Regenerative and Antibacterial Properties of Acellular Fish Skin Grafts and Human Amnion/Chorion Membrane: Implications for Tissue Preservation in Combat Casualty Care

Skuli Magnusson, BSc†; Baldur Tumi Baldursson, MD, PhD*‡; Hilmar Kjartansson, MD*‡; Ottar Rolfsson, PhD†; Gudmundur Fertram Sigurjonsson, MEng**

ABSTRACT Background: Improvised explosive devices and new directed energy weapons are changing warfare injuries from penetrating wounds to large surface area thermal and blast injuries. Acellular fish skin is used for tissue repair and during manufacturing subjected to gentle processing compared to biologic materials derived from mammals. This is due to the absence of viral and prion disease transmission risk, preserving natural structure and composition of the fish skin graft. Objectives: The aim of this study was to assess properties of acellular fish skin relevant for severe battlefield injuries and to compare those properties with those of dehydrated human amnion/chorion membrane. Methods: We evaluated cell ingrowth capabilities of the biological materials with microscopy techniques. Bacterial barrier properties were tested with a 2-chamber model. Results: The microstructure of the acellular fish skin is highly porous, whereas the microstructure of dehydrated human amnion/chorion membrane is mostly nonporous. The fish skin grafts show superior ability to support 3-dimensional ingrowth of cells compared to dehydrated human amnion/chorion membrane ($p < 0.0001$) and the fish skin is a bacterial barrier for 24 to 48 hours. Conclusion: The unique biomechanical properties of the acellular fish skin graft make it ideal to be used as a conformal cover for severe trauma and burn wounds in the battlefield.

INTRODUCTION

A shift in the nature of injuries from a traditional battlefield pattern to fighting against insurgents who use improvised explosive devices has brought burn injuries to the forefront of care for injured servicemen. Furthermore, new types of directed energy weapons are being developed that are likely to call for improved therapies for thermal wounds. As the fighting patterns evolve, the need for new and improved therapies in combat casualty care (CCC) for wounds and tissue repair that can be used immediately on the battlefield increases. Nearly, 90% of combat-related deaths occur before arrival at combat field hospitals or transfer to an echelon V hospital capable of providing definitive care.[1] The time it takes for wounded soldiers to be evacuated out of theater varies depending on location, evacuation from Iraq usually takes 48 to 72 hours.[2]

Cadaver skin is the preferred burn wound cover for severe burns and is frequently used in the hospital setting. The use of cadaver skin is, however, impractical in battlefield and other austere environments.[3] In order to respond to this unmet clinical need, attempts have been made to develop several new types of skin substitute in recent years. The main types of skin substitutes are either cellular or acellular. Cellular skin substitutes are not practical for use in a combat theater, as they either need special stabilization fluids with short shelf life or be frozen in liquid nitrogen.[3] Acellular mammalian-derived products possess prolonged shelf life as compared to cellular skin substitutes but per Food and Drug Administration requirements have to undergo "viral inactivation." Viral inactivation is performed with detergents that remove all soluble components from the tissue leaving behind an inert matrix of collagenous structure. The harsh viral inactivation processing removes lipids, glycans, elastins, hyaluronic acid, soluble collagen, and other important biological components from the tissue that potentially are beneficial to wound healing.[4]

Acellular fish skin is remarkably similar to human skin,[5] yet fundamentally different from mammalian-derived matrices, because of the preservation of structure, lipids, and other soluble components. Mammalian scaffolds require harsh chemical processing to reduce viral and prion transmission risk, such risk from the Atlantic cod (*Gadus morhua*) to humans is nonexistent. Fish skin grafts are subjected to gentle processing where structure and bioactive composition, including omega-3 polyunsaturated fatty acids is preserved.[6] Studies have shown that omega-3 fatty acids possess antiviral[7] and antibacterial[8] properties and also act as regulators of inflammation.[9]

Double-blind randomized clinical trials have shown that wound treatment with acellular fish skin allows for significantly faster wound closure than porcine small-intestinal-derived matrices.[10] The acellular fish skin is currently being used in a regulatory approved and patented wound treatment product being marketed in the United States and in Europe under the brand name Kerecis Omega3 (Kerecis, Isafjordur, Iceland). After grafting, the fish skin is incorporated into the damaged area and infiltrated by autologous cells that convert

*Kerecis Limited, Eyrargata 2, 400 Isafjordur, Iceland.

