

Research Article

Acellular Dermis as a Dermal Matrix of Tissue Engineered Skin Substitute for Burns Treatment

Arvind Shukla^{1*}, Nigamananda Dey², Prathanika Nandi^{1,2}, and Malika Ranjan³

¹School of Biotechnology and Bioinformatics, D.Y. Patil University, India

²Skin Regeneration Laboratory, National Burns Centre, India

³International Centre for Stem Cells, Cancer and Biotechnology (ICSCCB), India

Abstract

The current trend of burn wound care has shifted to a more holistic approach of improvement in the long-term form and function of the healed burn wounds and quality of life. This has demanded the emergence of various skin substitutes in the management of acute burn injury as well as post burn reconstructions. Skin substitutes have important roles in the treatment of deep dermal and full thickness wounds of various etiologies. At present, there is no ideal skin substitute in the market. Skin substitutes can be divided into two main classes, namely, biological and synthetic substitutes. The biological skin substitutes have a more intact extracellular matrix structure, while the synthetic skin substitutes can be synthesized on demand and can be modulated for specific purposes. Each class has its advantages and disadvantages. The biological skin substitutes may allow the construction of a more natural new dermis and allow excellent re-epithelialization characteristics due to the presence of a basement membrane. Acellular dermal scaffold are one of the types of biological skin substitutes which has the extracellular matrix (ECM) almost like the normal skin extracellular matrix. For regeneration and healing of deep burn wound, ECM plays a very important role in rapid healing with minimum scar. Although some acellular dermis based products are available in foreign market, their prohibitive high cost prevents the Indian patient population to use it. Hence, this Method was designed to prepare an acellular dermis, indigenously, suitable for clinical application. NaOH method was used to standardize the decellularization method. In our observation, six weeks of decellularization was sufficient to completely decellularize the skin as confirmed by H&E Staining. Also, it was found out to be biocompatible by cell culture assay.

ABBREVIATIONS

BM: Basement Membrane; DEJZ: Dermo-Epidermal Junction Zone; ECM: Extra Cellular Matrix; FGF: Fibroblast Growth Factor; GAGs: Glycosaminoglycan's; STSG : Split Thickness Skin Graft; ADM : Acellular Dermal Matrix; AD : Artificial Dermis; IHC : ImmunoHistoChemistry; RT : Room temperature; K-SFM : Keratinocytes Serum Free Medium; SCDM : Soyabean Casein Digest Medium; FTM : Fluid Thioglycollate Medium; HE : Hematoxylin and Eosin.

INTRODUCTION

The current trend of burn wound care has been shifted from merely achieving satisfactory survival rate to improvement in the long-term form and function of the healed burn wounds and quality of life. The change in the trend has demanded the

*Corresponding author

Arvind Kumar Shukla, School of Biotechnology and Bioinformatics, D.Y. Patil University, Plot No.50, Sector- 15, C.B.D. Belapur, Navi Mumbai, 400614, Maharashtra, India, Tel- 91-9768864441 /91-9702943189; Email: arvindkumarshukla10@gmail.com

Submitted: 29 April 2015

Accepted: 24 July 2015

Published: 26 July 2015

ISSN: 2378-9328

Copyright

© 2015 Shukla et al.

OPEN ACCESS

Keywords

- Acellular dermal scaffold
- Extracellular matrix (ECM)
- NaOH method
- Decellularization method

emergence of various skin substitutes in the management of burn injury. The timely restoration of skin protective functions is the key to the successful treatment of burn victims with various severity of damaged skin. Conventionally, autologous split or full-thickness skin graft have been recognized as the best definitive burn wound coverage, but it is constrained by the limited available sources, especially in major burns. Donor site morbidities in term of additional wounds and scarring are also of concern of the autograft application.

Skin substitutes are required in the acutely burned patients as well as in those requiring extensive post burn reconstructions. These have important impact in the care and outcome in the burn victims. Burn injury is one of the most serious of all injuries caused to the body. This is because it damages the skin – the primary protective barrier of the body. The significance

of the skin is realized moreover in this situation – the selective permeability of the delicate epidermis that provides a barrier against microbial entry and vapor loss. Also, the dermis is what provides the skin its toughness and elasticity. The hypodermis provides insulation to body from cold temperatures, acts as a shock absorbent and is also a storehouse of energy. Thus, burns expose the body to infection and other associated and resultant functional and aesthetic losses.

To find an effective solution to this problem, it is first necessary to understand the complexity of skin.

Biology of the Skin

Skin is a complex organ composed of three different layers [Figure 1].

1. The epidermis
2. The dermis
3. The hypodermis

Epidermis

1. The epidermis is the outer layer and protects against infection, evaporation of body fluid, ultraviolet light and provides thermal regulation. The epidermis is derived from fetal ectoderm. The principal cells of the epidermis are Keratinocytes. These cells are arranged into five progressively differentiated strata [Figure 2].

2. Stratum Corneum: It is the outermost layer which is a cornified layer of 15–30 sheets of non-viable, but biochemically-active cells called corneocytes.

3. Stratum Lucidum: Present in palms, soles, etc. (padded, non-hairy regions) beneath stratum corneum.

4. Stratum Granulosum: A 3–5 sheets granular layer of non-dividing keratinocytes producing granules of a protein called keratohyalin. These cells flatten as dividing cells below progressively push them to the skin surface. At the same time their cell organelles and nuclei break down and their cell membranes become increasingly impermeable.

5. Stratum Spinosum: Or 'spiny' layer containing 8–10 sheets of keratinocytes with some limited capacity for cell division. Also found here are bone marrow derived Langerhans Cells, which scour the skin for evidence of entry by 'foreign' entities.

6. Stratum Basale: The basal or dividing layer of the epidermis is called the S. basale or S. germinativum. These cells are attached to a non-cellular basement membrane that separates the epidermis from dermis. In addition to the differentiating (i.e., maturing/aging) keratinocytes, the S. basale also houses other cells with stem cell-like properties, the enigmatic Merkel cell (with apparent sensory functions) and melanocytes.

Cells of Epidermis

1. Keratinocytes : Keratinocytes in the stratum basale proliferate through mitosis and the daughter cells move up the strata changing shape and composition as they undergo multiple stages of cell differentiation to eventually become enucleated. During this process keratinocytes will become highly organized,

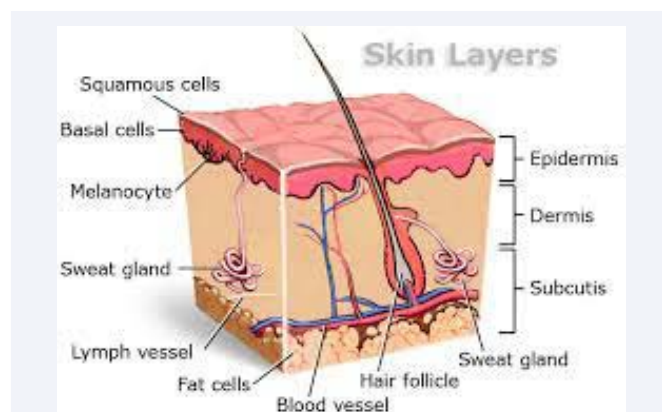


Figure 1 Layers of Skin.

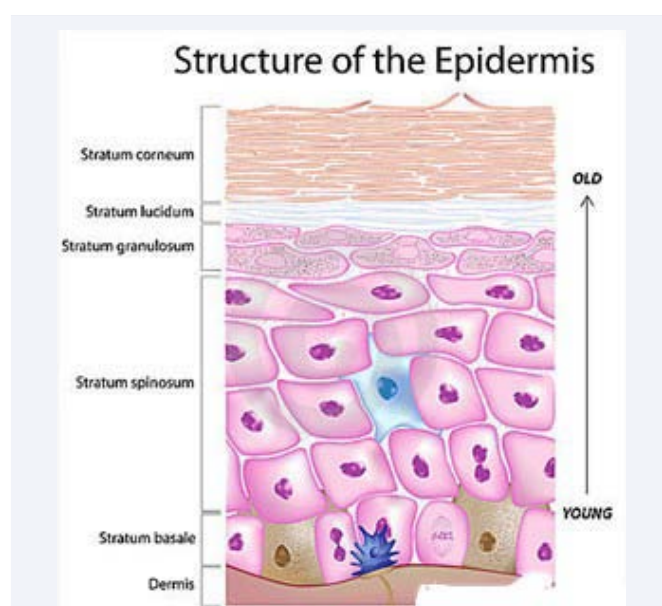


Figure 2 Structure of Epidermis.

forming cellular junctions (desmosomes) between each other and secreting keratin proteins and lipids which contribute to the formation of an extracellular matrix and provide mechanical strength to the skin. Keratinocytes from the stratum corneum are eventually shed from the surface (desquamation).

2. Melanocytes: Melanocytes are derived from fetal neural ectoderm and populate the interfollicular Stratum Basale and hair follicles. The melanosomes synthesize the pigment melanin through the action of the enzyme tyrosinase. The melanin provides the skin with its pigment and absorbs harmful ultraviolet radiation.

3. Langerhans cells: These cells are derived from bone marrow cells. They recognize, phagocytize, process, and present foreign antigens through their expression of class II antigens, and initiate the rejection process in skin transplantation.

4. Merkel cells: These are neuroendocrine cells that reside near hair follicles and nerve endings. These cells are transducers of fine touch.

Basement membrane (BM) Zone

The BM Zone is a complex region of the extracellular matrix connecting the basal cells of the epidermis with the papillary dermis. The dermal-epidermal junctions consist of protrusions of dermal papillae, which interdigitate with epidermal productions known as rete ridges. The functions and homeostasis of skin critically depend on the stable organization and cohesion between the epidermis and the dermis. These tissue layers are confined and inter connected by the dermo-epidermal junction zone (DEJZ), which comprises the basal keratinocytes, the dermo-epidermal basement membrane, and the uppermost, the papillary dermis [Figure 3].

Hemidesmosomes connect epidermis to dermis. On the dermal side of the basal lamina there are numerous anchoring fibrils. Collagen VII is a major component of anchoring fibrils that attach epidermis to dermis. Integrins are BM components that attach epidermal cells to BM via hemidesmosomes. Alpha 6 beta 4 integrin attaches to laminin on BM.

Dermis

Dermis is a layer of cells between epidermis and subcutaneous tissue which consist of cells and connective tissue (ECM) which cushions the body from stress and strain. It is divided into two layers which give the skin its durability and elasticity [Figure 4].

