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Research Article

Skin Grafts: Local quest for viable alternatives to autologous grafts using silk and acellular dermal matrices

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Abstract. The gold standard with regards to skin transplantation is the use of the patient's own skin obtained from a healthy donor site. Such grafts can be either full thickness skin or more commonly nowadays, split thickness skin. Various materials, having either natural and or synthetic origins, have been used in the engineering of skin substitutes to-date and these grafts are then confronted against autologous skin grafts. If proven to be successful, such matrices could be utilised in clinical applications such as in the treatment of burn wounds and in cases of skin ulcers amongst others.

In this study the primary cells used, keratinocytes and fibroblast, were obtained from donor skin and cultured. Scaffolds of xenogenic (raw silk) as well as of allogenic (acellular dermal matrices) origins were obtained via low-cost methods and seeded using the fibroblasts and keratinocytes so as to determine which gave the closest mimic to skin grafts.

Out of the matrices assessed, the raw silk matrix allowed the best colonisation with skin cells in our hands. The ADM matrice also showed some cell colonisation, but will need further experimentation.

Keywords Skin graft, silk and acellular dermal matrix (ADM).

1 Introduction

The skin is the largest single organ of the human body. It is composed of the epidermis, which is an epithelial layer of ectodermal origin, and the dermis, a layer of connective tissue, originated from the mesoderm. The epidermis consists of a stratified squamous keratinized epithelium, as well as less abundant cells types namely; melanocytes, Langerhans cells, and Merkel's cells. The keratinising epidermal cells are called keratinocytes. Amongst many of its complex roles, one of its major functions is to protect the body against environmental influences. Acute or chronic loss of this barrier necessitates the mechanisms of tissue repair for any organism to survive. The most important step of such a mechanism in the re-epithelialization of the wounded surface is the primary destination of skin wound healing (Slavin, 1996).

It was Revendin who introduced skin grafts for the first time in 1871. Transplantation of the patient's own skin from a healthy donor site, either as a full thickness or as a split thickness skin graft, is commonly practised nowadays and is regarded as the surgical "gold standard" to cover skin wounds. This is the reason every tissue engineered skin substitute is always confronted against the performance of autologous skin grafts.

Knowing that extensive wounds necessitate a barrier protection to prevent both infection and desiccation, as well as the need of cell guidance by dermal elements to maximize healing, has led to the evolution of both biologic and synthetic dressings and skin substitutes (Horch et al., 2001). In cases of substantial burns, the great extent of wound surfaces and considerable loss of skin necessitated the invention of various types of temporary or permanent skin substitutes. The clinical utility of cultured skin substitutes for wound closure has reduced the amount of donor skin required by at least 10 times when compared with conventional skin grafts, and has also reduced the number of surgeries required to harvest donor skin, while at the same time decreasing the time of recovery of those patients with severe burn injuries.

Theoretically, skin substitutes can be permanent or

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temporary; epidermal, dermal or composite; and biologic or alloplastic (synthetic). Biologic components are further subdivided into autogenous, allogenic, or xenogenic. Logically, research efforts of different groups are centred on several possible combinations of these traits, whereas practically, most designs rely on a permanent or temporary engraftment of the material. Researchers in this field have defined the common techniques according to their principal biological action in the patient (Horch et al., 2005):

- Temporary – in this case the material is designed to be placed on a fresh wound (partial thickness) and left until healed.
- Semi-permanent – here the material remains attached to the excised wound, and eventually replaced by autogeneous skin grafts.
- Permanent – in this type there is an incorporation of an epidermal analogue, dermal analogue, or both as a permanent replacement.

The increasing emphasis on rehabilitation and "quality of skin cover" has further valued this field. A skin substitute which has the properties of a dermis is the marker for gauging a permanent substitute. Allogenic or alloplastic skin substitute coverage as a temporary solution is necessary until definitive cover can be achieved (Gallico, 1990). Allogenic skin grafts may be completely integrated into the healing wound initially and bridge the critical time gap in the early phase of burn treatment, but over time they will irrevocably undergo immunogenic rejection (Zhao, 1992). In theory, the application of in vitro cultivated autologous skin substitutes is able to overcome this specific deficit of modern burn treatment and reconstructive surgery.