†Center for Systems Biology, University of Iceland, Sturlugata 8, 101 Reykjavik, Iceland.

‡National University Hospital of Iceland, Nordurmyri, 101 Reykjavik, Iceland.

doi: 10.7205/MILMED-D-16-00142

the graft into functional, living tissue while the graft slowly breaks down. The fish skin graft has been used to treat a large number of wounds of various etiologies, both acute and chronic.[11,12]

Here, we set out to investigate properties of the acellular fish skin that are important for tissue repair and regeneration. Employing microscopy-based techniques, we compared the fish skin to dehydrated human amnion/chorion membrane (dHACM) allograft, with respect to structure and ability to sustain fibroblast ingrowth in culture. Furthermore, we investigated the ability of the acellular fish skin to resist bacterial invasion.

METHOD

Cell Culture

To assess cell ingrowth capability of the biologic materials, the scaffolds were seeded with cells. We conducted all cell culture experiments at 95% humidity and with 5% CO_2. For all cell culture experiments, we used high glucose Dulbecco's Modified Eagle's medium (DMEM) (D6429; Sigma Aldrich, St. Louis, Missouri) with 10% fetal bovine serum (Cat. 10270-106; Thermo Fisher Scientific, Waltham, Massachusetts) and 1% Antibiotic-Antimycotic solution (Cat. 15240-062; Life Technologies, Carlsbad, California). Before seeding the biologic materials, we prehydrated them in DMEM for 24 hours and incubated in cell culture cabinets. The punches we made from the biologic materials were 6 mm in diameter, roughly the same size as the wells (0.32 cm^2) of a 96 well plate. We cultured NIH 3T3 mouse embryo fibroblast cell line (ATCC, CRL-1658). We split the cells when they reached 70 to 80% confluency and then seeded each biologic material with 16,000 cells in 50 μL volume of DMEM. After four hours, we added 150 μL of medium bringing the final volume to 200 μL in each well. Biologic materials that did not receive cells served as negative controls. After 24 hours, we transferred the biologic materials to 12-well plate and incubated in a cell culture hood for 12 days. Medium was changed every 3 days and wells checked for contamination. After 12 days, we harvested the samples and stained them after fixation.

Fixation

First we fixed the samples in 10% formalin for 48 hours. Next we placed the samples in a fixation machine (Tissue-Tek VIP 6; Sakura, Torrance, California) with the following program: 10% formalin: 2 hours, 70% alcohol: 2 hours, 90% alcohol: 2 hours, 100% alcohol: 2 hours, xylene: 2 hours. Lastly, we embedded the samples in paraffin for 2 hours. We cut 2 μm slices and put them on a slide in a 70°C heat cabinet for 30 minutes. Next, we embedded the slides in xylene for 15 minutes and washed with 100% alcohol for 5 minutes and water for 2 minutes.

Hematoxylin and Eosin Staining

We embedded slides with fixed samples with hematoxylin: 8 minutes, water: 2 minutes, 0.02 M HCL in 70% alcohol: 2 minutes, water: 4 minutes, eosin: 2 minutes, 100% alcohol: 14 minutes. Lastly, we glued cover slips on top of the slides.

Periodic Acid Schiff Staining

Samples are deparaffinized and hydrated in water then added to 0.5% periodic acid solution for 5 minutes and then Schiff's reagent for 15 minutes. We used Mayer's hematoxylin as a counter stain.

Masson Trichrome

Samples are fixed in 100% alcohol, 95% alcohol, 70% alcohol, Weigert's iron hematoxylin working solution, and Biebrich scarlet-acid in solution for 10 to 15 minutes. Sections are transferred to aniline blue solution and stained for 5 to 10 minutes. Lastly, the slides are rinsed briefly in distilled water and differentiated in 1% acetic acid solution.

Fluorescent Staining

Before staining, we used hydrophobic barrier pen to surround the fixed tissue on the slides. We diluted 6 μL of nuclear stain (NucBlue, Cat. R37605; Thermo Fisher Scientific) with 994 μL of 1× phosphate-buffered saline buffer with 1% Triton X-100 (Sigma Aldrich). We added 30 μL of the fluorescent staining solution to the slides and incubated at 37°C for 30 minutes. We washed the slides 3 times with 1× phosphate-buffered saline and lastly we added mouting medium (Fluormount, F4680; Sigma Aldrich) to the samples and glued cover slips on top.