1. **Papillary region (Stratum papillare):** The papillary region is composed of loose areolar connective tissue. This is named for its fingerlike projections called *papillae* that extend toward the epidermis and contain either terminal networks of blood capillaries or tactile Meissner's corpuscles. It is the superficial layer adjacent to the epidermis.
2. **Reticular region (Stratum reticulare):** The reticular region lies under the papillary region and is usually much thicker. It is composed of dense irregular connective tissue, and receives its name from the dense concentration of collagenous, elastic, and reticular fibers that weave throughout it. These protein fibers give

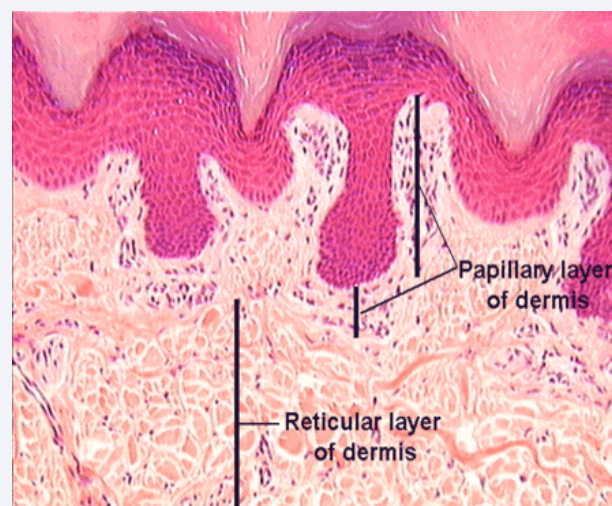


Figure 4 Structure of Dermis

the dermis its properties of strength, extensibility, and elasticity. The reticular regions are the roots of the hair, sebaceous glands, sweat glands, receptors, nails, and blood vessels. Deep thicker region is known as reticular region.

Cells of Dermis

- a. **Dermal fibroblast:** These are derived from mesenchymal stem cells within the body. Its proliferation can be stimulated by the presence of fibroblast growth factor (FGF). Fibroblasts do not appear to be fully differentiated or specialized. Dermal fibroblasts are responsible for creating the ECM which organizes the stratified squamous epithelial cells of the epidermis into a unified tissue. Dermal fibroblasts create long fibrous bands of connective tissue which anchor the skin to the fascia of the body. Therefore, without dermal fibroblasts, the largest and heaviest organ would not tightly adhere to body's frame.

Upon injury, dermal fibroblast can give rise to myofibroblast. Myofibroblasts are the cells with smooth muscle characteristic. When dermal fibroblasts express actin, the cells can slowly contract. This contraction plays a critical role in wound healing and fibrosis. By pulling tissues closed differentiated myofibroblasts, seal the skin after an injury (thereby, preventing infection but inducing scar formation. Interestingly, myofibroblasts can also be derived from non-fibroblast sources.

- b. **Cellular Matrix (ECM):** Collagen is the major structural matrix molecule, constituting of approximately 70% of the skin's dry weight. Elastic fibers account for approximately 2% of skin's dry weight and play an important role in maintaining the integrity of the skin after mechanical perturbation. Glycosaminoglycan's (GAGs) and adhesion molecules are the third major extracellular components of the dermis. These molecules help regulate intracellular and extracellular events upon binding by releasing, and neutralizing cytokines and growth factors. Adhesion molecules are crucial for cellular migration and chemotaxis in and out of the wound. The fibroblast is the principal cell of the dermis and is responsible for the synthesis and degradation of

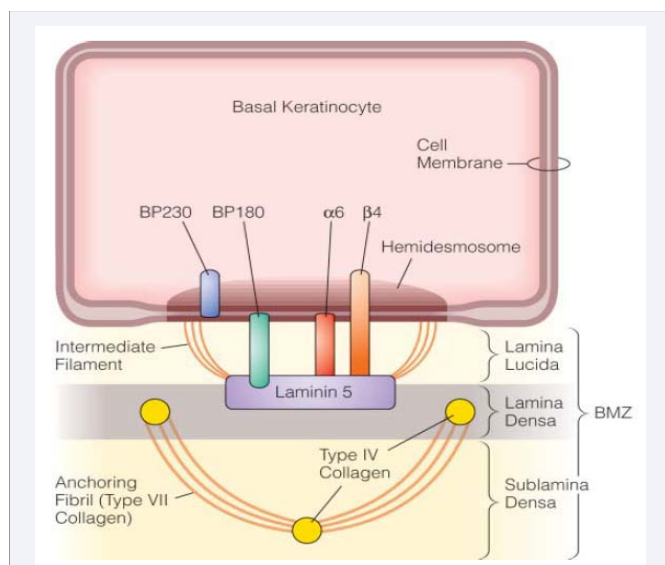


Figure 3 Basement Membrane (BM) Zone.

fibrous and elastic dermal proteins. The dermis also contains various bone-marrow derived inflammatory cells and other poorly understood mesenchymal stem cells, mast cells and cells associated with vascular, lymphatic and nervous tissue.

c. Function of Skin

I. Protection: Langerhans in the skin are the part of the adaptive immune system serving as the anatomical barrier from pathogens and damages between the internal and external environment.

II. Sensation: Skin contains a variety of nerve endings that jump to heat, cold, touch, pressure, vibration and tissue injury.

III. Heat regulation: It increases perfusions and heat loss, while constricted vessels greatly reduce cutaneous blood flow and conserve heat.

IV. Control of evaporation: Skin provides a relatively dry and semi-permeable barrier to fluid loss.

V. Storage and synthesis: It acts as a storage center for lipids and water.

VI. Absorption: Oxygen, nitrogen and carbon di-oxide can diffuse into the epidermis. In small amounts, some animals use their skin as their sole respiration organ.

VII. Water resistance: Skin acts as a water resistant barrier, hence essential nutrient is not washed out of the body. The nutrient and the oil, which plays a role in helping to hydrate our skin, which is being covered by epidermis. The water itself will not cause the elimination of oils on the skin.

Burns disrupts all these functions depending on the extent of burns as mentioned below

A. First Degree of Burn

Burns that affect only the superficial skin are known as superficial or first-degree burns. They include only the outer layer of skin, the epidermis. Skin is usually red and very painful equivalent to superficial sunburn without blisters and dry in appearance. Healing occurs in 3-5 days, injured epithelium peels away from the healthy skin. Hospitalization is for pain control and maybe fluid imbalance. Also therapeutic burn cream is made available in the market [Figure 5].

B. Second Degree of Burn

This can be classified as partial or full thickness. Partial thickness Blisters can be present Involve the entire epidermis and upper layers of the dermis. Wound will be pink, red in color, painful and wet appearing. Wound will blanch when pressure is applied Should heal in several weeks (10-21 days) without grafting, scarring is usually minimal. Ointments can be applied, ice should be avoided since it causes tissue damage and do not spray or use butter since it traps heat inside the skin. And using products such as alloderm, surederm and graft jacket can be of use. Full thickness can be red or white in appearance, but will appear dry and involves the destruction of the entire epidermal layer and most of the dermis. Sensation can be present, but diminished. Blanching is sluggish or absent Full thickness will most likely need excision & skin grafting to heal. Full-thickness

burns usually require surgical treatments, such as skin grafting [Figure 6].

C. Third Degree of Burn

Is referred to as a full thickness burn. This type of burn destroys the outer layer of skin (epidermis) and the entire layer beneath (the dermis). All layers of the skin are destroyed Extend into the subcutaneous tissues Areas can appear, black or white and will be dry Can appear leathery in texture Will not blanch when pressure is applied No pain [Figure 7].

D. Forth Degree of Burn

Extends through entire skin and enters the fats, muscles and bones. Since the entire part of the skin gets burnt. The Less possibility of survival remains. Since it includes the entire skin portion, it requires reconstruction and often amputation. Burn injury is often a devastating physical and psychosocial effect. Quality of life in burnt patients initially seems to be lower than

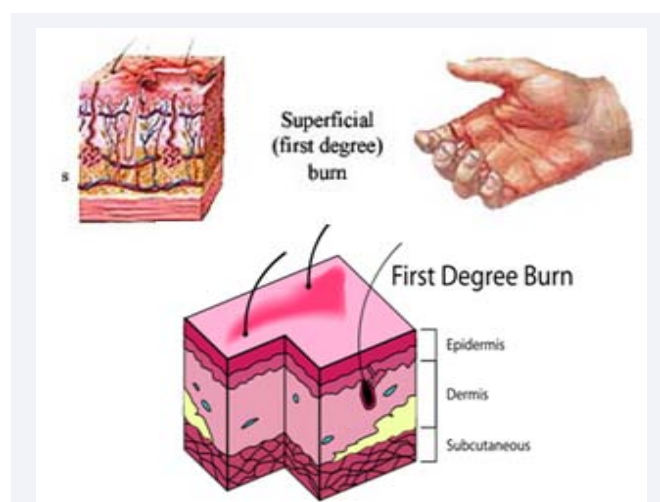


Figure 5 First Degree of Burn.

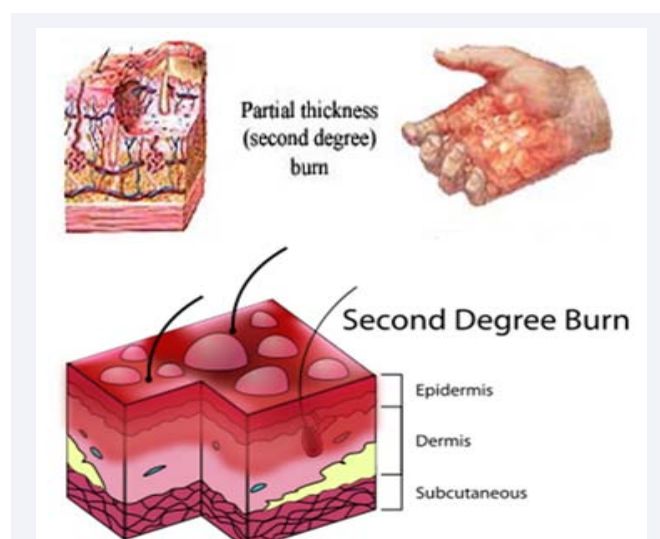


Figure 6 Second Degree of Burn.

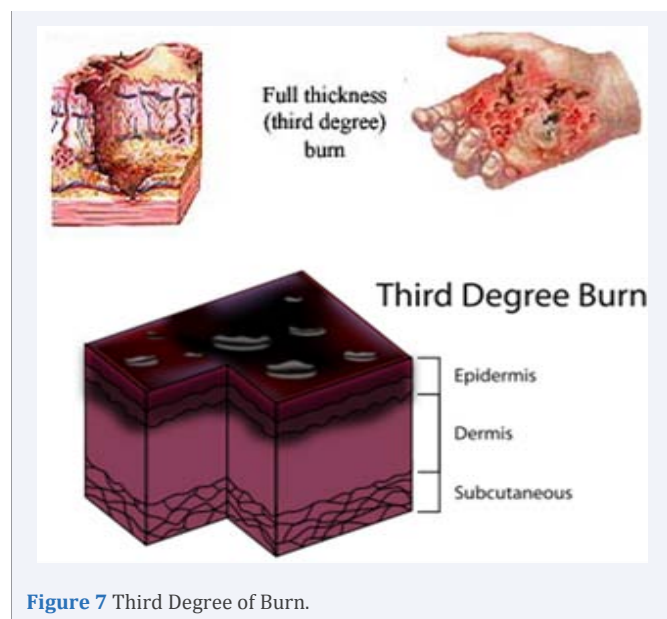


Figure 7 Third Degree of Burn.

the normal population. Problems in mental area are more troublesome than problem in physical areas. After a burning accident the person gets exposed to infection that travels through the blood stream and affects the whole body. It causes blood damage, loss of fluid through the body leading to breathing problems along with bones and joint problem. Treatment of burnt patients involves recovery of optimal survivors to fully participate in society, physically and psychologically [Figure 8].

