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In situ forming biomaterials as muscle void fillers for the provisional treatment of volumetric muscle loss injuries

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ABSTRACT

Volumetric muscle loss (VML) represents a devastating extremity injury which leads to chronic functional deficits and disability and is unrecoverable through normal healing pathways. When left untreated, the VML pathophysiology creates many challenges towards successful treatment, such as altered residual muscle architecture, excessive fibrosis, and contracture(s). As such, innovative approaches and technologies are needed to prevent or reverse these adverse sequelae. Development of a rationally designed biomaterial technology which is intended to be acutely placed within a VML defect – i.e., to serve as a muscle void filler (MVF) by maintaining the VML defect – could address this clinical unmet need by preventing these adverse sequelae as well as enabling multi-staged treatment approaches. To that end, three biomaterials were evaluated for their ability to serve as a provisional MVF treatment intended to stabilize a VML defect in a rat model for an extended period (28 days): polyvinyl alcohol (PVA), hyaluronic acid and polyethylene glycol combination (HA + PEG), and silicone, a clinically used soft tissue void filler. HA + PEG biomaterial showed signs of deformation, while both PVA and silicone did not. There were no differences between treatment groups for their effects on adjacent muscle fiber count and size distribution. Not surprisingly, silicone elicited robust fibrotic response resulting in a fibrotic barrier with a large infiltration of macrophages, a response not seen with either the PVA or HA + PEG. Taken together, PVA was found to be the best material to be used as a provisional MVF for maintaining VML defect volume while minimizing adverse effects on the surrounding muscle.

1. Introduction

Traumatic injuries to the extremities often involve damage to multiple types of tissues (e.g. bone, muscle, nerve, vascular) which complicates their treatment. As such, these composite tissue injuries (CTI) are a major cause of disability for affected patients and elicit a significant strain on the healthcare system. Gustilo-Anderson type III open fractures are particularly difficult to treat due to the severity of bone loss and the extensive damage to the adjacent soft tissue (e.g. volumetric muscle loss; VML). Staged surgical procedures have been shown to be effective for treating large bone defects [1,2], but current treatment strategies often neglect the soft tissue during this process beyond simply achieving closure or coverage, which leads to suboptimal healing and functional outcomes. Thus, there exists a current unmet need to develop a provisional muscle void filler (MVF) technology that can be used acutely during multi-stage surgical treatment strategies to stabilize the surrounding soft tissue and prevent pathological changes, specifically for the acute management of VML. Such an MVF could be used in CTI in

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parallel with a bone spacer in which the induced membrane technique [3] is needed.

Multi-staged treatment strategies may also be imposed by practical limitations, such as in a prolonged care scenario [4] where military personnel injured in a far-forward setting will have limited or delayed access to medical supplies and evacuation [5]. As such, access to the types of definitive treatments currently being investigated for treating VML will be delayed by days or weeks. Delayed treatment of VML is particularly problematic as its pathogenesis is known to include prolonged inflammation, fibrosis, loss of innervation potential, and contracture when left untreated. Currently, the majority of existing VML-focused therapies are designed for use in fixed facilities (e.g., Role IV) and are not suitable for use in far forward settings (i.e., Role I-II), much less within the projected austere prolonged care environment. Even in higher echelon settings, combat-related composite tissue injuries are, as a rule, contaminated to such a degree as to preclude attempts at acute repair, reconstruction, or reconstitution of VML injuries. As such, there exists an unmet need for acute management that can successfully temporize and minimize sequelae of VML injury for an extended period of time until a subsequent, definitive treatment can be administered. To this end, an MVF that can be used within a far-forward setting to temporarily stabilize the VML injury until it can be definitively treated at an appropriate medical facility is necessary to improve outcomes and preserve the feasibility of current and future reconstructive alternatives for VML.

An ideal MVF for VML would have the following properties: (1) conform to and maintain the shape and volume of the residual void after a VML injury for at least 4 weeks (an appropriate timeline for both multi-staged strategies [3] and evacuation from a prolonged care environment), (2) elicit a minimal host foreign body response, and (3) prevent negative downstream outcomes such as fibrosis and contracture. Additionally, to extend the utility of such an intervention to military-relevant prolonged care scenarios, the MVF should (1) be simple to transport, (2) easy to apply, and (3) have a long shelf-life over a range of temperatures [5]. By fulfilling these requirements, an acutely placed MVF would preserve a VML void in a way that is permissive of successful recovery after subsequent definitive treatment, and as such would be of great value for military and civilian applications alike.

Several polymeric biomaterials exist which may be suitable for use in this MVF application but they require comprehensive evaluation. One example is polyvinyl alcohol (PVA), a synthetic polymer that is versatile due to its ability to be easily modified and functionalized [6]. PVA has the ability to form a hydrogel with tissue-like mechanical properties under pre-defined conditions such as light exposure and does not cause significant irritation of surrounding tissue when implanted [7]. While PVA is degradable, the degradation rate is tunable; specifically, it can be tuned to be minimally degradable over the desired 4-week lifespan of an MVF. Furthermore, PVA lacks sites for cell attachment which may limit robust tissue in-growth and thus allow for an easy(r) removal from a muscle defect. For these reasons, PVA could be an optimal material for an MVF but needs comprehensive evaluation for this application.

Another polymer that has been a focus of recent research is hyaluronic acid (HA), which has anti-inflammatory properties [8] and can be combined with polyethylene glycol (PEG) to create tunable hydrogels without sites for cell attachment [9]. These composite hydrogels can be formed *in situ* via simple, pre-defined means such as light exposure. HA is naturally present in human tissue (e.g., cartilage) and has excellent biocompatibility [10]. These properties are advantageous for an MVF.