Microscopy

We used a Field Emission Scanning Electron Microscope (Zeiss Supra 25, Oberkochen, Germany). The software program used to run the microscope is Smart SEM. The unit we used to gold coat the specimens is an Edwards S150B sputter coater. We coated the specimens for 2 minutes and afterward we mounted them on aluminum stubs with carbon tabs. We used light microscope (Leica DM-I6000B; Leica-Microsystems, Wetzlar, Germany) to observe hematoxylin and eosin (H&E)-stained samples and confocal microscope (FV1200; Olympus, Tokyo, Japan) to observe samples stained with fluorescent stains.

Porosity and Thickness

We counted the number of pores per 100 μm of sample and measured the diameter of the pores using the software ImageJ (Wayne Rasband, National Institute of Health, Bethesda, Maryland) and scale bar on the image. We did not count pores with a diameter of 1 μm or less. ImageJ was also used to calculate the diameter of pores from the scanning electron microscope images.

Cell Ingrowth Quantification and Statistical Analysis

We quantified the cell ingrowth by counting fluorescently labeled cells within the biologic materials using the software

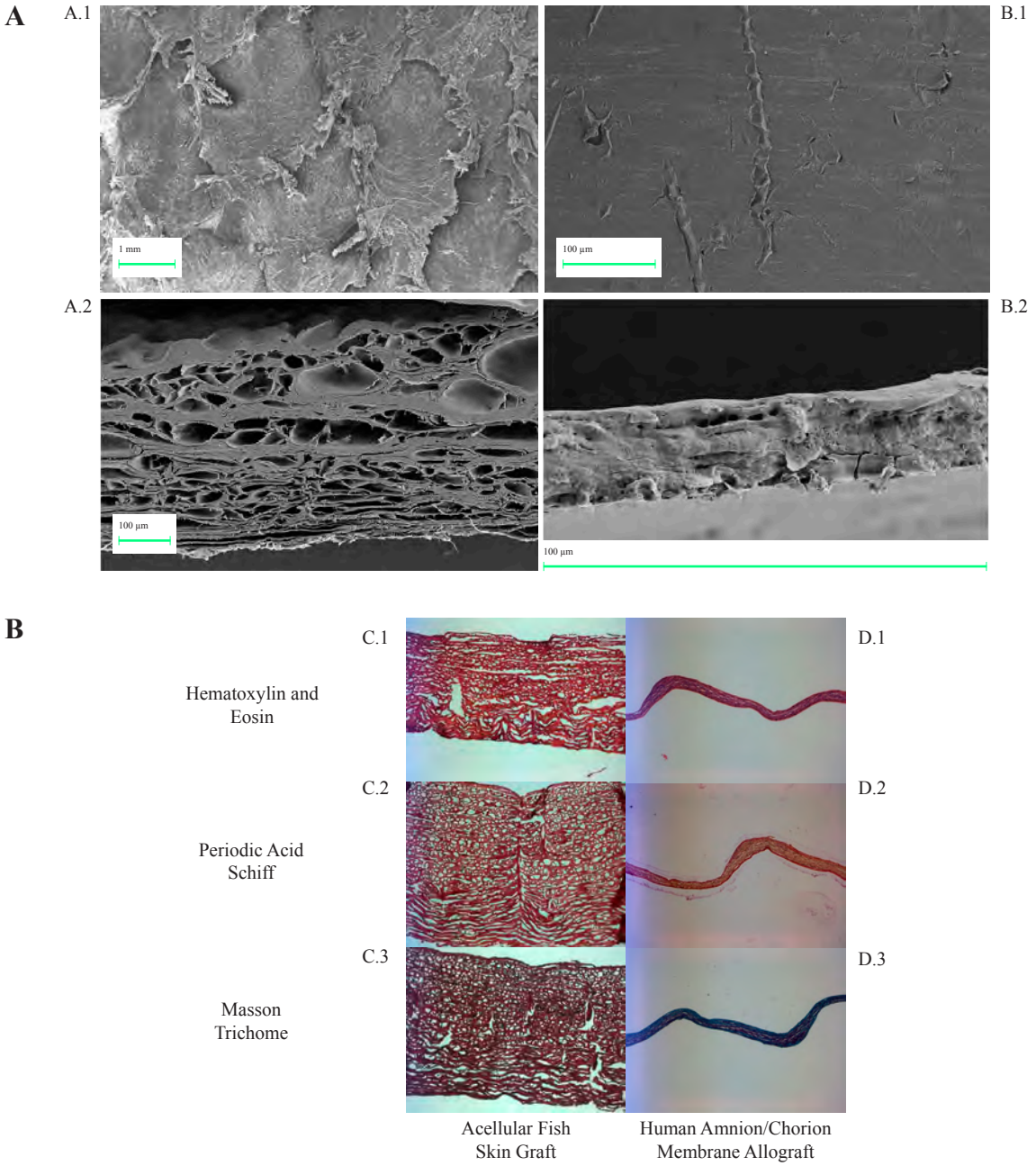


FIGURE 1. Scanning electron microscope images showing the thickness and porosity of acellular fish skin and amnion/chorion membrane allografts. (A) Acellular fish skin graft with: (A1) surface with 370× magnification. Scale bar is 1 mm and (A2) cross section of 300× magnification. Scale bar is 1 µm. (B) Human amnion/chorion membrane allograft: (B1) surface with 600× magnification and (B2) cross section of a 3,000× magnification. Scale bars are 100 µm. Histology of cross section of (C) acellular fish skin graft and the (D) dHACM allograft. H&E staining shows