Burns treatment involves initial covering of the wound to reduce infection and fluid loss. Skin is the best covering for skin. Hence, in early day case of Burn wound management and treatment was to increase the survival rate by early excision (removal of dead infected tissue), resulting in reduced mortality in burn patients. Then, in less extensive burns, autograft (STSG-Split Thickness Skin Graft) is taken, meshed and this acts as a permanent cover. In patients with extensive burns it is not possible to use thicker autograft because of increased donor site morbidity. The partial thickness dermis in this STSG and as the graft is meshed, the lack of dermis in the graft interstices are major causes of hypertrophic scar formation which further lowers the quality of life post burn.

Therefore, Cadaver skin is used. But, allograft is not a permanent covering as it has donor cells within it that are immunogenic to the recipient. Another option is to use cultured autologous keratinocytes, which takes about 3 weeks to reach the culture sheet grafts. During this tenure patient may succumb to infection, multi-organ failure, etc. Instead of which cultured allogeneic allografts can be used. But these cell cultured graft work best in superficial burns. In deep burns, application of only cell based therapy leads to lot of scar ring hence a very low post burn life.

The results can improve if a dermal substitute is made. It can be placed under split thickness skin graft for improved wound healing and less scar. The scaffold must be non-immunogenic (devoid of cells), biocompatible, rigid enough to withstand the proteolytic enzymes of the burn wound environment and have

considerable dermis or dermal components (to reduce scar and contractures). Thus, Acellular Dermal Matrix (ADM) can be used. Donated cadaver skin is one of the major sources of preparation of ADM. However skin of other animals such as pig, cow, frog, buffalo etc. But human cadaver skin is considered to be the best source for preparation of ADM. There are some products available from the skin of all the above mentioned animals.

The key to whole process remains with the decellularization process followed. It should completely throw- out the cells with minimum disintegration of the extracellular matrix. The decellularization process should maintain the proteins specially the integrin which helps in cell attachment. The process should not break the interlinking bond of ECM fibers and it should be non- toxic for clinical use, cost effective and consume less time.

Acellular Dermal Matrix (ADM)

ADM has been used as a soft tissue replacement and burns management since its introduction in 1994. At present, one of the treatments of choice for closure of full-thickness skin loss is to use a cultured epidermal autograft when skin loss is extensive. In this study, we investigated a simple method of processing surplus donated cadaver skin to produce an acellular, structurally intact, dermal matrix. First, the Acellular dermal matrix (ADM) was prepared from cadaveric human skin. ADM was then subjected to Immunohistochemistry technique to observe cell surface markers and to see to see cell viability. The matrix maintained the basement membrane complex and the extracellular matrix structure of the dermis despite cadaveric skin being used. Next, by doing primary culture of Keratinocytes, growing cells to number of generations, sub-culture was done and the cells were seeded on the acellular dermal matrix. The dermal matrix supported fibroblast infiltration.

These results suggest that skin processed by our simple method has the potential to be used as a dermal template together with the cultured epidermis in the closure of full-thickness wounds and can effectively direct regeneration of normal skin morphology. There are several types of skin substitute available in market .But, they are very costly and cannot be afforded by Indian Burns' patients that generally come from the lower, non-affording strata of the society.

So, this project aimed to use decellularization techniques so as to create a cost effective dermal matrix to be used for Indian burns patients.



Figure 8 Fourth Degree of Burn.

Skin substitutes

Skin substitutes have important roles in the treatment of deep dermal and full thickness wounds of various etiologies. At present, there is no ideal substitute in the market. Skin substitutes can be divided into two main classes, namely, biological and synthetic substitutes. The biological skin substitutes have a more intact extracellular matrix structure, while the synthetic skin substitutes can be synthesized on demand and can be modulated for specific purposes. The biological skin substitutes may allow the construction of a more natural new dermis and allow excellent re-epithelialization characteristics due to the presence of a basement membrane. Synthetic skin substitutes demonstrate the advantages of increase control over scaffold composition. The ultimate goal is to achieve an ideal skin substitute that provides an effective and scar-free wound healing [1].

Skin substitutes are heterogeneous group of wound coverage materials that aid in wound closure and replace the functions of the skin, either temporarily or permanently. There are several important factors that are taken into consideration in the decision to use the skin substitutes in burn wound management. These include the depth of burn, availability of donor site, likelihood of wound infection, sites of burn, likelihood of contracture, aesthetic outcome, relative cost, time consumption and experience of the burn surgeons.

The skin substitutes provide rapid wound coverage solution that may require less vascularised wound bed, increase in the dermal component of healed wound, reduce or removed inhibitory factors of wound healing, reduced inflammatory response and subsequent scarring [1].

The optimal skin substitute will provide for immediate replacement of both the lost dermis and epidermis, with permanent wound coverage. Other features of the ideal skin substitute should have the many features eg. resisting infection, preventing water loss, withstanding the shear forces and should be cost effective, widely available, have long shelf life, easy storage, lack of antigenicity, flexible in thickness, durable with long-term wound stability. Also it should be conformed to irregular wound surfaces and easy to be secured and applied.

There are several important factors that are taken into consideration in the decision to use the skin substitutes in burn wound management. These include the depth of burn, availability of donor site, likelihood of wound infection, sites of burn, likelihood of contracture, aesthetic outcome, relative cost, time consumption and experience of the burn surgeons.

The skin substitutes provide rapid wound coverage solution that may require less vascularised wound bed, increase in the dermal component of healed wound, reduce or removed inhibitory factors of wound healing, reduced inflammatory response and subsequent scarring. However, these skin substitutes generally necessitate higher cost, expertise and experience, durable with long-term wound stability and it can be conformed to irregular wound surfaces and easy to be secured and applied.

Till date, there is no ideal skin substitute available that fulfills all the above-mentioned features. Currently, tissue engineering and biotechnology are creating an optimal skin substitute like

naturally occurring or biological dressing substitute, synthetic dressing substitute, e.g. synthetic polymer sheet or spray, epidermal substitutes, e.g. cultured epithelial autograft (CEA), e.g. Apligraf®, dermal substitutes, bovine collagen sheet, e.g. Kollagen®, bovine dermal matrix, e.g. Matriderm® and human dermal matrix, e.g. Alloderm®.

Skin Grafts

Large, open wounds are highly susceptible to bacterial infections and if the body can't regulate its temperature and hydration, it will go into shock. For decades, the best treatment option for a severe burn has been a skin graft. Skin grafts sound like something straight out of a medieval torture manual, but they may save thousands of lives every year. More than two million people in the United States require treatment for burns every year and between three and four thousand die from their injuries.

A skin graft may be needed. Some skin grafts replace burned skin that will not heal. Other skin grafts help by temporarily covering and protecting the skin as it heals on its own. In a skin grafting procedure, a piece of healthy skin is taken from an unburned area of the person's body (autograft), a dead person (allograft), or an animal (xenograft). Autografts can be solid pieces of skin or meshed grafts. For a meshed graft, doctors use a tool to make multiple, regularly spaced, small incisions in the piece of skin. The incisions allow the donor skin to be stretched to cover a much larger area (often several times the area of the original piece of skin). Meshed grafts are used in areas where appearance is less of a concern and when burns involve more than 20% of the body surface and donor skin is scarce. Meshed grafts heal with an uneven grid like appearance, sometimes with excessive scarring. After any dead tissue is removed and the wound is clean, a surgeon sews or staples the skin graft over the burned area. Artificial skin can also be used. Autografts are permanent. Allografts and xenografts, however, are rejected after 10 to 21 days by the person's immune system, and artificial skin is removed. Although allografts and xenografts provide temporary protection to healing skin, an autograft eventually must be placed if the wound is full-thickness and is too large to heal by itself. Burned skin can be replaced anytime within several days of the burn.

Tissue engineering of skin helps patients with burns injuries

In most cases the body can heal itself remarkably well but when patients suffer extreme skin loss such as burns or have problems in wound healing because of poor blood supply or diabetes then tissue engineered skin can be of help to patients who have lost most of their skin through extensive burns and can't repair their skin fast enough and risk infection and death. In all cases a small piece of skin is initially taken from the patient and the skin cells are isolated from it and expanded in the laboratory at a rate much faster than they would normally grow on the patient. Dermal regeneration template, e.g. Integra® and Biobrane® is tissue engineered skin used to treat burns patients. From the practical point of view, the skin substitutes are best classified as temporary or permanent and synthetic or biological.

Temporary Skin Substitutes

Temporary skin substitutes provide transient physiologic

wound closure, including protection from mechanical trauma, physical barrier to bacteria and creation of a moist wound environment.

The Common uses for Temporary skin substitutes include

For dressing on donor sites to facilitate epithelialization and pain control, clean superficial wounds until epithelialization, it provides temporary physiological closure of deep dermal and full thickness wounds after excision while awaiting autografting, as sandwich graft technique over the widely meshed autograft.

Permanent Skin Substitutes

The permanent skin substitutes have the roles of permanently achieving wound closure, replacing the skin components and providing a higher quality skin replacement than the thin autologous skin graft. The purpose of these products is to replace full thickness skin loss as well as to improve the quality of the skin, which has been replaced after a severe burn. As opposed to the bilayer concept of the ideal temporary skin substitute, permanent skin replacement is much more complex. There are two approaches to developing a permanent skin substitute.

The first approach is the use of a bilayer skin substitute, with the inner layer being incorporated into the wound as a neodermis, rather than removed like a temporary product. The outer layer is either a synthetic to be replaced by autograft (epidermis) or actual human epithelial cells. The epithelial cells, which will form epidermis barrier function, is not often sufficiently developed at placement to act immediately as an epidermal barrier.

The second approach is the provision of either just an epidermal or a dermal analog, i.e. a one layer tissue. These products are technically not permanent skin substitutes as there is no bilayer structure.

Biological Skin Substitutes

The biological skin substitutes have a more intact and native ECM structure which may allow the construction of a more natural new dermis. They also allow excellent re-epithelialization characteristics due to the presence of a basement membrane. However, natural constructs can exhibit problems with slow vascularization of the material. The most widely used biological substitute worldwide is cadaveric skin allograft, porcine skin xenograft and amnion.

Xenograft

Xenografts are skin substitutes harvested from the animals for use as temporary graft in human. Xenografts are indicated for clean partial-thickness burns as temporary coverage.

Allograft

The cadaveric skin allograft application is one of the most commonly applied skin substitutes in burn wound all over the world. There are two main types of cadaveric skin allografts, cryopreserved allograft and glycerol-preserved allograft (GPA). The GPA is more popular and commonly used in clinical practice. The GPA is applied to cover the partial-thickness burn as a definitive biological dressing until the underlying burn wounds

have epithelialized. It is noted that GPA allows painless and easy dressing changes.

A viable skin allograft can re-vascularized by inosculation like autologous split skin graft. In addition, skin allograft can provide growth factors and essential cytokines while creating chemotaxis and proliferation at the wound beds, it has been used for burn wound bed preparation. The freshly excised burn wound could be optimized and conditioned to prepare for subsequent autografting by application of skin allograft.

Amnion

The amnion is a thin semi-transparent tissue forming the innermost layer of the fetal membrane. The amnion has been used as biological dressings for burns since 1910. It is one of the most effective biological skin substitutes used in burn wounds, with efficiency of maintaining low bacteria count.