Silicone has been used as a filler for numerous other soft tissue applications [11] and is widely available. Silicone exists in different forms, some with high mechanical properties that can make it minimally deformable. It is non-degradable and thus could potentially serve as an MVF, not only acutely but also indefinitely, if/when that may be desired. Silicone, like the other candidate materials, does not allow for cell attachment. However, the clinical use of silicone implants has shown adverse effects causing a negative outlook towards their use [12]. For these reasons, silicone has been included as a benchmark for an MVF that is widely utilized in other medical and reconstructive applications but is known to elicit a strong host foreign body response.

The purpose of this study was to evaluate candidate polymeric biomaterials that could serve as an MVF treatment approach for VML injuries. The chosen polymers are conceivably suitable for use in both conventional medical centers and far-forward, prolonged care settings. This study utilized these polymers with functional groups that allowed for crosslinking utilizing ultraviolet light. However, these polymers could be made with other functional groups that allow for different crosslinking mechanisms such as visible light or chemical initiator. The ability to polymerize *in situ* is highly valuable for making the MVF conform to a range of VML defect shapes and sizes. Finding an appropriate biomaterial and its development into a successful MVF would enable effective multi-staged treatment strategies for traumatic injuries with volumetric muscle loss.

2. Materials & methods

2.1. In vitro assessment of biomaterials

The biomaterial systems selected to be evaluated for use as MVFs are as follows: 1) an acrylamide-modified PVA at 10% in water (70 kDa; BioCure), 2) a composite of thiol-modified HA (50k Da; Creative PEGWorks) and methacrylate-modified polyethylene glycol (5 kDa; Creative PEGWorks) at 20 mg mL^{-1} in PBS (HA + PEG), and 3) a UV-curable silicone (Henkel). Both the PVA and HA-PEG required the addition of 1% v/v Irgacure 2959 at 1000 mg/mL in DMSO (Advanced Biomatrix) as the photoinitiator. Precursor solutions of each biomaterial solution were crosslinked using a UV LED Spot-Curing System (UVFAB) with 385 nm wavelength at 6800 mW cm^{-2}.

For swelling studies, a precursor of 150 μL for each biomaterial system was individually pipetted into a cylindrical mold and exposed to UV light for 2 min. Hydrogels were then blotted, weighed, and incubated in phosphate buffered saline (PBS) at 37 °C for 24 h. After incubation, samples were again blotted and weighed. Their swelling ratios determined by the following equation [13]:

$$Swelling\ Ratio = \frac{Wt_{swollen} - Wt_{nonswollen}}{Wt_{nonswollen}}$$

To assess the mechanical properties of the biomaterials, rheology was performed (TA Instruments Discovery Hybrid Rheometer, HR-20T). A biomaterial precursor solution of 50 μL for each biomaterial system was individually pipetted on the rheometer plate at 25 °C and an 8 mm test geometry was lowered to a gap of 1000 μm. The sample was exposed to UV light for 2 min prior to testing, and a frequency sweep was performed at 5% strain over 0.1–100 Hz [14].

Mechanical testing was further assessed via compression testing [15]. Biomaterials were polymerized in 24 mm diameter disks (PVA, HA + PEG) or 6 mm diameter disk (silicone) using UV light. The discrepancy in geometry was necessitated due to the difference in stiffnesses and the available load cells. Biomaterials were allowed to swell in PBS for 24 h. The disks were then subjected to compression testing on an UniVert tester (CellScale Biomaterials Testing) equipped with load cells ranging from 1 N to 50 N. Compression strain of 20% (PVA, HA + PEG) or 15% (silicone) was applied over a 60 s period. Young's modulus was obtained from the slope of a linear regression of the initial portion of stress versus strain values.

For scanning electron microscopy (SEM), crosslinked samples were swollen overnight as described above and then lyophilized for 24 h before coating with Au/Pd (60:40) using a Cressington 108 (Cressington) before imaging. Imaging was performed via FEI NovaNano SEM 450 (HV: 5 kV, magnification: 2000×) [16,17].

2.2. Surgical procedures

All animal procedures were approved by the Institutional Animal Care and Use Committee at the Uniformed Services University of the Health Sciences and were conducted in AAALAC-accredited facilities of the Department of Laboratory Animal Research. Adult male Lewis rats (10–12 weeks old, ~350 g; Charles River Laboratories) received unilateral VML injuries in the tibialis anterior (TA) muscle using previously established methods and were randomly allocated to experimental groups [18]. Prior to surgery, animals received a subcutaneous dose of Ethiqa XR (buprenorphine extended release; 0.65 mg kg^{-1} bodyweight) for analgesia, and a surgical plane of anesthesia was induced (5% isoflurane) and maintained (1–3% isoflurane). A lateral incision was created on the left hindlimb of the animal, reflecting the skin and underlying fascia to expose the TA muscle. A 6 mm, full-thickness biopsy was removed from the middle third of the muscle belly to create the VML injury. The defect was blotted from blood and then approximately 100 μL of the designated MVF precursor was pipetted in and immediately exposed to UV light for 1 min. The wound was then closed in layers and a topical antibiotic was applied to the site. Following surgery, animals were monitored daily for three days to assess wellness. All animals were exposed to a 12-h light/dark cycle and had *ad libitum* access to food and water. Rats were euthanized 1 day or 28 days following the VML surgery via intracardiac delivery of Euthasol (pentobarbital sodium and phenytoin sodium) while under anesthesia. The TA and extensor digitorum longus (EDL) muscles were harvested post-mortem, separately weighted, and snap frozen in optimal cutting temperature compound (OCT) via liquid nitrogen-cooled isopentane for histological analyses. A total of 30 rats were used in these experiments with n = 4 per experimental group with endpoint of 1 day and n = 6 per experimental group with endpoint of 28 days.