that the (C1) fish skin graft contains collagens, extracellular matrix components and no cells and (D1) dHACM contains nuclei and some extracelluar components. Periodic acid schiff stain shows glycoproteins and proteoglycans in the (C2) fish skin and (D2) dHACM. Masson trichrome staining shows connective tissue and collagens in the (C3) fish skin graft, connective tissue and nuclei is seen in (D3) dHACM.

ImageJ. We divided the images into columns with their width corresponding to 100 µm of sample and used the free hand tool in ImageJ to define the boundaries of the materials. Cells were considered ingrown when observed within the material. We only counted cells observed within the biologic materials but excluded the cells on top of the materials (Fig. 3. The mean values and standard deviations as well as all other statistical analysis were performed using the statistical application R.3.2.3 (Bell Laboratories, Murray Hill, New Jersey). All groups were tested for a normal distribution by the Shapiro–Wilk method and statistical methods were chosen according to those results and the group dynamics. Wilcoxon

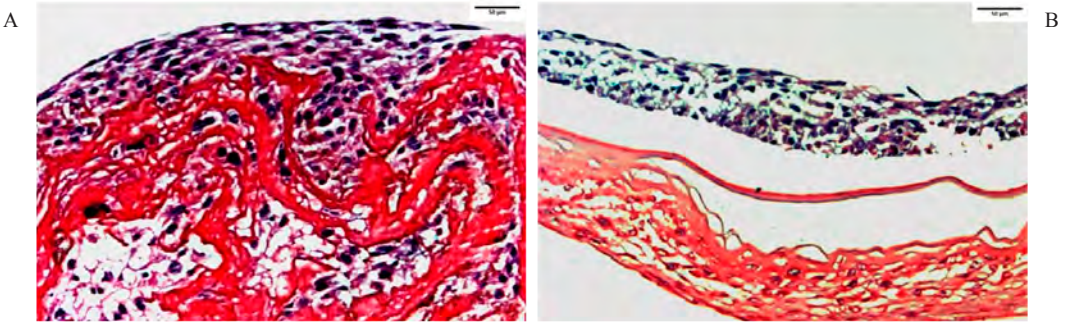


FIGURE 2. Fibroblasts infiltrate and remodel the fish skin graft but do not interact with human amnion/chorion membrane. H&E staining of (A) fish skin graft and (B) dHACM. Cross sections are shown. Fibroblast cells infiltrate and remodel the fish skin graft following 12 days of culture. Cells infiltrate dHACM material to a much lesser extent and are primarily observed on the top layer of the dHACM material. Scale bars are 50 μm.

rank sum test with continuity correction was used to analyze significant differences between groups when at least one of the groups did not contain normally distributed data.