It also has advantages of reducing loss of protein, electrolytes and fluids, decreasing the risk infection, minimizing pain, acceleration of wound healing and good handling properties. Amnion is primarily used for covering partial-thickness burns until complete healing. It is particularly useful for superficial partial-thickness facial burns.

Culture Epithelial autograft

The culture of Keratinocytes is an important advance in the burn care. CEA was first reported in the clinical use in 1981 in extensive full thickness burns. A large surface area of keratinocytes can be obtained from the relatively small biopsy of healthy skin from the patient. The autologous keratinocytes are isolated, cultured and expanded into sheets over periods of 3–5 weeks. CEA avoids the mesh aspect of split skin autograft and discomfort of donor site after skin harvesting. It is however limited by the fragility and difficulty of handling, unpredictable take rate and high cost.

Synthetic skin substitutes

Synthetic skin substitutes are constructed out of non-biological molecules and polymers that are not present in normal skin. These constructs should be stable, biodegradable and provide an adequate environment for the regeneration of tissue. It should maintain its three-dimensional structure for at least 3 weeks to allow ingrowths of blood vessels, fibroblast and coverage by epithelial cells. Biodegradation should preferably take place after this period. This process should occur without massive foreign body reaction as this process would increase the inflammatory response, which may be associated with profound scarring. It should also be composed of immunocompatible materials to avoid immunoreactive processes.

The artificial nature of these skin substitutes has some distinct advantages and disadvantages when compared to natural biological structures. The composition and properties of the product can be much more precisely controlled. Various additives such as growth factors and matrix components can be added to enhance the effect. These products could also avoid complications due to potential disease transmission. However, these synthetic skin substitutes generally lack basement membrane and their architecture. There are several synthetic

skin substitutes that are available for wound coverage. However, there are also substantial numbers of synthetic substitutes undergoing *in vitro* or animal testing. Amongst the synthetic skin substitutes available in the market are Biobrane®, Dermagraft®, Integra®, Apligraf®, Matriderm®.

Biobrane®

Biobrane® consists of an inner layer of nylon mesh that allows fibro vascular in growth and an outer layer of silastic that serves as a vapor and bacterial barrier. It has been used to give a good effect in clean superficial burns and in donor sites. When used to cover partial-thickness wounds, the mesh adheres to the wound until healing occurs beneath. Biobrane® should be removed from any full-thickness wound prior to skin grafting.

Biobrane® is an established synthetic dressing for burn wounds. When dressed with Biobrane®, patients with superficial partial-thickness burns experience less pain as compared to gauze and silver sulfadiazine dressing. Biobrane® also significantly reduces hospital stay, wound healing time and requirements of pain medications. There are reported applications in patients with toxic epidermal necrosis, chronic wounds, or following skin resurfacing. However, Biobrane® has been associated with permanent scarring in partial-thickness scald wounds.

Dermagraft®

Dermagraft® is a bioabsorbable polyglactin meshes seeded with allogenic neonatal fibroblast. Indications for the usage of dermagraft are in burn wounds, chronic wounds and diabetic ulcers. It can be used as a temporary or permanent covering to support the take of meshed split-thickness skin grafts on excised burn wounds.

Dermagraft® appears to produce results as good as allograft with regard to wound infection, wound exudate, wound healing time, wound closure and graft take.

Integra®

Integra® is a dermal regeneration template consisting of bovine collagen, chondroitin-6-sulphate and a silastic membrane. This product has gained widespread use in the clinical treatment of deep partial-thickness and full-thickness burn wounds, full-thickness skin defects of different aetiologies, chronic wounds and in soft tissue defects. The bovine collagen dermal analogue integrates with the patient's own cells and the temporary epidermal silicone is peeled away as the dermis regenerates. A very thin autograft is then grafted onto the neo-dermis. However, with regard to wound infection and graft take, Integra® did not produce as good a result.

Apligraf®

Apligraf® is a bilayered living skin equivalent. It is composed of type I bovine collagen and allogenic keratinocyte and neonatal fibroblast. It is indicated in partial to full thickness burns, skin graft donor sites, chronic wounds, diabetic ulcers and Epidermolysis Bullosa. It has to be applied "fresh" as it has a shelf-life of 5 days at room temperature. Apligraf® has been shown to accelerate wound closure.

Matriderm®

Matriderm® is a structurally intact matrix of bovine type I collagen with elastin. It is utilized for dermal regeneration. Its indications are full thickness or deep dermal burn wounds and chronic wounds. The matrix serves as a support structure for the in growth of cells and vessels. Its elastin component improves the stability and elasticity of the regenerating tissue. As the healing process advances, fibroblast lays down the extracellular matrix and the Matriderm. However, unlike Integra, Matriderm has been shown to be able to accommodate immediate split thickness skin grafting with no diminished take. In experimental models, the matrix reduces wound contracture, and histologically collagen bundles in the scar are more randomly orientated. Clinical trials with a long-term clinical evaluation showed no difference in scar elasticity between the described dermal substitute and split thickness grafts alone.

Artificial dermis (AD)

This has been developed as the dermal regeneration template and became an effective method of treatment for full-thickness skin defects as reported by Yannas and Burke in 1980. In favor of treatment with AD, it is pointed out that good tissue quality resembling full thickness skin grafting can be obtained by thin spit-skin grafting, of the treated wound site, so that donor site morbidity is reduced. However, to achieve this task, two surgical procedures where the AD grafting and another secondary skin grafting, epidermal coverage, are required. With this method, 2-3 weeks time is required before the secondary skin grafting can be performed. Investigators studied the histological phases of the vascularization of the dermal template in acute burns and others [2]. Moiemmen et al. reported that the neovascularization of the templates starts at the end of the second week in cases other than acute burns such as scar contracture. Therefore, the period between the AD grafting and the secondary skin grafting should be shortened. Chu *et al.*, 2002 [3] reported the one-step operative procedure using Integra1 in a rat experimental model. Treatment with platelet derived wound healing factor (PDWHF), combined with cultured endothelial cells and fibroblasts, and accelerated wound angiogenesis. By this method, one-step grafting procedure of the artificial dermis and the skin is possible. [4] Thus considering the review it is observed that even preparation of artificial skin needs a natural base of components and support for the efficient wound healing. Hence the decellularization of cadaveric skin usage as a scaffold for autologous skin substitute is ideal scaffold material for the support of cells and apt for its cost effective application purpose.

Effective Repair and Regeneration

Effective repair and regeneration of injured tissues and organs depends on early reestablishment of the blood flow needed for cellular infiltration and metabolic support. Implantable biomaterials designed to replace damaged or diseased tissues must act as supports (i.e., scaffolds) into which cells can migrate and establish this needed blood supply [5]. Repair and regeneration of damaged tissues uses intact extracellular matrix (ECM) obtained from animal tissues as the growth support for host cells. ECM is the non-cellular part of a tissue and consists of protein and carbohydrate structures secreted by the resident cells. The most common constituent of the ECM is

the structural protein, collagen. When harvested from the tissue source and fabricated into graft prosthesis, these ECM materials may be referred to as naturally occurring polymeric scaffolds, bioscaffolds, biomatrices, ECM scaffolds, or naturally occurring biopolymers. These materials, though harvested from several different body systems, all share similarities when processed into a graft material. Specifically, since they are subjected to minimal processing after they are removed from the source animal, they retain a structure and composition nearly identical to their native state. The host cells are removed and the scaffolds are implanted acellularly to replace diseased or damaged tissues [6]. Many synthetic biopolymers, such as hyaluronic acid based polymers, support the growth of cells in dedifferentiated or incompletely redifferentiated states [7] or, like poly(glycolic) acid, lead to tissue deposition that is less than optimal [8]. Various composition of scaffold matrix are used all around the world as mentioned above which has its own benefits and disadvantages. Thus, preparation of a scaffold which is highly biocompatible, natural and mimics the originality is the most wanted criteria.

Acellular matrix products can be used in a wide variety of applications, including burns and reconstructive surgery, soft tissue and abdominal wall repair and as internal implants for orthopedic use in joint resurfacing and tendon repair. Acellular matrices (or scaffolds) are also used in hard-to-heal wounds such as diabetic foot ulcers, venous leg ulcers and pressure ulcers [9]. In many chronic wounds, increased levels of inflammatory cells lead to elevated levels of proteases that appear to degrade the ECM components, growth factors, protein and receptors that are essential for healing [10]. Different types of tissue-engineered products exist and may be classified as skin substitutes, xenografts, allografts or collagen dressings. Alternatively, these products may be described as biological dressings in that they function as a protective wound cover. However, while most wound dressings need to be changed frequently, matrices provide a scaffold for tissue repair and therefore must remain in the wound for a sufficient length of time [9]. While tissue-engineered products offer increasingly important strategies for managing complex wounds, potential drawbacks include the risks of infectious agent transfer and immunological rejection. [11] Acellular matrices are engineered using a range of chemical and mechanical processes. The ultimate goal is to remove all cellular components using a non-damaging process that maintains the structure and function of the source tissue. The more compatible the final product is to host ECM, the less likely it will elicit an adverse reaction [12]. Ulcers related to numerous underlying etiologies may present particular challenges for clinicians and are costly to treat. These wounds are often slow to heal and associated with high levels of pain, inflammation and tissue necrosis [13]. Removing epidermal and dermal cells leaving the matrix components of the dermis will remove the antigenicity of the allograft but leaves the matrix structures of the dermis. Applied on a full thickness wound such cell-free dermis has been shown to act as a template for the reconstitution of a viable dermis [14]. Data from animal models also suggest that if a matrix is flexible rather than rigid, cells can migrate more rapidly and proliferate in organized manner similar to normal tissue regeneration [15]. This is of particular importance in heavily burnt patients as the nature of the injury leads to a temporary suppression of cell-mediated and humoral

immunity [16]. Preparation of a proper acellular scaffold is not only about biocompatibility but also depends on the promotion of secretion of ECM and should be immunologically inactive to avoid rejection and other complications as mentioned above.

Wounds can be divided into epidermal, superficial partial-thickness, deep partial-thickness and full-thickness with increasing depth of the injury. Treatment approaches differ accordingly [17]. Cells migrate either from the wound edge, hair follicle or from sweat gland remnants that lie in the deeper dermis, which has been preserved in this depth of injury [18]. Reserve of stem cells located in the bulge region of the follicle, which are capable of self-renewal [19]. All full thickness skin wounds which are more than 1 cm in diameter require skin grafting as they cannot epithelialize on their own and may lead to extensive scarring, resulting in limitations in joint mobility and severe cosmetic deformities [17]. Hyalomatrix PA material served as a temporary dressing to stimulate wound regeneration after dermabrasion and was reported to be a good and feasible approach for such wound treatments [20]. It is also reported to give favorable outcomes in deep pediatric burns treatment [21]. For instance, Ahlfors & Billiar in 2007 have developed a rapid method for producing a fibroblast-derived matrix that promotes further organized growth of cells. Mechanical testing of the final product using an inflation method similar to that of Billiar *et al.* (2005) [22], in terms of ultimate tensile strength showed it to be superior (313 kPa) to collagen and fibrin gels, but still not as strong as native skin (713 kPa). True regeneration in vertebrates is seen in *Xenopus* and *axolotl* where the entire limb is restored [23]. Examples of tissue regeneration in humans consist of the regeneration of the digit tip in a child [24]; regeneration of the liver [25], and teeth [26]. Human skin does not regenerate post-natally. However, during the early antenatal period, when injured; all skin layers and appendages are regenerated. Fetal wound healing does not result in scar formation after injury [27].