2.3. Histology

Frozen samples were sectioned at a thickness of 7 μm and fixed with formalin for downstream processing. All tissue sections were cross-sections of the TA in the middle of the VML defect where the MVF was its largest. Picrosirius red (PSR) staining was performed with PSR kit (Abcam, ab150681) per the manufacturer's protocol. Slides were sealed with Micromount® Mounting Medium (Leica, 3801730). PSR cross sections were used to calculate rectangularity (ratio of the area of hydrogel to the area of minimum bounding rectangularity) [19]. For fibrosis quantifications, the collagen staining was extracted through color deconvolution [20] and zones of 25 μm thickness were made from the border of the MVF radiating into the muscle [21,22]. Hematoxylin (ThermoScientific, 7211) and eosin (ThermoScientific, 71204) staining was performed using a Tissue TEK Prisma Stainer automated system. For immunofluorescence staining, samples were blocked in 10% normal goat serum and then incubated with primary antibody for CD68 (1:500; Bio-rad, MCA341R), wheat germ agglutinin (WGA) with Alexa Fluor™ 488 conjugate (1:500 Invitrogen, W11261), or solution with no antibody (negative control) overnight in 4 °C. The next day, slides were washed and those stained with the CD68 antibody were incubated with Alexa Fluor™ 594-AffiniPure donkey anti-mouse IgG (1:200; Jackson Immuno, 715-585-150) for 1 h at room temperature. The slides were then washed and mounted with VECTASHIELD® Vibrance™ Antifade Mounting Medium with DAPI (Vector, H1800). All slides were imaged at 20× magnification with an Axio Scan Z1 (Zeiss; Oberkochen, Germany).

The percentage of collagen in each of the 25 μm zones was analyzed through ImageJ using the binarized image to calculate the percentage of positive collagen stain to the total area of the zone. The percentage of area of DAPI was calculated in a similar way from the isolating the DAPI signal from images stained with both DAPI and CD68. Percentage of area of CD68 was calculated through ImageJ by thresholding the positive intensity values to be above the intensity seen in the negative controls (with the same threshold value set for all images) as a percentage of the total tissue area as manually drawn. Analysis of fiber count and size were obtained through automatic analysis of WGA-stained images via the Myosoft plugin for ImageJ [23].

2.4. Statistical analysis

All data are represented as mean with standard deviation (SD). Results were statistically analyzed through GraphPad Prism 9 software. Data comparing two groups were analyzed through the Student's t-test. Data comparing multiple factors were analyzed through a 1- or 2-way ANOVA including interaction effects. Statistically significant ANOVAs then underwent a Holm-Sidak post-hoc test. Statistical tests for the compression testing were run on log transformed data in order to make the data more homoscedastic. Myofiber diameter data, which was non-normally distributed, was analyzed through Kruskal-Wallis test. Statistical significance for tests were determined by p-values less than 0.05.

3. Results

In vitro evaluation of each biomaterial demonstrated that all were able to qualitatively crosslink via UV light to form solid structures (Fig. 1A). SEM imaging of the polymerized biomaterials revealed different mesh and pore structures for each biomaterial (Fig. 1A). PVA had a smooth, continuous surface with a porous interior while HA + PEG appeared to be mesh-like with pores on its surface and interior that are. In contrast, the polymerized silicone was solid throughout. HA + PEG exhibited a greater swelling capacity (0.990 ± 0.057) than PVA (0.625 ± 0.014) and both hydrogels exhibited more swelling than silicone (0.005 ± 0.008) which predictably did not swell (Fig. 1B). With respect to the mechanical properties of the biomaterials, HA + PEG was softer than PVA with storage moduli of 2.807 ± 0.360 kPa, and 7.020 ± 1.193 kPa, respectively ($p = 0.0058$), and Young's moduli of 3.216 ± 0.0040 kPa (PVA), 1.130 ± 0.1249 kPa (HA + PEG), and 860.2 ± 43.86 (silicone) ($p < 0.0001$ for all comparisons) (Fig. 1D and E). Reliable rheology data could not be performed with the silicone biomaterial due to adherence with the plate.

In vivo experimentation revealed that each of the candidate MVF materials conformed to the VML defect volume, maintained the space/shape upon implantation (Fig. 2A), and remained in the defect region for 28 days (Fig. 2B). Histological examination of cross sections of harvested tissues in the middle of the VML defect revealed that the stiffest biomaterials - PVA and silicone - had consistently rectangular cross-sectional shapes at both 1 (D1) and 28 (D28) days after implantation (Fig. 3A). The calculated rectangularity for PVA were 0.74 ± 0.07 (D1) and 0.70 ± 0.06 (D28), and for silicone were 0.63 ± 0.09 (D1) and 0.65 ± 0.05 (D28) (Fig. 3B). HA + PEG exhibited signs of deformation from the surrounding musculature at 1 day after implantation (rectangularity = 0.63 ± 0.02) which was further exacerbated by 28 days (rectangularity = 0.53 ± 0.11). Cellular infiltration into the HA + PEG hydrogels, particularly the deep portion, was observed through the H&E and DAPI stained images (Fig. 3A, S1). Analysis of TA fiber count peripheral the MVF showed that all VML injured/MVF treated cohorts had less than half the number of fibers of an uninjured TA muscle (Fig. 3C-D, S2). Amongst the experimental groups at 28 days, TA muscles treated with HA + PEG presented with more myofibers than silicone at 28 days (5803 ± 2200>1629 ± 851, $p = 0.0082$). No differences in median fiber minimum Feret diameter were observed between any of the MVF groups from tissue cross-sections at the middle of the VML defect (Fig. 3E).