Bacterial Barrier

We prepared an overnight culture of *Staphylococcus aureus* (ATCC 25923) and diluted it after thorough mixing for 60 seconds on a vortex mixer. From the overnight culture, we diluted 1.0 mL into 9.0 mL of broth repeatedly to make 10-fold dilutions from the maximum recovery diluent (MRD). From each dilution, we transferred 1.0 mL into separate petri plates and added 20 mL of melted Tryptone soya agar at 45°C. We incubated the plates at 35.0 ± 1.0°C for 48 hours. After the incubation, we counted colonies on the plates and calculated the number of colony-forming units in the initial suspension. We transferred 200 μL of precultured bacteria

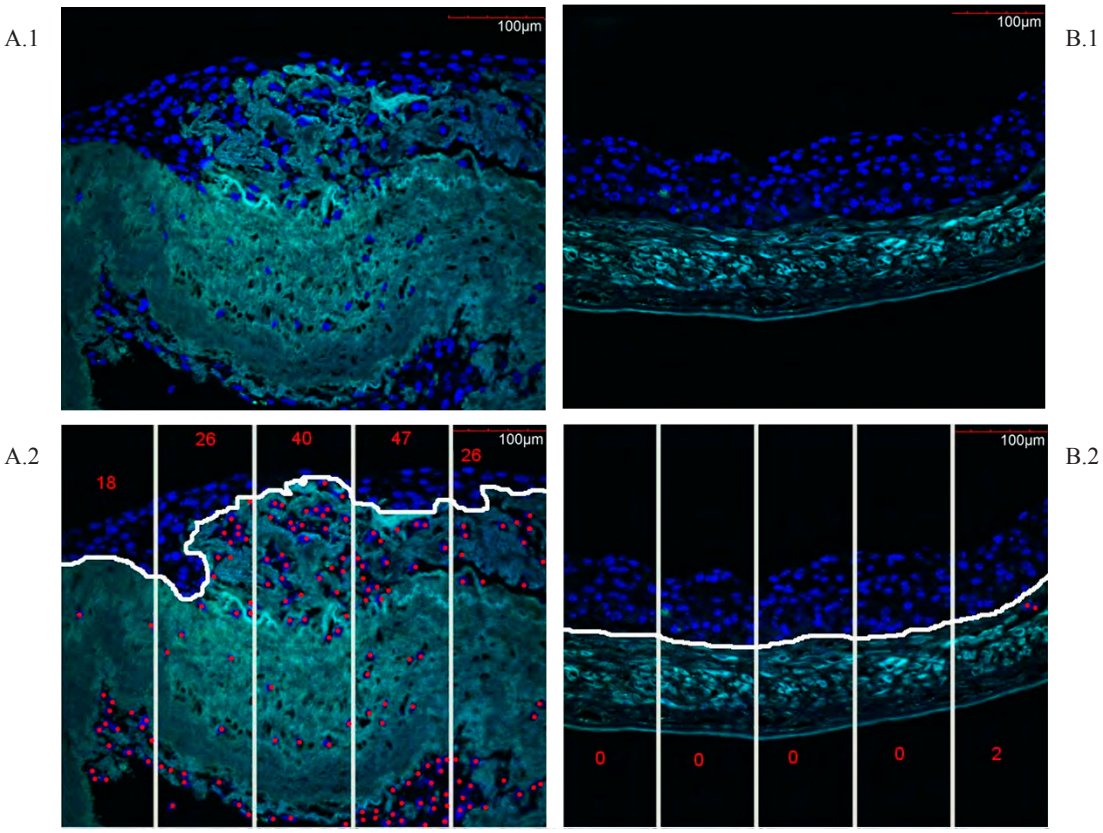


FIGURE 3. Cell ingrowth is clearly seen into the fish skin graft but to a much lesser extent to the human amnion/chorion membrane allograft. Fibroblasts were seeded onto fish skin graft and dHACM that were then imaged following 12 days of culture in the presence of a fluorescent antibody against nuclei (blue). Both the fish skin graft and the dHACM emit green autofluorescence at certain wavelengths. Cells are clearly observed to infiltrate the thicker (A1) fish skin graft, whereas fewer are observed in (B1) dHACM. The lower row (A2 and B2) shows cellular quantification in the respective grafts that are summarized in Figure 4. Scale bars are 100 μm.