Currently available tissue-engineered products for skin substitution, including dermal and epidermal constructs, although not perfect, occupy a specific niche within a complex approach to treat full-thickness extensive burns, improving patients' survival rates and their quality of life after injury [28]. Biologically active dressings and structural molecules necessary for wound healing while the patient's own skin regenerates to be used for serial auto grafting. Products based on autologous cultured keratinocytes and fibroblasts are more likely to contribute to actual skin substitution and results of clinical trials are encouraging [29]. However, no one will agree that these products at the current level of sophistication can fully replace damaged skin [30]. Any bioengineered product based on 'natural' mechanisms of wound healing will result in scarring as well as limited functionality, rather than giving fully functional skin regeneration [31]. However, it has been shown that grafting cultured epithelial auto grafts to the remaining tissue after rejection of the allograft will accelerate biologic events in skin regeneration [32]. Understanding the mechanisms by which fetal wounds heal could result in a real breakthrough in adult wound healing with the possibility of real skin regeneration rather than defective and inferior scar-like skin repair [33]. In order to make a successful bioengineering skin scaffold apart from immune compatibility and other major characteristics, it is also highly

essential to promote the regenerative capacity of the patients own cells as well as newly formed once by adopting different mechanics above.

Clinical Studies on use of ADM

The first ADM were processed by trypsin, freeze-thawing, or long incubations with enzymes. Most of those matrix remained highly antigenic, which lead to poor graft survival. Later, more effective decellularisation techniques were undertaken so as to cause minimal damage to ECM. Several different products (natural biological origin) are currently available for wound care. Following are some well known products and their clinical studies results:

1. Derma Matrix™ (Synthes, Inc., West Chester, PA) [20, 21] is human donor skin processed using a combination of detergent and acid washes and is then freeze dried. It is especially commercialized for reconstructive surgery, but clinical studies in wound care remain to be published.

2. Glya Derm™ (Euro Skin Bank, Netherlands) is another acellular dermal collagen-elastin matrix. The elastin matrix is not damaged by this manufacturing and preservation method, which lead to a more durable effect. [22, 23] Additional advantages of glycerol preservation include inactivation of viruses and ease of storage and handling. It is intended to be cost-effective, enabling widespread application. Clinical studies in burn care are yet to be established.

3. AlloDerm® (Lifecell Corp., Branchburg, NJ) is an acellular human de-epidermized dermis product that is a semi permanent skin substitute. It is a cryopreserved and lyophilized allodermis that acts strictly as a dermal replacement. It has no protective epidermal analogue. It has been successful alone and in combination with cultured autografts (two-steps procedure) in the treatment of burn wounds and dermal defects. Additionally, AlloDerm® is procured by cryopreservation which may affect the integrity of the elastin matrix, and its manufacturing is expensive.

Culturing Keratinocytes on ADM

Rheinwald and Green developed an in-vitro technique to culture human keratinocytes into epidermal sheets suitable for auto-transplantation. In order to obtain the reconstructed epidermis *in vitro*, melanocytes and keratinocytes need to be reproduced on a substrate. Hence dermis is used, which was named de-epidermized human dermis, following the technique described by by Pruniéras et al. [34]. Functional keratinocytes and melanocytes that are correctly positioned, equivalent to epidermis *in-vivo*. The extent of the stratification and keratinization of human epidermis reconstructed *in vitro* had the same characteristics as found *in-vivo* Jussara Rehder et al. [35]. The transplantation of cultured autologous keratinocytes is the most advanced area of tissue engineering and it has an important application in the restoration of skin lesions such as burns and chronic ulcers (Terskikh VV, Vasiliev AV 1999) [36] epidermal sheets with keratinocytes by themselves do not provide a satisfactory skin cover on full thickness burns due to subsequent skin layer.

Wound contraction, fragility of the grafted area and poor

cosmetic result. The wounds covered with cell-free dermis and keratinocytes showed a complete epidermal coverage in two weeks after transplantation, in contrast to the wounds covered with un-seeded dermis which only showed epidermal coverage at the wound edges. There was also a marked difference concerning fibroblast in-growth and angiogenesis. Autologous keratinocytes can be seeded on a cell-free dermis and transplanted as a keratodermal graft which stimulates re-epithelialization as well as fibroblast in-growth and angiogenesis in the wound. As judged by the histological appearance of the cell-free grafts as well as the high degree of keratinocyte adherence also the basement membrane complex was maintained after the treatment; however this should be investigated more extensively in the future. Autologous keratinocytes will both shorten the time until cultured grafts are available and improve the quality of the final result with a better biomechanical strength as well as a better cosmetic appearance (C.-J. Gustafson., 1999). Cultured autologous keratinocytes are considered as a gold standard in re-epithelialization procedure when comes to extensive skin burn and ulcers. Cultured conditions have to be thoroughly scrutinized and standardized up-to clinical grade in-order to carry out transplantation surgical procedures.

Keratinocytes Culturing

The use of autologous keratinocytes in the treatment of cutaneous defects, such as full thickness burns and venous leg ulcers, is established as a method for improving epithelial closure in patients suffering extensive skin damage (Boyce et al. 2002) [37].

The rapid expansion of keratinocytes from a small skin biopsy is essential in the tissue engineering of new skin, especially when large numbers of cells are necessary to repopulate and repair extensive areas of cutaneous damage. Rheinwald and Green (1975) [38] were the first to establish the culture of keratinocytes from skin biopsies. The method established by Rheinwald and Green is still currently used, including in the preparation of keratinocytes for clinical use (Moustafa et al. 2007; James et al. 2010) [39].

The use of collagen layer facilitating the attachment and proliferation of keratinocytes in culture, this being due to the collagen is part of extracellular matrix protein. The natural biological structure of acellular-Dermis plays an important role in keratinocyte proliferation and formation of epidermal architecture *in vitro* as well as *in vivo*. The bioactivity of acellular-Dermis was studied by a cell culture assay. We analyzed growth and differentiation of human keratinocytes cultured *in vitro* on acellular-Dermis, and we compared the results with formation of neoepidermis in the deep dermal wounds treated with acellular-dermis. Keratinocytes cultured on acellular-dermis submerged in the culture medium achieved confluence in 7–10 days. After lifting the cultures to the air-liquid interface, the keratinocytes were stratified and differentiated within one week, forming an epidermis with basal, spinous, granular, and stratum corneum layers. Immunohistochemical detection of high-molecular weight cytokeratins (HMW CKs), CD29, p63, and involucrin confirmed the similarity of organization and differentiation of the cultured epidermal cells to the normal epidermis. The firm natural structure of Acellular-dermis stimulates proliferation

and differentiation of human primary keratinocytes and by this way it aids in improving wound healing (Robert Zajicek et al. 2012) [40]. The proliferative keratinocytes are small cells that are on the top of the basal membrane and normally present a low division rate. The new cells serve to substitute the ones that suffer terminal differentiation and desquamation.

It is necessary 3 to 4 weeks to the new cell goes from basal stratum to the corneum stratum [38]. Cultured cells for the treatment of disease: The successful growth of human skin cells in culture has made it possible to restore epidermis after severe burns and other forms of damage [41]. The area of skin that is necessary to the keratinocytes culture is small. A 1 cm biopsy can be expanded more than 5000 in 3 to 4 weeks, generating sufficient epithelium to the adult body surface coverage. The wound coverage is permanent when the human acellular dermis with autologous cultured human keratinocytes (GraganiA., et al., 2003) [42].

Seeding of Culturing Keratinocytes on ADM

Autologous skin grafting is the gold standard for definitive wound coverage; it is difficult to find suitable donor areas in a patient with extensive burns. Dermal skin substitutes may be used to handle the problem of donor site shortage when dealing with major skin loss. Indeed, recent developments in the multidisciplinary field of tissue engineering have yielded many novel tissue replacements and implementation strategies [43]. Donor skin allografts preserved in 85% glycerol are often used as a temporary coverage for large burn wounds, as glycerol does not affect the structural integrity of the skin. Indeed, the glycerol-preserved skin allograft, although devitalized, does retain its morphological structure and, therefore, can be used as a temporary skin substitute, or grafted as a dermal template. Glycerol-preserved allografts, that have advantageous biomanual properties, are being investigated as scaffolds for tissue engineering applications. The aim of the research reported herein was an *in vitro* construction of a skin substitute made up of an alloplastic acellular glycerolized dermis (AAGD) scaffold directly seeded with low-density keratinocytes [44]. The glycerolized allografts were de-epithelialized with three different methods and used as a scaffold for keratinocyte growth and differentiation. The first method used dispase II, which cleaves the anchoring system between the dermis and epidermis. The second used trypsin which breaks-down the single cells, producing a cellular suspension. The third was a manual method without the use of any enzyme. Specific markers and immunohistochemistry assessed the basement membrane of all the three types of dermis. The scaffold was then nebulized with keratinocytes and cell adhesion analyzed by immunohistochemistry and histology (Carlotta Castagnoli. et al. 2010) [45].

The newly formed epithelium was analysed to evaluate the keratinocyte differentiation and maturation, compared to that of healthy skin. The immunohistochemistry results did not differ between primary and uncultured cells. The choice of all plastic dermis as a scaffold is determined by the fact that it retains almost all healthy mechanical skin properties, being compact and elastic, able to take into the bed wound, providing a barrier against invading organisms: it is considered, to date, the best skin substitute available (Mara Fumagalli et al., 2010) [46].

Moreover it has been demonstrated that alloplastic dermis is a good support for the transplant of *in vitro* cultured keratinocytes (M.R.Herson., et al., 2001) [47]. In addition glycerol preserved skin allografts maintain intact tissue morphology and show a strong decrease in tissue antigenicity compared to cryopreserved ones (C. D. Richters., et al., 1996) [48].

A cell-free dermis seeded with autologous keratinocytes stimulates the formation of a neo-dermis at the same time, as the wound is re-epithelialised by the transplanted keratinocytes. We believe that this approach with a combination of a matrix which stimulates the regeneration of dermis with non-confluent autologous Keratinocytes will both shorten the time until cultured grafts are available and improve the quality of the final result with a better biomechanical strength as well as a better cosmetic appearance.

MATERIALS AND METHODS

Skin Harvesting , Processing and Storage

Cadaver skin is harvested within 6 hours after death with due consent for use in research. Upon harvesting of the skin, place it in sterile 50% glycerol containing streptomycin and penicillin. Then skin upon reaching the skin bank should be kept at 4-8 °C and further processing must occur within 2 - 24 hours, Processing takes place in a safety cabinet. Make sure the bio safety cabinet is operating for at least 30 minutes before to Select a skin piece of at least about 5×5 cm of 0.4-0.6 mm thickness and Place this skin into a sterile bottle containing 85% glycerol with streptomycin and penicillin. Incubate at 33 °C for 3 hours at 100 rpm. Further, store at 4 - 8 °C for 21 days and after 21 days, Place one small piece each in Soya bean casein medium and fluid Thioglycollate medium for microbiological analysis.