Numerous studies have been conducted to date with the aim of solving important hurdles in skin transplantation. Such a problem is posed by the need for large amounts of skin in cases of burns and chronic skin ulcers, with scarce donor sites. In certain trials, cells were cultured in vitro, where the use of both autologous (Terskikh and Vasiliev, 1998) and non-autologous (Bolivar-Flores and Kuri-Harcuch, 1999) cells were assayed. Also, the use of dermal substitutes or their equivalents containing bio-degradable bovine collagen, in association with autologous cells cultured in vitro, has been studied by Jansson and colleagues in 2001.

The matrices looked at in this study, were composed of the following;

1.1 Protein Matrices

In general, around 60 percent of the polypeptide chain exists as two regular secondary structures, namely a helices and the β-sheets. The remainder of the molecule is in random turns and coils. Thus these helices and sheets are the major internal supportive elements of the protein itself.

Proteins having structural roles in cells, and thus having to span over a large distance, generally have an elongated three dimensional structure and are commonly referred to as fibrous proteins.

1.1.1 Silk

Silk derived from the silkworm, Bombyx mori, has been utilised as a biomedical material for applications such as sutures for many decades. According to (Altman et al., 2003), silk has distinctive mechanical characteristics that may be utilised in the field of clinical repair options with many possible applications. Although bioincompatibility of these fibres has been the major draw back in their utilisation; it has been demonstrated that this was most likely due to some residual proteins which may have contaminated the silk, not to the nature of the fibres themselves.

Many structural proteins are composed of multiple layers of pleated sheets that provide toughness. Silk fibres consist mostly of stacks of antiparallel β-sheets. The fibres are flexible because these β-sheets can slip over one another. Also, their resistance to breakage is derived from the fact that the silk's peptide backbone is aligned with the fibre's axis. In fact, core silk fibroin has been shown to exhibit biocompatibility both in vivo and in vitro comparable with that of other widely used biomaterials such as collagen and polylactic acid (Altman et al., 2003). Also, this study underlines the fact that silk fibres posses numerous different side chains to which growth factors, as well as adhesion factors, could be added. Another advantage of silk fibres is that these may be genetically tailored, making silk even more viable for biomedical utilisation which is in fact what is presently ongoing.

The use of spider silk has also been intensively assessed by (Yager, 1997), amongst many others, in order to determine its structure and physical as well as chemical properties so that such silk could also be used as an application in the future as a matrix for tissue engineering.

Genetic engineering techniques have been utilised in the synthesis of recombinant spider silk fibroin-mimetic polymers that have been proven to possess excellent mechanical properties (Guerette et al., 1996), as well as an improved cell adhesion capacity, as shown by (Bini et al., 2006).

Applications of silk include being scaffolds for tissue engineering, especially after the recent results obtained in trying to produce both ligament and bone formation in vitro. (Altman et al., 2003) thus conclude that such studies support the great potential of silk as a biomaterial for future clinical applications. Other studies have underlined the fact that silk is very versatile with respect to biocompatabilty, biodegradation, controllable

degradation rates (Rice et al., 2005; Urist, 1965) and the potential of being turned into a number of different formats including gels, porous structures and fibres (Preda et al., 2013).

1.2 Acellular Dermal Matrix (ADM)

There have been many attempts to produce a dermal substitute. The most successful of these substitutes seem to be ones derived from either full or split thickness skin treated to remove all epidermal and dermal cellular components and appendages such as keratinocytes, fibroblasts, hair, sweat glands and smooth muscle (Walter et al., 1998).

This substitute should exhibit three very important properties namely; low antigenicity, the ability to vascularise rapidly and to be stable as a dermal template.

2 Methods used

2.1 Isolation of Fibroblasts and Keratinocytes

The skin (full thickness skin) was collected through informed consent of the patients during surgeries such as circumcisions, tummy tucks and facelifts. The skin was then cleaned and collected into 50ml tubes that had been filled prior to this with RPMI 1640 (10% FBS, 100µg/ml of streptomycin, 100U/ml of penicillin, 2.5µg/ml amphoteracin B) and stored at 4°C. These samples were processed within 36 hours under clean conditions. The method used was adopted from an established protocol by (Jones et al., 1996) and separation of epidermis and dermis was achieved overnight by dispase digestion at 4°C. The epidermis was cultured in Keratinocyte Growth Medium (KGM) (100µg/ml of streptomycin, 100U/ml of penicillin, 2.5µg/ml amphoteracin B) to obtain keratinocytes. The dermis was cultured in DMEM F12 (10%FBS, 100µg/ml of streptomycin, 100U/ml of penicillin) to obtain fibroblasts.