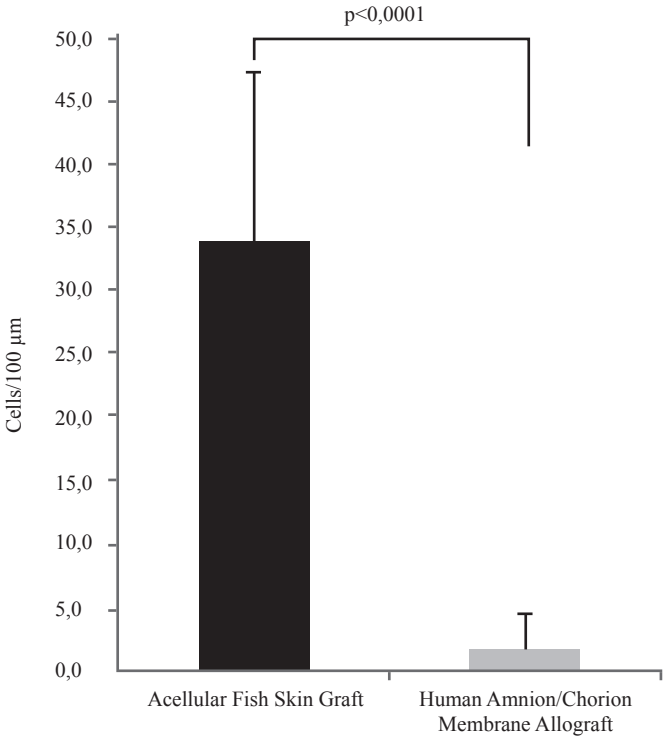


FIGURE 4. Significantly more cell ingrowth into acellular fish skin compared to human amnion/chorion membrane. Comparison of cellular infiltration into acellular fish skin graft compared to human amnion/chorion membrane allograft. There's significant difference between cell ingrowth into the fish skin graft as compared to the dHACM material ($n = 7$) ($p < 0.0001$).

corresponding to 7,000 cfu into 1 of the 2 chambers unit. Pieces of membranes made of fish skin to be tested were cut to cover the insert with sterile scissors and placed between the insert units and a clamp was used to screw the 2 inserts tightly together. The cut was 1 mm outside the edge of the insert. We injected sterile tryptic soy broth (TSB) into 1 of 2 inserts and TSB with a bacterial strain into the other. Control samples containing only TSB were prepared. The chambers were incubated at 37.0 ± 1.0°C. Each sample was analyzed in triplicate as were the control samples. Recovery tests were done after 4, 24, and 48 hours. From the originally sterile TSB chambers, 1.0 mL was transferred into 9.0 mL of maximum recovery diluent and further 10-fold dilutions were made as described earlier. The plates were incubated at 35.0 ± 1.0°C for 48 hours. After the incubation, the colonies on the plates were counted and the number of colony-forming units in the initial suspension calculated. The same amount of sterile TSB was added into the unit to secure that the surface was always the same.

RESULTS AND DISCUSSION

Microstructure of acellular fish skin is highly porous and the microstructure of human amnion/chorion membrane is mostly nonporous. Initially, we wanted to compare the microstructure of the biologic materials and evaluate if the porosity was well suited for cell ingrowth. The fish skin graft is highly porous compared to human amnion/chorion membrane. The fish skin contains 16.7 holes ($n = 6$) per 100 µm on average, whereas the dHACM contains 1.7 holes ($n = 6$) per 100 µm on average (Fig. 1). The average diameter of the pores in the fish skin graft is 16.1 µm ($n = 5$) and 1.3 µm in the dHACM ($n = 3$). The thickness of the dHACM is 20.1 µm ($n = 6$) on average and the thickness of the fish skin graft is 450 µm ($n = 6$) on average (Fig. 1).

Acellular fish skin graft shows superior cell ingrowth when compared to human allograft. After evaluating the porosity of the biologic materials, we hypothesized that the fish skin grafts are well suited to support cell ingrowth since the pore size is within the range of typical cell size. Hematoxylin and eosin staining indicates that the fibroblasts do remodel the fish skin graft as they migrate and proliferate into the graft. The fibroblasts, however, form a layer on top of the dHACM allograft material and do not interact with the graft to the same extent (Fig. 2). The acellular fish skin grafts showed significantly ($p < 0.0001$) more 3-dimensional ingrowth of cells when compared to the dHACM (Figs. 3 and 4).