In addition to maintaining the VML defect space, an ideal MVF should avoid causing adverse effects in the surrounding musculature such as increased fibrosis and inflammation which occurs both as part of the pathophysiology of VML and response to a foreign body (i.e. the MVF). From the PSR stained cross-sections, it is evident that a robust layer of collagen formed around the silicone biomaterial by 28 days. Meanwhile, both the PVA and HA + PEG hydrogels caused only a relatively thin layer of collagen around its borders (Fig. 4A). The

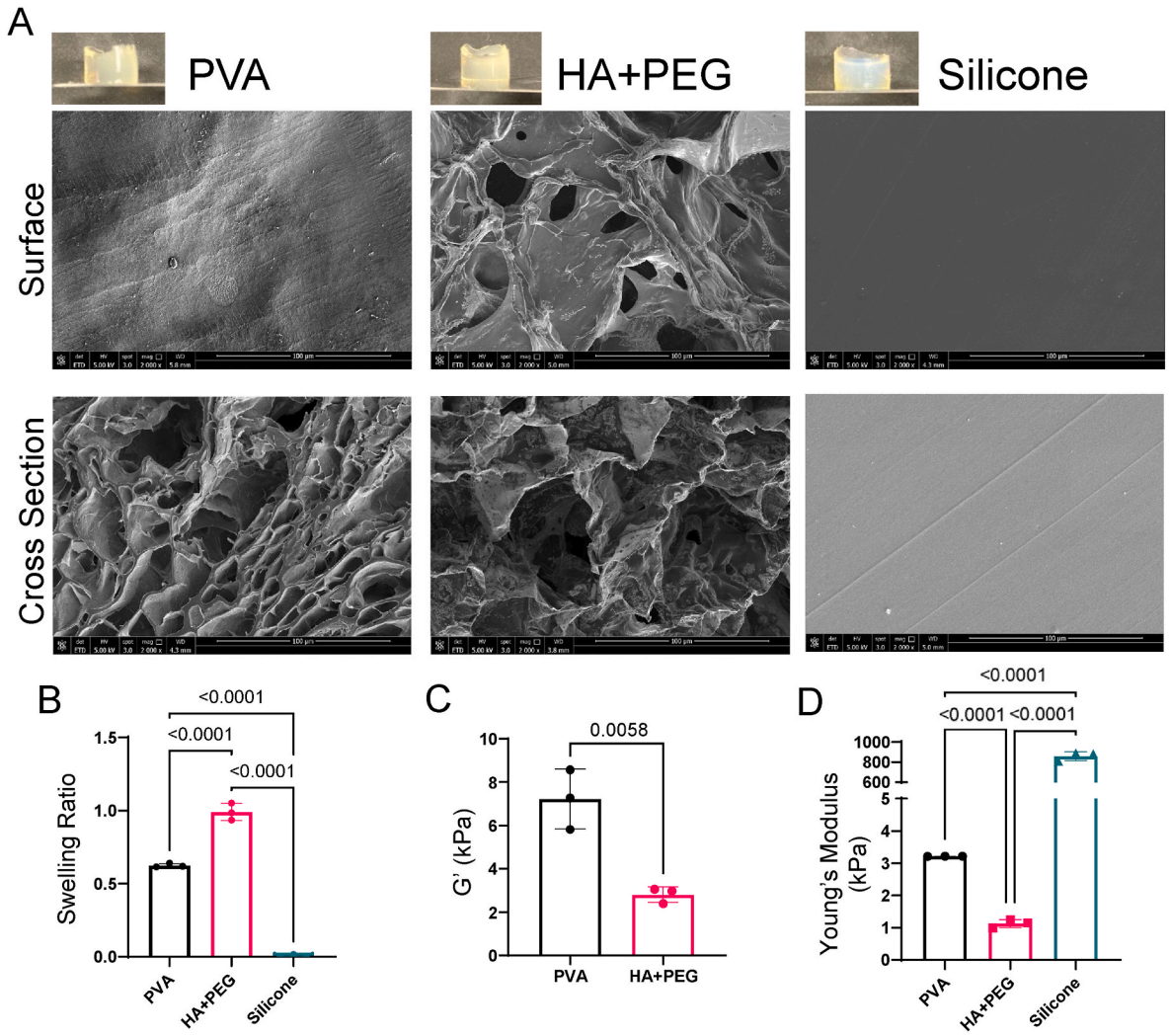

Fig. 1. (A) Digital and SEM images of swelled biomaterials formed *in vitro*. (B) Calculated swelling ratio of *in vitro* formed biomaterials 24 h after incubation in PBS at 37 °C. (C) G′ of *in vitro* formed biomaterials. (D) Young's Moduli of *in vitro* formed biomaterials. Data analyzed by ANOVA followed by Holm-Sidak post hoc test (B,D) or analyzed by unpaired *t*-test (C). p-values less than 0.05 are listed for all comparisons. All data represented by mean ± SD.

