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Testing a Novel Nanofiber Scaffold for Utility in Bone Tissue Regeneration

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Summary

Many variables serve to alter the process of bone remodeling and diminish regeneration including the size and nature of the wound bed and health status of the individual. To overcome these inhibitory factors, tissue engineered osteoconductive scaffolds paired with various growth factors have been utilized clinically. However, many limitations still remain, for example, bone morphogenetic protein 2 (BMP2), can lead to rampant inflammation, ectopic bone formation, and graft failure. Here we studied the ability for a nanofiber scaffold (Talymed) to accelerate BMP2 growth factor induced bone healing compared to the traditional absorbable collagen sponge (ACS) delivery system. 155 adult wild-type mice were arranged in 16 groups by time, 4 and 8 week, and treatment, ACS or Talymed, loaded with control, low, medium or high dosages of BMP2. Skulls were subjected to microCT, biomechanical, and histological analysis to assess bone regeneration. The use of Talymed within the defect site was found to decrease the bone volume, bone formation

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5. Conflict of Interest Statement

The authors have no conflicts of interest to declare.

rate, and ALP activity compared to ACS/BMP2 combinations. Interestingly, though Talymed regenerated less bone, the regenerate was found to have a greater hardness value than that of bone within the ACS groups. However, the difference in bone hardness between scaffolds was not detectable by 8 weeks. Based on these results, we found that the nanofiber scaffold generated a better quality of bone regenerate at 4 weeks but, due to the lack of overall bone formation and the inhibition of normal remodeling processes, was not as efficacious as the current clinical standard ACS/BMP2 therapy.

Keywords

absorbable collagen sponge; BMP2; Bone regeneration; Talymed; Tissue Engineered Scaffold; nanofiber

1. Introduction

Bone has the innate capacity to heal after injury due to its dynamic and highly vascularized nature, which sustains remodeling throughout life. Despite this regenerative ability, fractures and defects resulting from trauma or invasive surgical procedures present a major clinical obstacle. In cases with significant mechanical and/or metabolic restrictions (Dayer, Badoud, Rizzoli, & Ammann, 2007) or impaired health (Aspenberg et al., 2010; Sansone & Sansone, 2012), bone is unable to regenerate, requiring surgical intervention to augment natural fracture repair (Mountziaris & Mikos, 2008). Therapeutic strategies used to treat such cases include the use of grafts (Bauer & Muschler, 2000), implantable medical devices such as plates and pins, tissue engineering strategies, or a combination of these therapies. The field of bone tissue engineering focuses on the development of alternative treatment strategies to overcome failures in the treatment of bone wounds (Grabowski & Cornett, 2013). Current areas of research focus on adjuvant cell therapy, biomaterials/scaffolds, and the manipulation of osteogenic signaling pathways (Amini, Laurencin, & Nukavarapu, 2012; Giannoudis, Dinopoulos, & Tsiridis, 2005).

The synergistic combination of biomaterials and growth factors for their regenerative capacities in fracture models has been a particularly robust area of study. Bone morphogenetic protein 2 (BMP2) is one of the most commonly used growth factors in orthopedic application (Einhorn & Gerstenfeld, 2015; Park, Kim, Lee, Kim, & Kim, 2015) and is United States-Food and Drug Administration (US-FDA) approved for procedures such as maxillary sinus lift and spinal fusion. Despite the clinical efficacy of BMP2 in bone regeneration due to its ability to recruit osteoprogenitor cells and induce osteogenic differentiation (Agrawal & Sinha, 2017), BMP2 products have evoked controversy due to aberrant clinical events (Boerckel et al., 2011; Carragee, Hurwitz, & Weiner, 2011; Govender et al., 2002). Both preclinical *in vivo* experimentation (Cray et al., 2014; Zara et al., 2011) and clinical data (Lewandrowski, Nanson, & Calderon, 2007; Tannoury & An, 2014) suggest BMP2 may precipitate rampant inflammation resulting in poor quality bone regenerate. Research efforts are ongoing to enhance our understanding of BMP2 biology and expand clinical utility of this peptide. Areas of continued investigation are aimed at

exploring dosages, release rates, inflammation, and alternative scaffolding to better deliver BMP2.