The acellular fish skin grafts can withstand bacterial invasion for up to 48–72 hours. In addition to regenerative capabilities, biologic materials used on battlefield wounds should, ideally possess antibacterial properties. Therefore, the fish skin graft was tested for its ability to resist bacterial invasion. The bacterial properties of the fish skin graft were tested by constraining the material between a 2-chamber unit and adding medium with bacteria on the other side and medium only in the second chamber. The fish skin graft can act as a bacterial barrier for up to 48–72 hours, $n = 3$ and when the fish skin graft supplemented with additional amount of omega-3, its bacterial barrier properties are augmented (Table I). Previously, it has been demonstrated that omega-3 PUFA have antibacterial properties against multiresistant bacteria[8] and they might play a key role for the ability of the fish skin graft to act as a bacterial barrier.

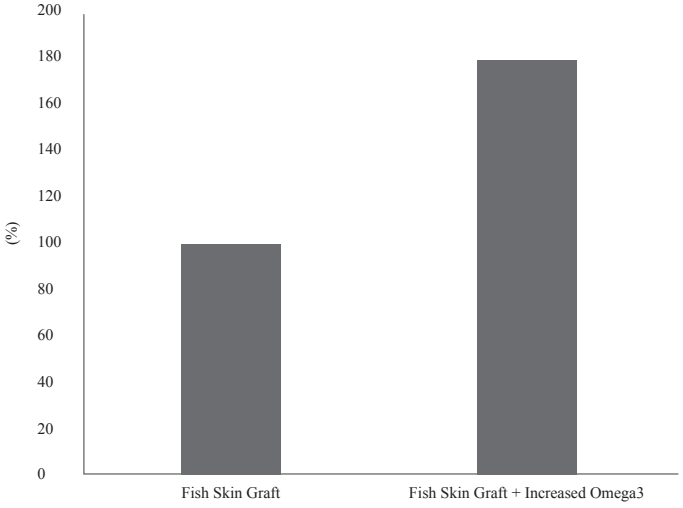
Acellular fish skin has the potential to become a battlefield wound treatment of choice. The first few hours of CCC is vital for saving lives. An improvement in advanced field trauma wound treatment may play a large role in reducing morbidity, increased the viability of injured tissues. Burn injuries are often associated with significant complications such as localized or systemic infections, cosmetically unacceptable scar formation, and incomplete wound healing.[13] Thermal injuries due to improvised explosives were the most serious threat to soldiers operating in Afghanistan and Iraq. Those burn patients face many complications, including supplementary injuries from multiple fragment wounds, high rate of infection, and long rehabilitation and hospitalization time.[14] Cadaver skin is the preferred burn wound cover for severe burns and is frequently used in the hospital setting. The use of cadaver skin is however impractical in battlefield and other austere environments.[15]

Overall, the results presented here show that acellular fish skin grafts act as a bacterial barrier and possess superior

TABLE I. The fish skin graft withstands bacterial invasion for up to 48–72 hours ($n = 3$)

n	24 Hours	36 Hours	48 Hours	72 Hours
1	0	0	4.7	NA
	0	0	0	8.3
2	0	0	8.1	NA
	0	0	7.9	NA
3	0	8.2	NA	NA
	0	7.4	NA	NA

This table summarizes the results from the 2-chamber assay with the fish skin graft, given in log(colony forming units/mL). The fish skin graft was able to act as a bacterial barrier for up to 48–72 hours. By increasing the omega-3 content of the fish skin graft the bacterial barrier properties are augmented.



ability to stimulate 3-dimensional cell ingrowth compared to dHACM allograft. Cellular ingrowth is a fundamental first step of tissue regeneration. The porosity of the fish skin structure and natural omega-3 content likely plays a key role in its regenerative and bacterial barrier properties.