percentage of collagen in successive 25 μm zones from the MVF border show rapid reductions in collagen content by 100 μm for both the PVA and HA + PEG groups dropping from 75.7% to 81.1% respectively within the first 25 μm to 29.3% and 41.2% within 75–100 μm range. In contrast, the percentage of collagen in each region for silicone was 93.2% (0–25 μm), 92.42% (25–50 μm), 92.3% (50–75 μm), and 89.3% (75–100 μm). The last of three regions for silicone were higher than both of the other biomaterials. In addition to collagen content, cellularity adjacent to the biomaterial was analyzed using DAPI stained images (Fig. 4B). While all biomaterials induced cellularity immediately adjacent to the MVF perimeter, there were no differences in cell content between experimental groups or as a function of distance from the implant. However, using $CD68^{+}$ expression as an indicator of immune cell infiltration within the surrounding muscle, (Fig. 4C, S3), we observed a significant increase in macrophage infiltration into the muscle tissue in silicone treated VML injuries than HA + PEG (3.63 ± 1.71% > 1.27 ± 0.89%, $p = 0.0335$). No difference in infiltration was observed relative to PVA (1.83 ± 1.56%, $p = 0.0893$). There were nearly identical macrophage amounts between the PVA and HA + PEG treated injuries ($p = 0.5079$).

4. Discussion

The goal of this investigation was to characterize different biomaterials for their utility as an MVF to provisionally treat and temporize VML. Three biomaterials — chosen due to their potential to conform to the defect volume, degradation profile, and biocompatibility — were examined *in vitro* and tested within a rat model of VML for the ability to maintain the defect volume and minimize adverse effects. The experiments conducted herein point to PVA being the most capable of being an MVF.

When polymerized *in vitro*, each biomaterial was able to maintain a cylindrical shape unsupported, indicating the ability to hold the theoretical shape of our surgically-induced VML defect when solely under the force of gravity. However, rheological testing and compression testing showed differences in material rigidity. In addition, the biomaterials had notedly different swelling and morphological properties as seen through SEM imaging. While the rigidity of filler for use in skeletal muscle has not been studied and optimized, it is likely to play an important role for a successful MVF in the setting of VML. An ideal MVF would be rigid enough to maintain the defect volume and resist deformation and displacement from contracture of the surrounding tissue and pressures from muscle contraction. However, the capacity to have some flexibility and elastically return to its original shape is likely beneficial

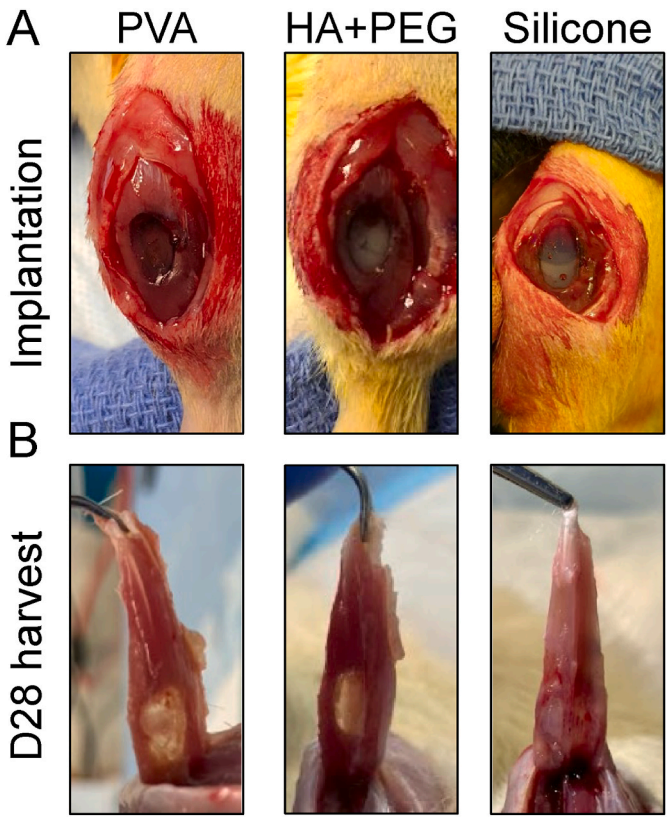

Fig. 2. Digital photographs taken of the MVF *in situ* (A) immediately after formation and (B) at time of harvest 28 days after implantation (photographs taken from deep side of tibialis anterior with superficial fascia intact).

to allow for unimpeded contraction of the surviving musculature. The optimal balance point is likely different for small animals versus humans due to the magnitude of forces subjected to in each. It is not yet known what the upper and lower limits are for if/when rigidity could have a detrimental impact on the ability for an MVF to fulfill its function. Lower amount of MVF swelling is likely beneficial to allow for a more accurate amount of the biomaterial to be placed without undue swelling causing greater external pressures or expanding into unwanted compartments. Swelling and storage modulus are inversely related through the degree of crosslinking of the biomaterial, as a greater crosslinked biomaterial swells less and has a higher storage modulus. Porosity can play a role in the future development of a bioactive MVF. Degree and size of porosity can affect the storage and release of potential therapeutics contained within an MVF such as growth factors, antibiotics, and/or nanoparticles containing these. At present the optimization and acceptable ranges of these parameters is in its nascent phase.

The stiffest material tested was silicone, which has been commonly used for other soft tissue filler applications, such as tissue expanders and breast implants [11]. Silicone quickly polymerized in the VML defect region and experienced no degradation or deformation over a 28-day period. Silicone, however, did elicit a robust fibrotic response. Furthermore, silicone resulted in greater macrophage infiltration into the remaining tissue mass of the affected muscle. These adverse effects are in line with other reports using silicone as a soft tissue void filler [24,25]. While silicone maintained the VML defect space, its deleterious effects on the adjacent muscle would likely make it a poor candidate for use as an MVF, particularly in muscles whose function depends on contractile cellular elements as opposed to extracellular matrix.

