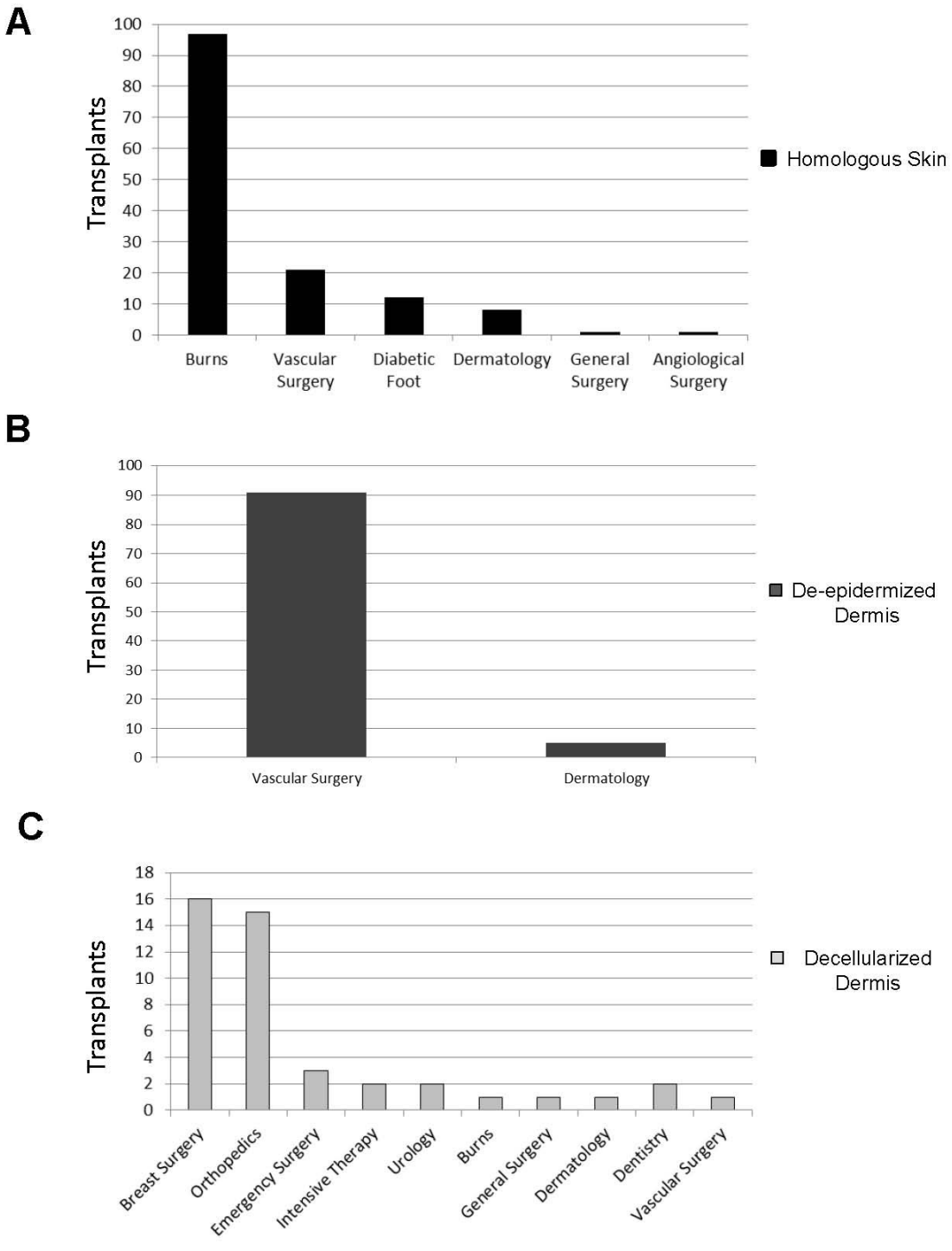

A
Transplants
100
90
80
70
60
50
40
30
20
10
0
Burns
Vascular Surgery
Diabetic Foot
Dermatology
General Surgery
Angiological Surgery
Homologous Skin
B
Transplants
100
90
80
70
60
50
40
30
20
10
0
Vascular Surgery
Dermatology
De-epidermized Dermis
C
Transplants
18
16
14
12
10
8
6
4
2
0
Breast Surgery
Orthopedics
Emergency Surgery
Intensive Therapy
Urology
Burns
General Surgery
Dermatology
Dentistry
Vascular Surgery
Decellularized Dermis

Fig 4

Table 1 Numeric values corresponding to structural integrity of cutaneous tissue

Values	Structural integrity of the tissue
1	Insufficient
2	Sufficient
3	More than sufficient
4	Adequate
5	More than adequate
6	Good
7	More than good
8	Excellent

Table 1