Sterility Check

This is to detect and confirm absence of any viable form of microbes in the ADM. The principle behind is that the testing looks for both aerobic and anaerobic contamination of the product undergoing the sterility test. It is carried out in two medium that is Thioglycollate Broth and Soyabean Casein Digest medium. Thioglycollate Broth medium allows the differentiation of obligate aerobes, obligate anaerobes, facultative, anaerobes, microaerophiles, and aerotolerant organisms and Soyabean Casein Digest medium is used for sterility testing by membrane filtration or direct inoculation. It is suitable for culturing of fungi and aerobic bacteria.

De-epidermization by using NaCl

Removal of epidermis from skin is called de-epidermization . The cadherins and integrins of the basement membrane layer that hold together the epidermis and dermis are affected in 1M NaCl solution. Thus, epidermal dermal adhesion becomes loose and they get separated from each other. Place the remaining skin into a sterile beaker containing 0.9% NaCl and rinse well to remove all the glycerol using a pair of sterile forceps. This is further rinsed in 0.9% NaCl by incubation at RT at 40 rpm for 5 minutes. Total three such rinsing to be done. Then, set the shaking incubator at 37 °C. Then transfer this skin onto a petri plate (epidermis facing upside). Make a cut on the right hand top side using a sterile

scissor (to differentiate between the epidermal and dermal side of the skin post de-epidermization). Place this skin in ~ 200 ml 1M NaCl with antibiotics (streptomycin and penicillin) for 2-8 hours at 37 ° C at 150 rpm (i.e. continuous shaking). Check after 2, 4, 6, 8 hours to confirm de-epidermization. After 8 hours, within the biosafety cabinet, the epidermis and dermis are gently separated using sterile forceps. The dermis is then rinsed in 0.9% NaCl by keeping it in this solution and incubating at RT at 40 rpm for 5 minutes. Total three such rinsings to be done and then keep one small piece in 85% glycerol for characterization study.

De-epidermization by using NaOH

Cadaveric skin is decellularised by treatment of NaOH at optimal concentration which removes the cells from dermis by osmotic lysis. When the cells are in a hypotonic environment, the water tends to move into the cell. When the cell membrane cannot hold the excessive influx of water, the cell membrane ruptures. Then transfer the DED (De-Epidermised Dermis) into a sterile plastic jar containing ~ 200 ml 0.06M NaOH. Incubate at RT at 120 rpm for 1 hour. After 3 days, again change the NaOH solution with fresh solution and shake the bottle in incubator for 1 hour, continue for six weeks. After treatment, every week, place one small piece each in Soyabean Casein and Fluid Thioglycollate medium for microbiology testing then every week place one small piece in 85% glycerol for characterization study. After six weeks treatment with NaOH, perform neutralization using 0.1 N HCl for 10 min, followed by washing in 0.9% NaCl thoroughly and preserve in 85% glycerol and rinse with 0.9% NaCl before use.

Immunohistochemistry

Immunohistochemistry (IHC) is a wide-used biological technique that combines anatomy, physiology, immunology and biochemistry. Developed from the antigen-antibody binding reaction, immunohistochemistry can be considered as a method that visualizes distribution and localization of specific antigen or cellular components in separated tissues, or tissue sections. Compared to other bio-techniques that are based on the antigen-antibody reaction such as immunoprecipitation, or western-blot, immunohistochemistry provides in situ information which promises a more convincing experimental result. The major components in a complete immunohistochemistry experiment to find out binding of primary antibody to specific antigen, formation of antibody-antigen complex after incubation with a secondary, enzyme-conjugated, antibody in presence of substrate and chromogen, where enzyme catalyzes to generate colored deposits at the sites of antibody-antigen binding. By antigen antibody reaction complement fixation, take tissue and place in 10% NBF (~5ml) tube and keep at 4°C for 24 hrs and next day keep tubes at room temperature (RT), discard solution And Add fresh 10% NBF, again keep at 4°C for 1-2 hrs. Then proceed for tissue processing.

Tissue Processing by dehydration , Clearing and Infiltration

Tissue is Processed by to allow NBF fixed pancreatic tissue to come at RT then discard NBF solution, place tissue in glass vial and add 30% alcohol (Methanol) and keep on shaker for 1 hr. at RT (timing depends on size and type of tissue) (Pancreatic tissue

require 1 hr. incubation as tissue is big and complex). After 1 hr., change 30% alcohol with fresh 30% alcohol and again keep for 1 hr. on shaker. Similarly two washes for 50% alcohol are given each for 1 hr. Then, two washes for 70% alcohol are given each for 1 hr. Once in 70% alcohol, tissues can be stored at RT for days without affecting tissue quality. Next day, 70% alcohol is replaced by fresh 70% alcohol and kept for 1 hr. on shaker. Similarly proceed with alcohol series. Two washes 80% alcohol, 1 hr. each. Two washes 90% alcohol, 1 hr. each. Two washes 100% alcohol, 1 hr. each. Discard 100% alcohol, add Xylol (1:1, Xylene: alcohol), and then Keep it for 30 min. on shaker. Replace xylol with Xylene and observe the tissue till it appears translucent. Pancreatic tissue normally takes ~ 40-45 min. discard xylene, add fresh xylene and molten wax (paraffin) in 1:1 ratio and discard within a minute. After this add fresh wax and keep on hot plate for 15 min. ensuring that wax doesn't get solidified. Then discard wax, add fresh wax and keep for 15 min, and discard wax, add fresh wax and keep for 30 min. and after this, store at RT till block preparation. Keep wax embedded tissue on hot plate for wax to melt. Meanwhile, apply glycerol to inner side of mold so that blocks are easily prepared. Arrange mold in proper shape depending on tissue, add liquid wax in mold and immediately add tissue in it in proper orientation before wax solidifies. After wax has been solidified, remove molds and thus blocks are ready. for sectioning. They are placed on microtome and sectioning is done and then High profile microtome blades (Leica, Microsystems) were used and 5 µm sections of pancreatic tissues were cut and taken slides.

H and E Staining

Most cells are colorless and transparent, and therefore histological sections have to be stained in some way to make the cells visible. H&E contains the two dyes haematoxylin and eosin. Eosin is an acidic dye, it is negatively charged (general formula for acidic dyes is: Na⁺dye⁻). It stains basic (or acidophilic) structures red or pink. Thus the cytoplasm is stained pink by H&E staining. And Haematoxylin can be considered as a basic dye (general formula for basic dyes is: dye⁺ Cl⁻). Haematoxylin is actually a dye called hematein (obtained from the log-wood tree) used in combination with aluminium ions (Al³⁺). It is used to stain acidic (or basophilic) structures a purplish blue. (Haematoxylin is not strictly a basic dye, but it is used with a 'mordant' that makes this stain act as a basic dye. The mordant (aluminium salts) binds to the tissue, and then Haematoxylin binds to the mordant, forming a tissue-mordant-Haematoxylin linkage.) Thus the nucleus is stained purple. For *H and E Staining* required Hematoxylin, eosin stains, Xylene, 100% alcohol and 1% acid alcohol (1% HCl & 70% ethanol). In the method of *H and E Staining*, first deparaffinise the slides by heating on hot plate then Plunge into the xylene coupling for 5 min. with 2 washes, each of 5 min. After air-dry the slides (optional) then Pass through alcohol grades series (100%, 90%, 70%), each for 5 min. and keep the slides under running tap water for 5 min. then Stain with Haematoxylin for 5 min. then dip in acid-alcohol (just 1 dip). (Acid-alcohol: - 500µl of conc. HCL + 50 ml of 70% methanol), Keep the slides under running tap water for 5 min. and dip in Eosin- 1 dip (~15 sec.) then Pass through alcohol grades series (70%, 90%, 100%) - just 1 dip in each series, air-dry the slides and Plunge into xylene for 5 min. (2 times each of 5 min.) then mount the slides with cover slip

and DPX.

Isolation of Keratinocytes from Epithelial layer

The Sample is collected by Human skin biopsies of 2×2 cm from the operation theater of National Burns Centre (NBC), Airoli, Navi Mumbai, India, after taking due informed consent. Primary Culture is done by isolated epithelial cells from the biopsies of patient from the OT of NBC, Airoli. Keratinocytes culture proved valuable for cell biology study, including organotypic culture. For the culture of keratinocytes culture serum free media (K-SFM) were used because serum have TGF-β factor which induce the keratinocytes differentiation. Keratinocytes culture require more care to avoid issues such as contamination, fibroblast overgrowth, apoptosis in low density, differentiation and senescence when reaching confluence.

The Isolation of Keratinocytes from Epithelial layer is required Dispase II, Dulbecco's Phosphate Buffer Saline (with antibiotics, Hi-Media), TrypLE Select (synthetic trypsin) and Keratinocytes serum free medium (K-SFM). Sample was collected in DPBS and was kept at 4°C until further use. The sample was placed in Petridis and washed with DPBS thrice. Sample was cut into three pieces and placed in dispase II overnight then take the overnight sample which was placed in dispase, with the help of forceps. Slightly remove the dermis from the epidermis. Scrap the dermis slightly for removing the remaining Keratinocytes, put the one piece of epidermis in approximately 5-7ml of TrypLE solution, dispase solution & in Trypsin solution and keep it in CO₂ incubator for 15-20 min. Then shake the tube at interval of 5min. Remove the tube from the incubator and with the help of forceps remove the epidermis and place it into Petridis separately, Add 1ml of triple solution to the epidermis and scrap it with the help of scalpel to remove the attached cells from it. Add 1ml of K-SFM to it. Place a cell strainer on another centrifuge tube and filter triple solution along with the scraped epidermis then Centrifuge it at 1200rpm for 10min at 37 °C. Place the tubes slowly and without disturbing the cell pellet discard the supernatant and dissolve the pellet in 1ml of medium and add appropriate volume of media to it.

Now with the help of haemocytometer check the cell viability using 1:1 mixture of cell suspension and trypan blue dye then accordingly calculate the total no cells to be seeded in T-Flask. The Seeding density: - T 25 = 1 Million, T 75 = 3 Million, T 175 = 7 Million and Seed the calculated volume of cell suspension and add 8, 15, 30 ml of K-SFM to T25, T75 and T175 flask respectively. Further incubate the flask in CO₂ incubator at 37°C with 5% CO₂ tension.

Cell Counting

Trypan blue is vital stain, it is excluded from live cells because plasma membrane of live cells is selective permeable and trypan blue is not non selective for live cells. Thus live cells appear colorless under phase contrast whereas dead cells appear blue. The cell counting formula is, Total no. of cells = average no. of cells in $1^2 \times 02 \times 10^4$. Where 02 is dilution factor and 10^4 is conversion factor to convert 10^{-4} to 1 ml. and Average no of cells in one square = total no. of cells in all 5² / 5.

The Equipment and Reagents is Phase contrast inverted

microscope, Haemocytometer, Cover slip, 10 microlitre pipettes, Trypan blue (Hi-Media) and Cell suspension. For Cell counting fist, Cover slip and haemocytometer were cleaned with IPA. Cover slip was affix to the haemocytometer chamber. 10 micro liter of trypan blue and 10 micro liter of cell suspension was mixed well. After mixing, the trypan blue/cell suspension mix was run under cover slip and visualize under the phase contrast microscope. The dead and live cell was counted.