HA + PEG hydrogels were tested due to the reported effectiveness of HA at being anti-inflammatory [26]. HA + PEG created a porous hydrogel that was able to conform to the muscle defect. HA + PEG created the least stiff of the tested biomaterials. However, this low stiffness caused the material to deform, a finding which was especially apparent by 28 days, likely by the pressures of the surrounding musculature during contractions. Cellular infiltration was seen into the HA + PEG hydrogels, especially the deeper portion. Currently it is unknown how this cellular infiltration may affect its ability to serve as an MVF. However, the HA + PEG hydrogels caused minimal fibrosis and immune cell infiltration into the injured muscle. As such, it is possible that HA + PEG hydrogels may be useful as MVFs in which the material does not need to resist high external pressures from swelling, repeated muscle contractions, or healing contracture.

The last hydrogel tested, PVA, had twice the storage modulus of the HA + PEG hydrogel. The PVA hydrogel was able to maintain the VML defect similar to that of the silicone biomaterial. However, it appears that PVA was able to maintain this defect shape while eliciting a reduced amount of a fibrotic border (<100 μm) and similar amounts of macrophage infiltration as the HA + PEG hydrogels. This favorable host response is likely indicative of a permissive environment that would require little to no debridement following removal of the MVF and prior to a secondary treatment [27]. Given that a robust inflammatory response is a key to the pathobiology of VML [28], a MVF that avoids eliciting a heightened acute inflammatory response prior to a secondary regenerative treatment could be a major advantage. For these reasons, it appears that PVA is the best candidate MVF of those tested. Further, this PVA system is easily tunable to vary its physical and chemical properties [29].

This work has shown the feasibility for a synthetic biomaterial to serve as an ideal MVF by maintaining a VML defect with minimal adverse effects. This work diverges from other biomaterial approaches for use in VML. Previous attempts using biomaterials have attempted to use them as scaffolds to regrow muscle tissue in the defect [30,31]. Other soft tissue filler applications have also started to look towards self-integrating or biodegradable materials [32–35]. However, results of using biomaterials in isolation for VML as scaffolds have shown poor outcomes with satellite cells failing to migrate more than 0.5 mm into scaffolds [36]. While these biomaterials may be effective with a cellular component, the significant time needed to expand, reprogram, and/or differentiate autologous cells inhibits their availability to use when a patient is first being treated. Rather than letting an implanted scaffold fill with fibrosis and immune cell infiltrate during the time needed to obtain the needed cells, it would likely be better to prevent any infiltration into the defect via a MVF until implantation of the cells with the necessary scaffold.

Due to this lack of similar approaches, we do not yet know the optimal or acceptable limits for many of parameters likely important for an MVF. The current predominant perspective of VML sees heightened inflammation as a contributor to pathophysiology [37]. Thus, an optimal MVF is likely one that elicits a minimal immune response on its own, or even exhibits anti-inflammatory properties. An MVF that can prevent fibrotic deposition would maximize available volume for regeneration during the definitive treatment as well as unimpeded interface with the surviving musculature. Additionally, this would minimize the positive feedback loop caused by cells and signals within the fibrotic region expanding into the defect once the MVF is removed. While collagen formation around the MVF is an important consideration, it is possible to debride tissue upon removal of the MVF before administering the definitive treatment. Likely the most important aspects for an MVF are maintaining the health of the surviving musculature and maximizing the available volume for regenerating tissue.

Future work can build upon these platforms with incorporation of bioactive payload, such as anti-inflammatory and/or pro-regenerative agents, to further sustain a healthy environment for a delayed regenerative treatment. Another beneficial payload could be antibiotic drugs to mitigate the potential risk of infection, which is deleterious for regeneration and potentially for an MVF.

A clinical application for an optimized MVF is within current paradigms of multi-staged treatments for traumatic CTI injuries. Currently