2.2 Silk

The silk matrices were made from layers of raw silk peeled off the cocoon of the mulberry moth Bombyx mori (with the aid of a scalpel blade no.24). These layers were cut into small pieces (5mm by 5mm), then wrapped in foil and autoclaved.

2.3 Acellular Dermal Matrices

First the epidermal layer of formalin-fixed cadaveric skin was scraped off using a scalpel. A modified version of the method described by (Walter et al., 1998) was then followed. Briefly, the skin was incubated overnight in 1M NaCl at 37°C with continuous agitation. Next the skin was placed in 0.5% sodium dodeycl sulphate (SDS) solution for 1 hour at room temperature, again with continuous shaking. Both solutions used contained 0.02% sodium azide so as to prevent microbiological growth. The acellular dermal matrices were then extensively washed using sterile PBS and were then transferred using sterile forceps into cryovials. Then, 1ml of sterile PBS was added and the sample was frozen at −20°C.

2.4 Seeding of the Matrices

The matrices were seeded, under sterile conditions, using a fibroblast suspension of around 1.5×10^6 cells/ml. Two weeks following the seeding of the silk matrix with fibroblast, the matrices were seeded with keratinocytes. In the case of the ADM, seeding with keratinocytes was undergone after four weeks. At this stage, a suspension of keratinocytes at a concentration of 1x106 cells/ml was used. The matrices were kept under microscopic observation for the following weeks.

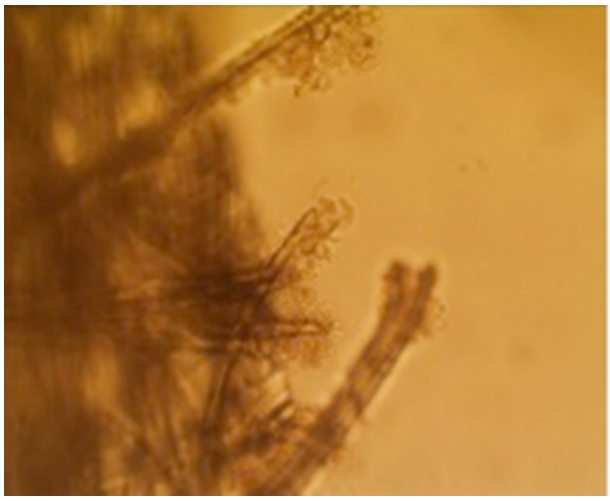

Figure 1: Cells attached all round silk fibres. (Magnification ×200).

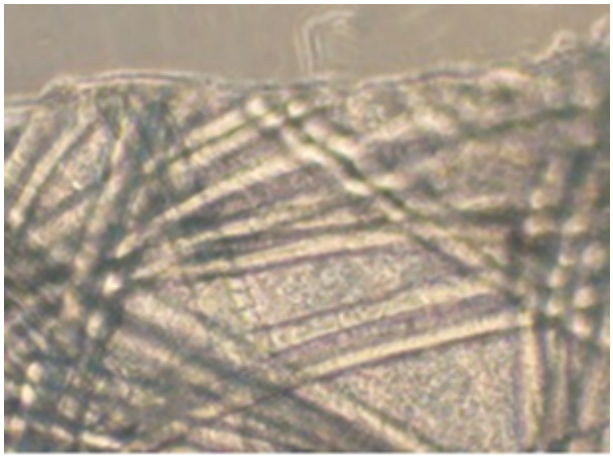

Figure 2: Spaces between silk fibres are bridged with cells. (Magnification ×400).

3 Results

3.1 Silk Matrix

Cells were seen to attach very rapidly into the silk fibres (Figure 1). At week six, that is, 30 days after the seeding of keratinocytes, cells were seen to spread out onto the silk fibres. Extended bridging between a relatively wide area of silk fibre was also observed (Figure 2).