RESULTS AND DISCUSSION

Acellular Dermis

After incubation in 1M NaCl, de-epidermization was checked at every 2 hour interval. Complete de-epidermization was observed between 6-8 hours. Dermis was not degraded under such salt conditions for such a short span of time (Figure: 9, 10).

Sterility Check

To detect and confirm absence of any viable form of microbes in the ADM. The absence of turbidity in the medium shows that there is no contamination in the ADM prepared (Figure: 11, 12, Table 1).

Haematoxylin and Eosin (HE) Staining of Acellular Dermis

After doing H and E Staining the acellular dermal matrix

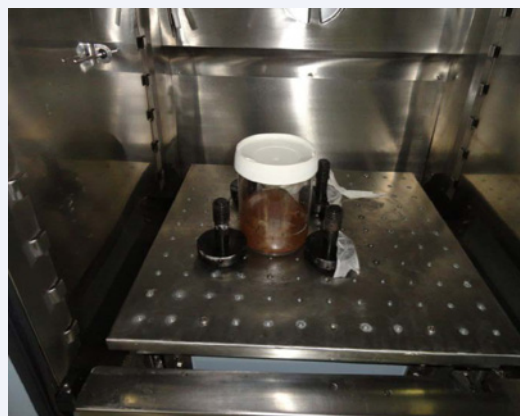


Figure 9 Agitation of ADM.

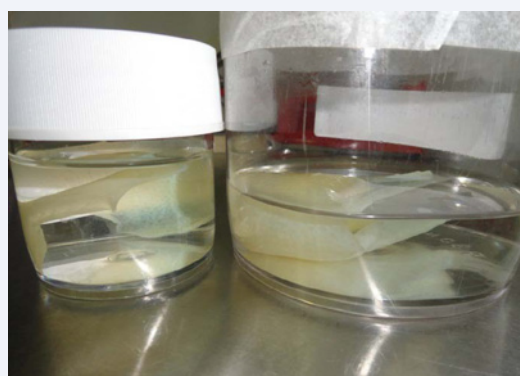


Figure 10 ADM after 42 days NaOH Treatment.

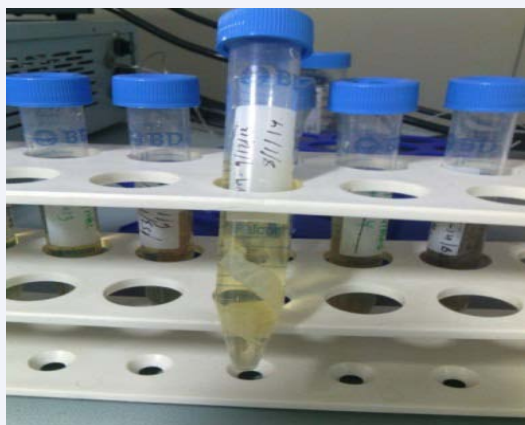


Figure 11 Soyabean Casein Digest Medium (SCDM).



Figure 12 Fluid thioglycollate Medium (FTM).

Table 1: CHECK Sterility Check on SCDM and FTM medium.

Tube Name	Medium	Incubation	Result	Medium	Incubation	Result
Before Deepidermization	SCDM	25°C	No Growth	FTM	33°C	No Growth
After Deepidermization	SCDM	25°C	No Growth	FTM	33°C	No Growth
After 2 weeks NaOH Treatment	SCDM	25°C	No Growth	FTM	33°C	No Growth
After 4 weeks NaOH Treatment	SCDM	25°C	No Growth	FTM	33°C	No Growth
After 6 weeks NaOH Treatment	SCDM	25°C	No Growth	FTM	33°C	No Growth

did not show any cells on it as compared to that of normal skin (Figure : 13, 14). The dermal scaffold was prepared using 42 days NaOH treatment protocol , H and E staining patterns of normal skin before decellularization and ADM are shown above and H and E staining of ADM did not show any purple stains (that of cell nuclei) while pink background (that of cytoplasm and ECM) was present on comparing with staining of normal skin before decellularization. Since the Haematoxylin is not stained it clearly shows the absence of Nucleus, thereby correlating with the above result of complete decellularization. Thus complete decellularization of de-epidermized dermis was observed after 42 days of NaOH treatment.

Basement Membrane study

The less green fluorescence shows that very less alpha 6 integrin was observed in Basement Membrane, but as this is present in BM zone, keratinocytes when seeded can grow on ADM (Figure 15).

Collagen and Elastin in Dermis

The highly green fluorescence shows that presence of intact Collagen, Elastin in the dermis and presence of intact Vimentin in the ADM was confirmed by IF technique (Figure 16, 17).

Keratinocytes Culture

Primary keratinocytes revived from cryopreserved keratinocyte cell bank in the lab was cultured till 3 to 7 days and thereafter seeded onto the ADM for biocompatibility studies (Figure 18, 19).

Seeding of Keratinocytes on ADM

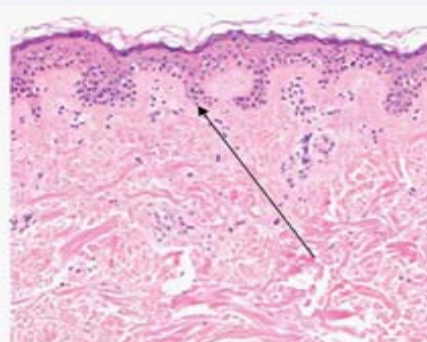


Figure 13 H and E Staining of Normal Skin.

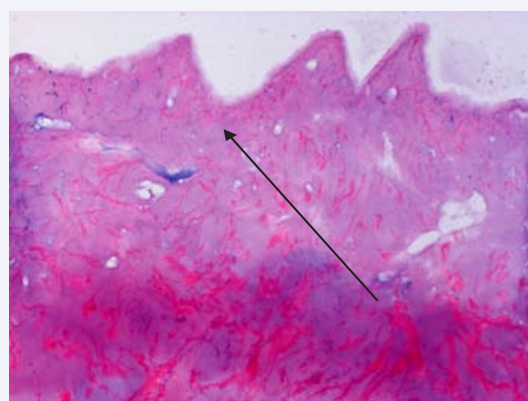


Figure 14 H and E Staining of ADM

As the ADM upon IF study, showed presence of basement membrane component – Alpha 6 Integrin. It signifies that the ADM possesses Basement membrane component. Thus, keratinocytes can be seeded onto this dermis for studying epidermis regeneration and biocompatibility invitro. The ADM when seeded with keratinocytes showed absence of turbidity in the surrounding solution. This signifies the absence of contamination and the attachment of cells onto the ADM (Figure 20, 21).

DISCUSSION

Analyzing the result, it can be assumed that the 'low concentration of NaOH' de-cellularization method is a low cost and effective method which can be adopted for making Acellular dermis. In one of the experiments it was observed that there was less expression of one of the Basement Membrane (BM) protein-alpha 6 beta 4 integrin. These proteins play a very important role in the epithelial cell attachment. Hence, further experiments needs to be done with slight modification of concentration of NaOH to achieve complete restoration of BM proteins. Further, the cell stratification study can be done on this ADM so as to

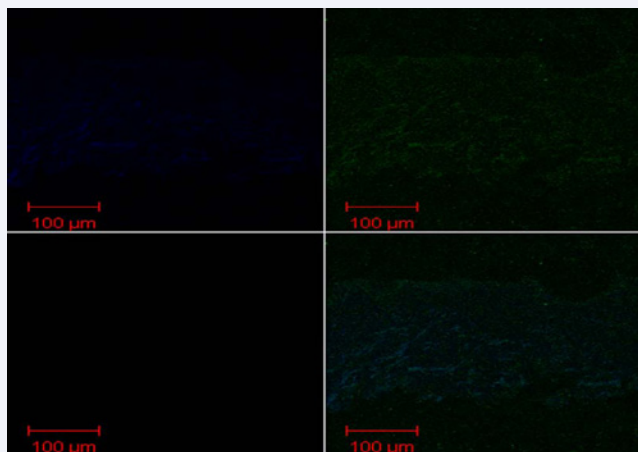


Figure 15 Alpha 6 Integrin Staining of ADM.

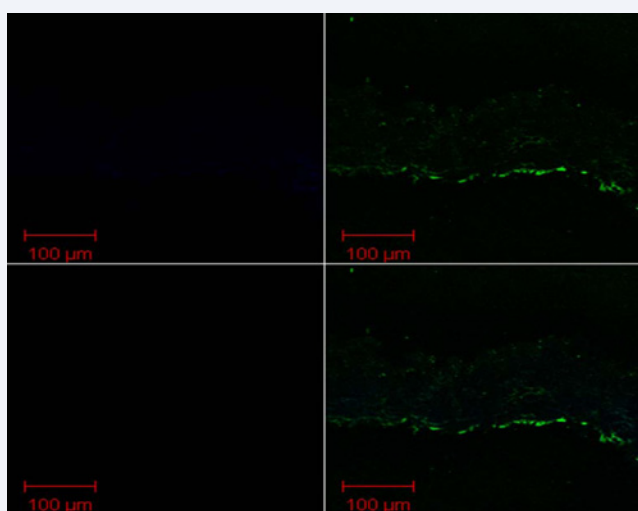


Figure 16 Collagen and Elastin in ADM.

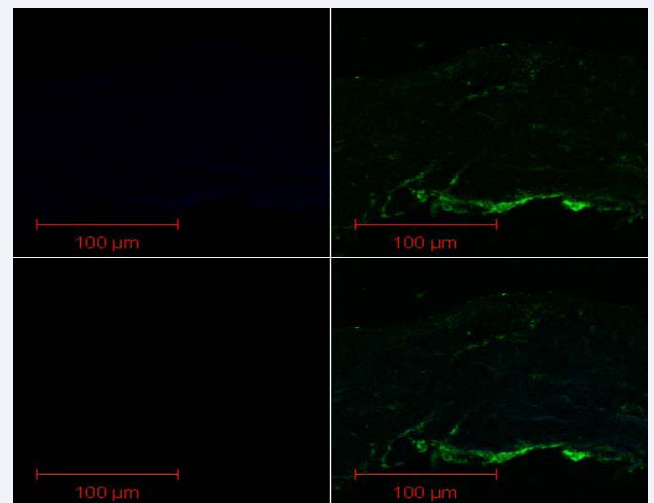


Figure 17 Vimentin in ADM.

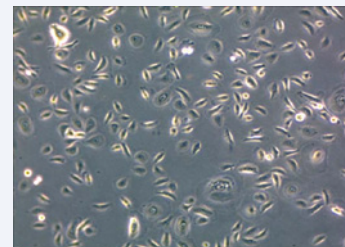


Figure 18 3 Days post seeding keratinocytes Culture.

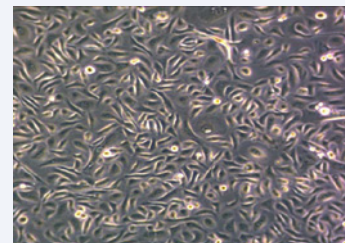


Figure 19 7 Days post seeding keratinocytes Culture.



Figure 20 Keratinocytes seeding culture in ADM.