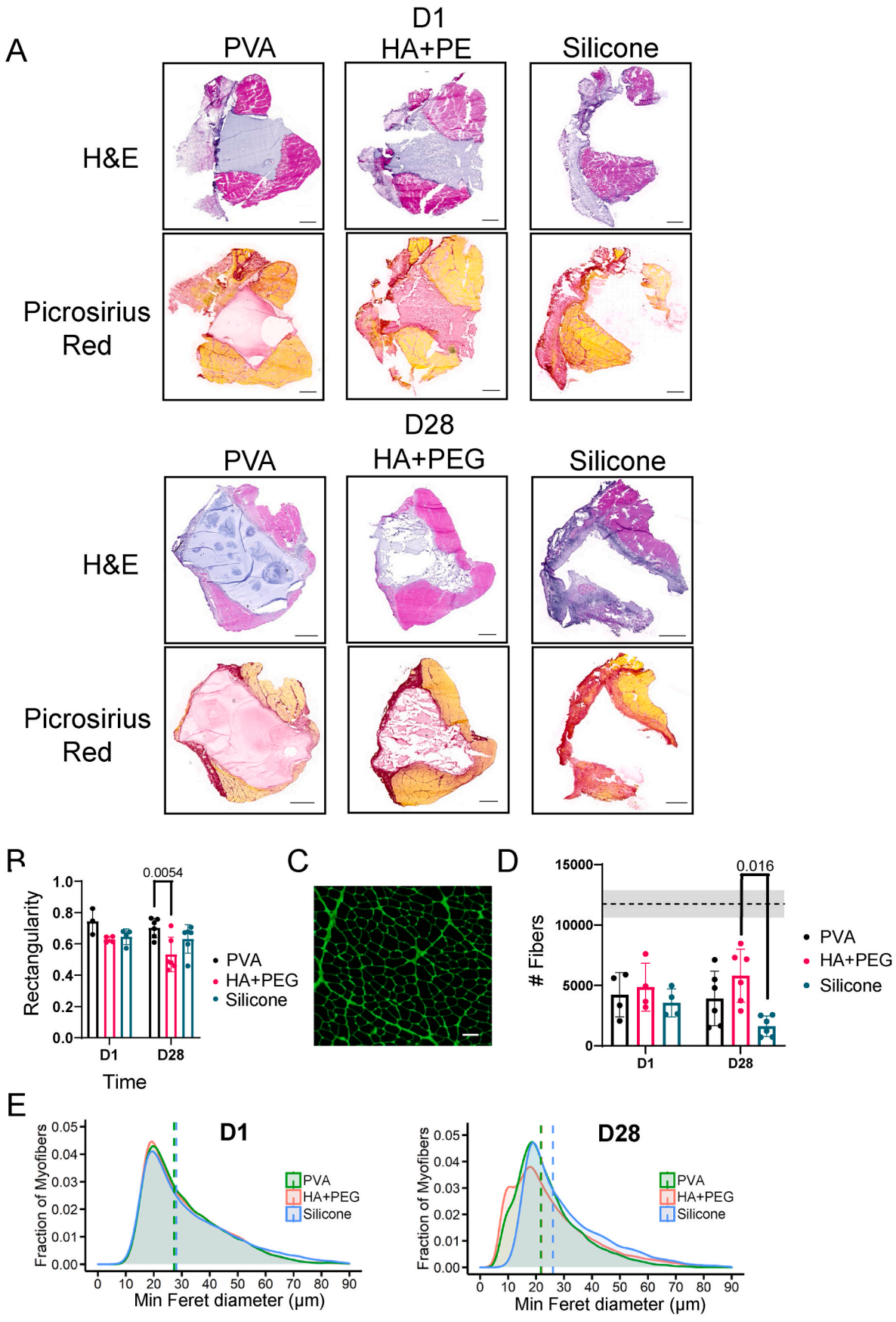

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Fig. 3. (A) Representative cross sections of H&E and picrosirius red stained tissue 1 day and 28 days after MVF implantation (scale = 500 μm). (B) Rectangularity measurements of implanted MVF. (C) Representative close-up image from WGA-stained muscles (scale = 50 μm). (D) Muscle fiber count of muscles with MVF implantation 1 day and 28 days after implantation. Dotted line and shaded region represents the mean ± SD for fiber number from healthy tibialis anterior muscles. (E) Kernel density estimations of the distribution of minimum Feret diameters 1 and 28 days after MVF implantation. Data in panels B and D were analyzed first via a 2-way ANOVA followed by Holm-Sidak post hoc test. Median data in panel E were analyzed via Kruskal-Wallis test. Post hoc p-values less than 0.05 are listed for all comparisons. Data represented as mean ± SD. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