New generation of highly destructive thermal weapons will present new challenges for CCC to treat extreme trauma, amputations, and burn wounds.[16] The need to develop new medical technologies to counteract the fast development of high-tech destructive weapons is great. The fish skin graft can be used as a conformal cover for a severe battlefield wounds preserving and stimulating the flesh and protecting it from bacterial infection until the injured soldier is brought to medical facility. Furthermore, the graft has the potential to impede eschar formation following burns given prompt wound treatment. The fish skin graft integrates into a wound bed in 7–10 days.[10] In that phase it provides a potential for a physical barrier, allowing for safer transfer to an Echelon V facility. Fish skin grafts therefore show promise as a future standard-of-care product for burn treatments upon the battlefield.

There is an unmet gap and the potential of the fish skin graft exists for the development of better treatments, which will significantly improve functional and cosmetic outcomes in military service member suffering from severe burn or trauma wound victims resulting in reduced morbidity and mortality and better quality of life. Here, we have described the characteristics of the acellular fish skin graft that has the potential to fill this unmet gap.

ACKNOWLEDGMENTS

We want to thank the staff of the government research institute Matis for their contribution on the bacterial studies. This research was funded by the Office of Naval Research and Kerecis Limited, Eyrargata 2, 400 Isafjordur, Iceland.

REFERENCES

1. Eastridge BJ, Mabry RL, Seguin P, et al: Death on the battlefield (2001-2011): implications for the future of combat casualty care. J Trauma Acute Care Surg 2012; 73(6 Suppl 5): S431–7.
2. Murray CK: Epidemiology of infections associated with combat-related injuries in Iraq and Afghanistan. J Trauma 2008; 64(3 Suppl): S232–8.
3. Calota D, Nitescu C, Florescu I, Lascar I: Surgical management of extensive burns treatment using allografts. J Med Life 2012; 5(4): 486–90.
4. Crapo PM, Gilbert TW, Badylak SF: An overview of tissue and whole organ decellularization processes. Biomaterials 2011; 32(12): 3233–43.
5. Rakers S, Gebert M, Uppalapati S, et al: "Fish matters": the relevance of fish skin biology to investigative dermatology. Exp Dermatol 2010; 19(4): 313–24.
6. Magnusson S, Baldursson BT, Kjartansson H, et al: Decellularized fish skin: characteristics that support tissue repair. Laeknabladid 2015; 101(12): 567–73.
7. Imai Y: Role of omega-3 PUFA-derived mediators, the protectins, in influenza virus infection. Biochim Biophys Acta 2015; 1851(4): 496–502.
8. Mil-Homens D, Bernardes N, Fialho AM: The antibacterial properties of docosahexaenoic omega-3 fatty acid against the cystic fibrosis multiresistant pathogen Burkholderia cenocepacia. FEMS Microbiol Lett 2012; 328(1): 61–9.
9. Serhan CN: Pro-resolving lipid mediators are leads for resolution physiology. Nature 2014; 510(7503): 92–101.
10. Baldursson BT, Kjartansson H, Konradsdottir F, Gudnason P, Sigurjonsson GF, Lund SH: Healing rate and autoimmune safety of full-thickness wounds treated with fish skin acellular dermal matrix versus porcine small-intestine submucosa: a noninferiority study. Int J Low Extrem Wounds 2015; 14(1): 37–43.
11. Trinh TT, Dünschede F, Vahl C-F, Dorweiler B: Marine omega3 wound matrix for the treatment of complicated wounds. Phlebologie 2016; 45: 93–8.
12. Yang CK, Polanco TO, Lantis II JC: A prospective, postmarket, compassionate clinical evaluation of a novel acellular fish-skin graft which contains omega-3 fatty acids for the closure of hard-to-heal lower extremity chronic ulcers. Wounds 2016; 28(4): 112–8.
13. Rowan MP, Cancio LC, Elster EA, et al: Burn wound healing and treatment: review and advancements. Crit Care 2015; 19(1): 243.
14. Kauvar DS, Wolf SE, Wade CE, Cancio LC, Renz EM, Holcomb JB: Burns sustained in combat explosions in Operations Iraqi and Enduring Freedom (OIF/OEF explosion burns). Burns J Int Soc Burn Inj 2006; 32(7): 853–7.
15. Ramasamy A, Hill AM, Clasper JC: Improvised explosive devices: pathophysiology, injury profiles and current medical management. J R Army Med Corps 2009; 155(4): 265–72.
16. Sprangle P, Hafizi B, Ting A, Fischer R: High-power lasers for directed-energy applications. Appl Opt 2015; 54(31): F201–9.