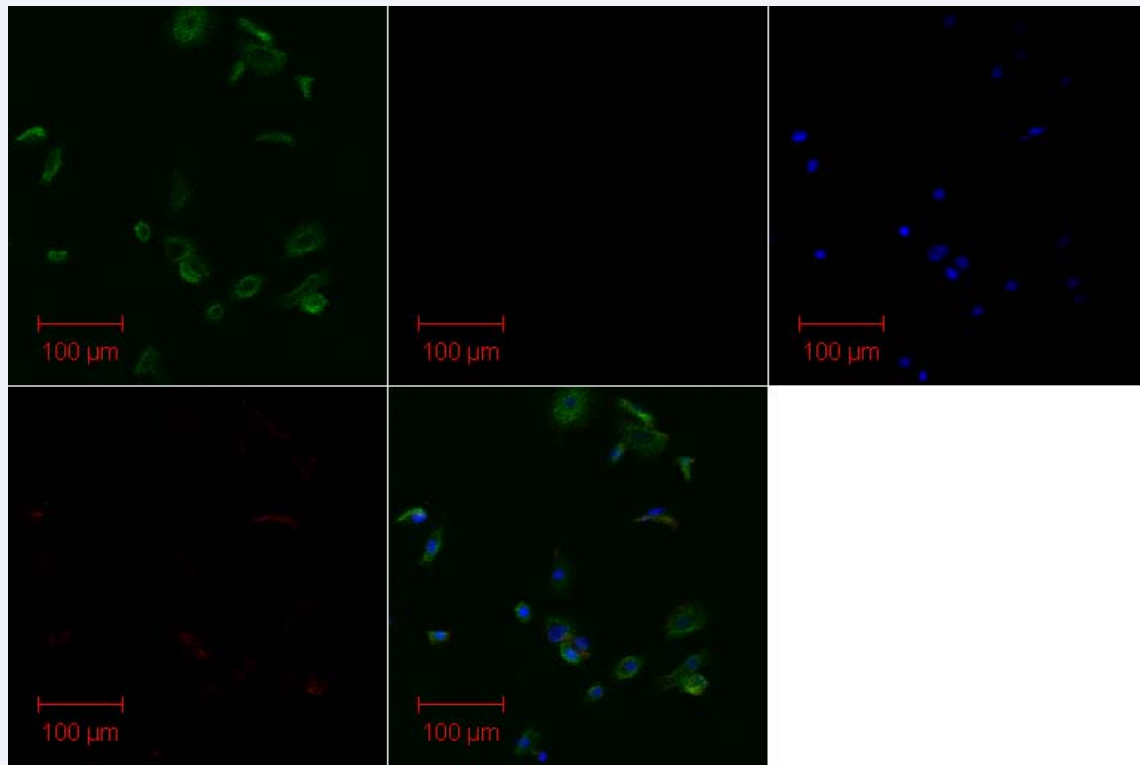


Figure 21 Keratinocytes seeding culture in ADM – Presence of K18 and Vimentin (Merge Image)

confirm that ADM seeded with autologous epidermal cells when applied on burn wound can regenerate the new skin epidermis.

I hope this project was just the first step in the direction of making a cost- effective but clinically effective acellular dermal scaffold which can be the further used to construct tissue engineered skin burn wound resurfacing.

CONCLUSION

After performing the experiments, I came to the conclusion that NaCl and NaOH combination could successfully decellularize the cadaver skin without disintegrating the dermal matrix. The presence of collagen, elastin and vimentin strongly indicates that the decellularization method could preserve the structural integrity of the ADM. The negative microbial growth of the ADM sample proved that the glycerol method of storage of cadaver skin is useful in limiting the microbial contamination. The second most important criteria for any dermal matrix intended for clinical use is to be non-toxic & bio-compatible.

To prove the above points, primary keratinocytes were seeded on to the ADM. We could observe that the cells could grow on the ADM surface. This indicates that this ADM has the structure compatible for cellular division and stratification when applied in-vivo. However further study needs to done.

ACKNOWLEDGEMENTS

I would like to gratefully and sincerely thank the deepest gratefulness to my thesis and Project adviser , Dr. Sunil Keswani

(MEDICAL DIRECTOR) and Ms. Nigamananda Dey (Fellow Researcher) , who gave the golden opportunity to do wonderful project , me and my collaborator researcher in National Burns Centre (NBC) , Airoli, Navi Mumbai - 400 078.

REFERENCES

1. Ahmadi H, Williams G. Permanent scarring in a partial thickness scald burn dressed with Biobrane. *J Plast Reconstr Aesthet Surg.* 2009; 62: 697-698.
2. Shores JT, Gabriel A, Gupta S. Skin substitutes and alternatives: a review. *Adv Skin Wound Care.* 2007; 20: 493-508.
3. Shakespeare P, Shakespeare V. Survey: use of skin substitute materials in UK burn treatment centres. *Burns.* 2002; 28: 295-297.
4. Sheridan RL, Moreno C. Skin substitutes in burns. *Burns.* 2001; 27: 92.
5. Kumar P . Classification of skin substitutes. *Burns.* 2008; 34: 148-149.
6. Khoo TL, Halim AS, Saad AZ, Dorai AA. The application of glycerol-preserved skin allograft in the treatment of burn injuries: an analysis based on indications. *Burns.* 2010; 36: 897-904.
7. Bujang-Safawi E, Halim AS, Khoo TL, Dorai AA. Dried irradiated human amniotic membrane as a biological dressing for facial burns--a 7-year case series. *Burns.* 2010; 36: 876-882.
8. Wood FM1, Kolybaba ML, Allen P. The use of cultured epithelial autograft in the treatment of major burn injuries: a critical review of the literature. *Burns.* 2006; 32: 395-401.
9. Van der Veen VC, Van der Wal MB, van Leeuwen MC, Ulrich MM, Middelkoop E. Biological background of dermal substitutes. *Burns.* 2010; 36: 305-321.
10. Blackwood KA, McKean R, Canton I, Freeman CO, Franklin KL, Cole D,

- et al. Development of biodegradable electrospun scaffolds for dermal replacement. *Biomaterials*. 2008; 29: 3091-3104.
11. Powell HM, Boyce ST. Engineered human skin fabricated using electrospun collagen-PCL blends: morphogenesis and mechanical properties. *Tissue Eng Part A*. 2009; 15: 2177-2187.
 12. Keong LC, Halim AS. In vitro models in biocompatibility assessment for biomedical-grade chitosan derivatives in wound management. *Int J Mol Sci*. 2009; 10: 1300-1313.
 13. Demling RH. Burns. *N Engl J Med*. 1985; 313: 1389-1398.
 14. Whitaker IS, Prowse S, Potokar TS. A critical evaluation of the use of Biobrane as a biologic skin substitute: a versatile tool for the plastic and reconstructive surgeon. *Ann Plast Surg*. 2008; 60: 333-337.
 15. Gerding RL, Imbembo AL, Fratianne RB. Biosynthetic skin substitute vs. 1% silver sulfadiazine for treatment of inpatient partial-thickness thermal burns. *J Trauma*. 1988; 28: 1265-1269.
 16. Mandal A. Paediatric partial-thickness scald burns--is Biobrane the best treatment available? *Int Wound J*. 2007; 4: 15-19.
 17. Hansen SL, Voigt DW, Wiebelhaus P, Paul CN. Using skin replacement products to treat burns and wounds. *Adv Skin Wound Care*. 2001; 14: 37-44.
 18. Hansbrough JF, Mozingo DW, Kealey GP, Davis M, Gidner A, Gentzkow GD. Clinical trials of a biosynthetic temporary skin replacement, Dermagraft- Transitional Covering, compared with cryopreserved human cadaver skin for temporary coverage of excised burn wounds. *J Burn Care Rehabil*. 1997; 18: 43-51.
 19. Purdue GF, Hunt JL, Still JM Jr, Law EJ, Herndon DN, Goldfarb IW, et al. A multicenter clinical trial of a biosynthetic skin replacement, Dermagraft-TC, compared with cryopreserved human cadaver skin for temporary coverage of excised burn wounds. *J Burn Care Rehabil*. 1997; 18: 52-57.
 20. Burke JF, Yannas IV, Quinby WC Jr, Bondoc CC, Jung WK. Successful use of a physiologically acceptable artificial skin in the treatment of extensive burn injury. *Ann Surg*. 1981; 194: 413-428.
 21. Heimbach D, Luterman A, Burke J, Cram A, Herndon D, Hunt J, et al. Artificial dermis for major burns. A multi-center randomized clinical trial. *Ann Surg*. 1988; 208: 313-320.
 22. Pham C, Greenwood J, Cleland H, Woodruff P, Maddern G. Bioengineered skin substitutes for the management of burns: a systematic review. *Burns*. 2007; 33: 946-957.
 23. Peck MD, Kessler M, Meyer AA, Bonham Morris PA. A trial of the effectiveness of artificial dermis in the treatment of patients with burns greater than 45% total body surface area. *J Trauma*. 2002; 52: 971-978.
 24. Waymack P, Duff RG, Sabolinski M. The effect of a tissue engineered bilayered living skin analog, over meshed split-thickness autografts on the healing of excised burn wounds. The Apligraf Burn Study Group. 2000; 26: 609-619.
 25. Ryssel H, Gazyakan E, Germann G, Ohlbauer M. The use of MatriDerm in early excision and simultaneous autologous skin grafting in burns--a pilot study. *Burns*. 2008; 34: 93-97.
 26. Schneider J, Biedermann T, Widmer D, Montano I, Meuli M, Reichmann E, et al. Matriderm versus Integra: a comparative experimental study. *Burns*. 2009; 35: 51-57.
 27. Kolokythas P, Aust MC, Vogt PM, Paulsen F. [Dermal substitute with the collagen-elastin matrix Matriderm in burn injuries: a comprehensive review]. *Handchir Mikrochir Plast Chir*. 2008; 40: 367-371.
 28. Eisenberg M, Llewelyn D. Surgical management of hands in children with recessive dystrophic epidermolysis bullosa: use of allogeneic composite cultured skin grafts. *Br J Plast Surg*. 1998; 51: 608-613.
 29. Still J, Glat P, Silverstein P, Griswold J, Mozingo D. The use of a collagen sponge/living cell composite material to treat donor sites in burn patients. *Burns*. 2003; 29: 837-841.
 30. Myers SR, Partha VN, Soranzo C, Price RD, Navsaria HA. Hyalomatrix: A temporary epidermal barrier, hyaluronan delivery, and neodermis induction system for keratinocyte stem cell therapy. *Tissue Eng*. 2007; 13: 2733-2741.
 31. Oliver RF, Grant RA, Kent CM. The fate of cutaneously and subcutaneously implanted trypsin purified dermal collagen in the pig. See comment in PubMed Commons below *Br J Exp Pathol*. 1972; 53: 540-549.
 32. Grillo HC, Mckhann CF. The acceptance and evolution of dermal homografts freed of viable cells. transplantation. 1964; 2: 48-59.
 33. Wainwright D, Madden M, Luterman A, Hunt J, Monafó W, Heimbach D, et al. Clinical evaluation of an acellular allograft dermal matrix in full-thickness burns. *J Burn Care Rehabil*. 1996; 17: 124-136.

Cite this article

Shukla A, Dey N, Nandi P, Ranjan M (2015) Acellular Dermis as a Dermal Matrix of Tissue Engineered Skin Substitute for Burns Treatment. *Ann Public Health Res* 2(3): 1023.