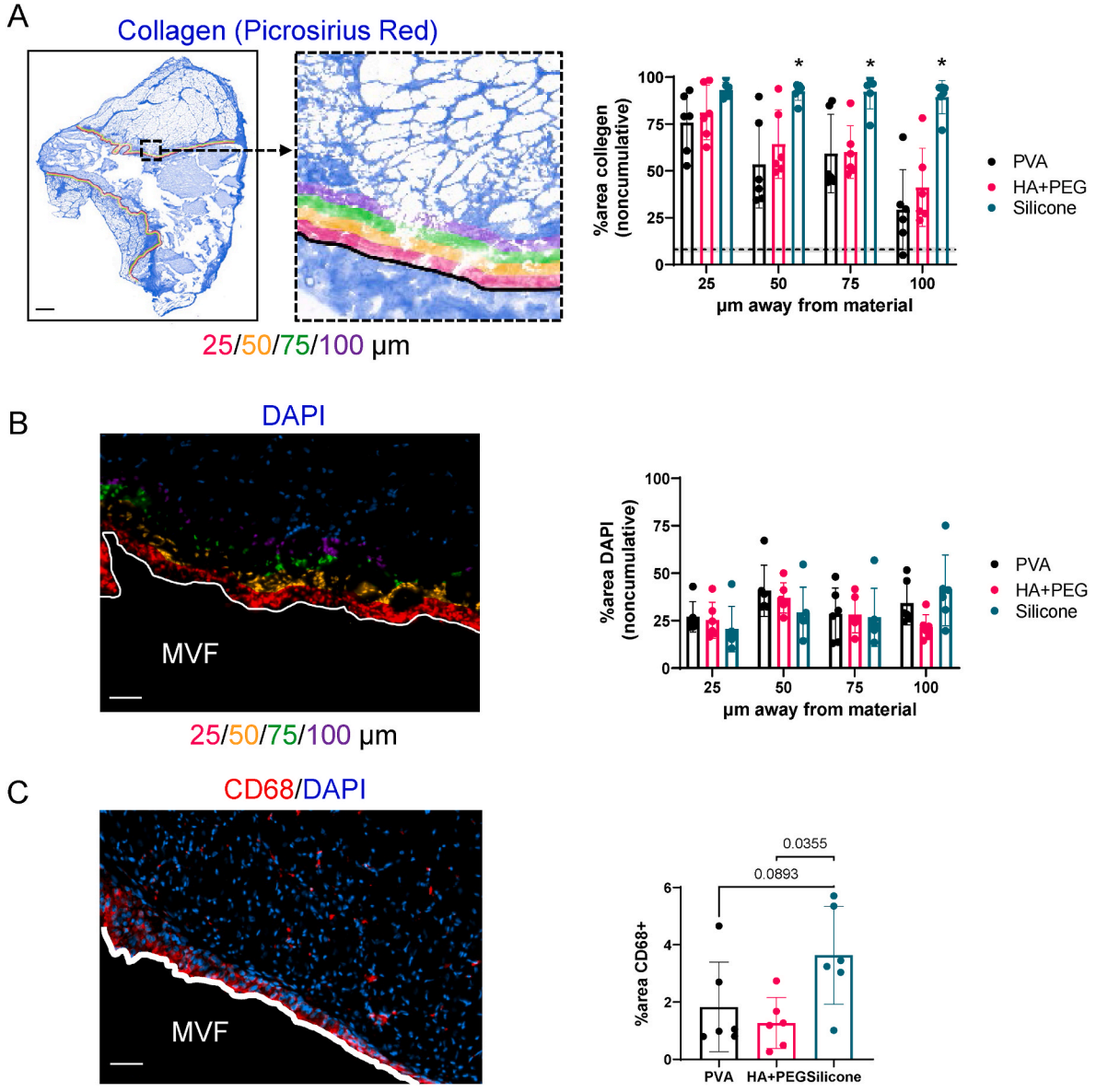

Fig. 4. (A) Color deconvolution of picrosirius red stained sections to isolate stained collagen. Zones of 25 μm thickness were made radiating out from the MVF-muscle border, pseudo-colored, and percent area of zone that consisted of collagen was quantified. Graph's dotted line and shaded region represents the mean ± SD for area of collagen in a healthy tibialis anterior muscle. *$p < 0.05$ for silicone vs PVA and silicone vs HA + PEG. (Scale = 500 μm) (B) Nuclei stained with DAPI and zones of 25 μm thickness were pseudo-colored and percent area of zone that consisted of positive DAPI signal was quantified (Scale = 50 μm). (C) Pan-macrophage marker CD68 was stained to look at cellular infiltrate. Analysis of total CD68 signal in cross section was quantified (Scale = 50 μm). All data were analyzed first via a 1 or 2-way ANOVA followed by Holm-Sidak post hoc test. Post hoc p-values less than 0.05 are listed for all comparisons. Data represented as mean ± SD. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

multi-staged treatments exist for large bone defect (e.g., induced membrane technique), however these injuries are often accompanied with skeletal muscle loss which is often neglected during treatment. An MVF would allow for a parallel treatment of skeletal muscle along with the bone. For example, with the induced membrane technique, a spacer is placed within the bone defect, during which time the MVF can be implanted within the skeletal muscle. When the bone spacer is removed and definitively treated, the MVF can be removed and definitively treated as well. Currently there is a lack of clinical treatments for VML outside of surgical techniques such as flaps. While preclinical treatments are in the pipeline, it appears a successful treatment will likely involve a cellular component [38], which often takes time to prepare. This

multi-staged approach may prove advantageous for VML to allow time for preparation of such a cell-based therapy. Currently it is unknown how receptive a VML-injured muscle is over the course of time to regenerative treatments as most preclinical research has focused on acute treatments [39–42]. However, it is possible that allowing the acute sequalae to subside would be beneficial for a cell-based therapy [43,44].

In addition to the above clinical uses, an ideal MVF will also be viable for injury management for those with limited access to treatment options. One such scenario is the prolonged field care, in which military soldiers in the front line have limited access to medical supplies. Ideally, and MVF would be able to be carried by a field medic into far-forward, austere environments and be used to stabilize battlefield injuries until evacuation to sophisticated military facilities. Preventing pathological changes and the secondary sequelae of battlefield injuries may increase outcomes and decrease the number/time/complexity of treatments needed to reconstruct and heal the injury [45].

5. Conclusion

This work characterized the ability of three biomaterial systems (PVA, HA + PEG, silicone) to act as an MVF for the provisional treatment of a VML injury. PVA appeared to be the superior biomaterial capable of maintaining the defect region with minimal adverse effects. The stability and ease of access for PVA makes it easy to translate into clinical practice if found efficacious. Future efforts which seek to further tailor a MVF as a platform technology are warranted, including evaluation of its efficacy at facilitated improved functional outcomes within a delayed regenerative treatment strategy.

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Statement of significance

Multi-stage surgical treatments can be effective for treating complex composite tissue injuries (CTI), including under circumstances where medical resources are limited (e.g., austere military settings). A multi-staged treatment approach for a CTI which includes volumetric muscle loss (VML) would require initial stabilization of the defect region with the goal of maintaining the defect volume and surrounding tissue architecture to prevent adverse effects (e.g., contracture, fibrosis). An unmet need exists for a material product suitable for such applications (i.e., a muscle void filler; MVF). Three biomaterials that could potentially serve as a provisional MVF to temporize VML defects and allow for staged treatments were evaluated. This work has the potential to greatly improve the care and outcomes for affected patients.

Disclaimer

The opinions or assertions contained herein are the private ones of the author/speaker and are not to be construed as official or reflecting the views of the Department of Defense, the Uniformed Services University of the Health Sciences, The Henry M. Jackson Foundation for the Advancement of Military Medicine, Inc., or any other agency of the U.S. Government.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

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Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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