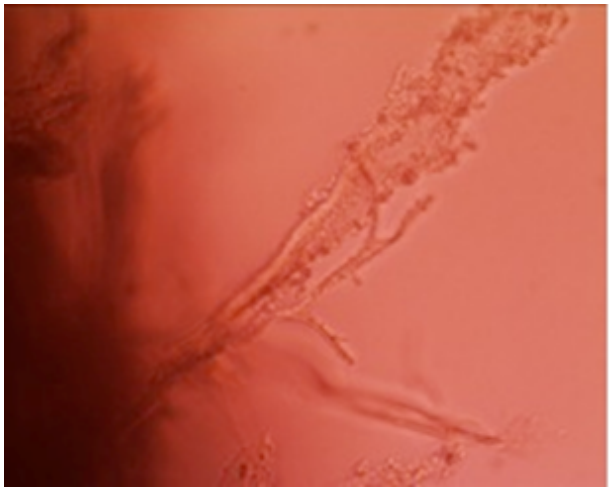

Figure 3: Cells attached onto the ADM's collagen strands. (Magnification $\times 400$).

3.2 Acellular Dermal Matrix

Cells were seen to grow compactly and uniformly onto collagen strands that spread out from the matrix's margins (Figure 3).

Next, the ADMs were stained with a Haematoxylin and Eosin (H&E) stain so as to investigate to what extent the fibroblasts and keratinocytes seeded attached onto the outer layer of the ADMs and whether the cells migrated deep into the inner layers of this collagen matrix.

4 Discussion

4.1 The Silk Matrix

The cells attached to the silk fibres relatively quickly when compared to the acellular dermal matrix. Also, over time the clusters of cells were seen to grow along the silk fibrils with frequent bridging between two or more of these structures. The cells were in fact seen to bridge even between not-so-close fibrils, over a period of four weeks, cells were seen to bridge over a relatively large span between multiple silk fibres, which was not seen in hair matrix cultures. Thus, silk seems to be relatively better in acting as a matrix onto which large films of cells form.

4.2 Formalin fixed Acellular Dermal Matrix

Unlike in the case of (Walter et al., 1998), who used fresh skin (stored at $-20°$C and thawed before the decellularisation process), formalin-fixed skin was used in this study since this was freely available in the department. Another study conducted by (Gibbs et al., 2006) prepared ADMs from glycerol-preserved door skin, thus formalin preserved ADMs might have been viable to achieve.

Our experience showed that both the decellularisation process, as well as the successive cell-seeding, were markedly less efficient with formalin-fixed skin and that this resource may need some treatment to reverse the effects of formalin (similar to the microwave antigen retrieval process for immunocytochemistry) to be a suitable source. Non-fixed skin (possibly derived from cosmetic procedures) is obviously a resource which should be investigated further.

With regard to cost effectiveness, although the acellular dermal matrices were cost-free to obtain, the reagents needed to process the matrices were both costly as well as relatively hazardous. While in the case of the silk, the cocoons were obtained at a very low cost and processing was practically cost-free. These facts helped make silk an ideal candidate for studies involving its utilisation as a matrix onto which skin grafts are moulded. Another point in this matrix's favour is the fact that core silk fibroin has been shown to exhibit biocompatibility both in vivo and in vitro, comparable with that of other widely used biomaterials such as collagen and polylactic acid (Altman et al., 2003). Combination matrices made from processed skin dermis, together with a silk framework or other related structure, may be future avenues to explore.

5 Conclusions

In the case of the acellular dermal matrix, being derived from formalin-fixed cadaveric skin, the formalin may have altered the protein surfaces rendering the adhesion of cells more difficult with respect to other matrices investigated.

The silk matrix seemed to be a good candidate for the development of skin structures, although studies have outlined the fact that raw silkworm silk can be immunogenic due to the protein sericin and that a degumming process should be carried out first (Kearns et al., 2008). Unfortunately, this in turn distorts the structure of the silk fibrons themselves and effects the mechanical properties (Langer and Vacanti, 1993), while sericin coated materials have been shown to aid cell adhesion and growth of human fibroblasts (Vacanti, 2001).

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