The current US-FDA approved scaffold for BMP2 delivery is absorbable collagen sponge (ACS; INFUSE Bone Graft, Medtronic TM). However, other scaffolds may prove to be more effective at precipitating bone growth (Herberg, Kondrikova, et al., 2014; Herberg, Susin, et al., 2014; Kinsella et al., 2011; Ma, Jing, Sun, & Liu, 2015; Perez, Kim, Buitrago, Wall, & Kim, 2015; Quinlan, Thompson, Matsiko, O'Brien, & Lopez-Noriega, 2015; Ribeiro, Gomez-Benito, Folgado, Fernandes, & Garcia-Aznar, 2015; Tang et al., 2016; Taniyama et al., 2015), because ACS releases BMP2 at a rate that is unable to sustain quality bone growth throughout the lengthy healing process (Uludag, Gao, Porter, Friess, & Wozney, 2001). As ACS is only osteoconductive, strategies to enhance the osteoinductive properties of BMP2 and promote local retention of the growth factor within the fracture sight are required. Ideally, a scaffold capable of binding BMP2 with inherent properties that enhance wound repair would be most effective to augment bone regeneration. To this end our group and others have published on the use of the US-FDA approved pGlcNAc nanofiber scaffold (Talymed; Marine Polymer Technologies, Danvers, MA) for augmentation of wound healing (Fischer, Nichols, Scull, Smith, & Demcheva, 2011; Gorapalli et al., 2012; Lindner et al., 2015; Pietramaggiori et al., 2008). This biomaterial is a polysaccharide derived from a marine diatom. In clinical application of treating cutaneous wounds, Talymed has been shown to reduce scar formation, allow for increased alignment of collagen fibers, increased collagen tensile strength, and stimulate activation of toll-like receptors 4 (TLR4) without an accompanying TLR4 induced nuclear factor kappa-light-chain-enhancer of activated B cells (NFkB) dependent inflammatory response. These same properties are particularly attractive for augmentation of BMP2-mediated bone wound healing due to the possible positive effects on collagen assembly and reduction of negative side effects often observed with BMP2 administration (i.e. rampant inflammation, ectopic bone formation, etc.) (James et al., 2016).

The medical need for augmentation of bone wound healing is growing and outpacing industry and academic discovery. There is a continued need for novel delivery systems for tissue based therapeutics including bone. In the present study, the novel Talymed scaffold was utilized to investigate its innate properties that predict successful bone wound repair compared to the traditional ACS delivery system in conjunction with BMP2 treatment. Due to the innate healing qualities of Talymed, we hypothesized that bone regenerated using Talymed as a BMP2 delivery system would possess improved collagen matrix formation and subsequent bone regeneration as compared to healing augmented by the current clinical standard scaffold, ACS.

2. Materials and Methods

2.1 *In vitro* BMP2 carrier release characterization

Retention of BMP2 to ACS and Talymed was determined using radiolabeled BMP2 based on Cooper et al (Cooper, Miller, et al., 2010) with modification. Briefly, BMP2 was iodinated using a Chloramine T method. Approximately 10 ng equivalent of ¹²⁵I-BMP2 in 20 µl 10 mM phosphate buffer, pH 7.4 was applied to approximately 2×4 mm pieces of either ACS or Talymed carriers. Carrier samples were incubated in 1.5 ml Eppendorf tubes at 37C. After 1

hr, samples were rinsed 3 times with 0.5 ml PBS at 23°C. Carriers were transferred to 12×75 mm polypropylene tubes along with 1 ml of simulated body fluid (SBF:alpha medium, 25 mM HEPES, 0.02 % sodium azide, 1 % penicillin/streptomycin, 10% calf serum). Tubes were counted in a gamma counter to determine ^{125}I -BMP2 bound. This initial bound radioactivity represented the zero binding timepoint and this bound cpm was normalized to 100 % ^{125}I -BMP2 bound. Tubes were then incubated at 37 °C. At indicated timepoints, SBF was aspirated and replaced with fresh SBF after which tubes were immediately counted in a gamma counter to determine radioactivity remaining bound. Any released radioactivity was removed with the aspirated SBF. Radioactivity was corrected for ^{125}I decay. Binding experiments were conducted using quadruplicate sample carriers for each carrier type in two separate experiments representing two separate iodinations. Data is presented as mean $\pm$ SD of a total of 8 independent samples for each carrier type.

2.2. BMP2 carrier preparation for *in vivo* implementation

Under sterile conditions, 5 mm biopsy punches of Talymed and, due to ACS shrinkage after loading, biopsy punches of 6 mm were obtained and stored in a 24 well plate lined with sterile gauze. Fifteen minutes prior to implantation, matrices were loaded with control (sterile H₂O), low (110 ng), medium (325 ng), or high (542 ng) doses of BMP2 resuspended in sterile water consistent with manufacturer's recommended method of preparation (Infuse, Medtronic, Memphis, TN) (Herberg et al., 2015).

2.3. Critical-sized calvarial defect model

155, 8 week old, male and female C57BL6 mice (Jackson Laboratory, Bar Harbor, ME) were randomized into one of 8 surgical groups implanted with ACS or Talymed matrix soak loaded with control (sterile H₂O), low (110 ng), medium (325 ng), or high (542 ng) doses of BMP2. A critical-sized calvarial defect was performed as previously described (Cooper, Mooney, et al., 2010; Herberg et al., 2015; Herberg, Kondrikova, et al., 2014). Briefly, the mice were anesthetized with isoflurane (Bethlehem, PA, USA) and a midline scalp incision was used to expose and remove the periosteum. A 5-mm craniectomy defect was trephined using a slow-speed hand drill. Each craniectomy defect was filled with the assigned exposure (Table 1). After the application of experimental treatment, the incision was closed with 6×0 polypropylene suture (removed 2 weeks later). Animals were weighed and monitored two days post-surgery for any signs of pain or distress, with daily monitoring continuing until time of sacrifice. The mice were euthanized at 4 and 8 weeks post-surgery by asphyxiation using CO₂ gas followed by secondary cervical dislocation, at which point skulls were collected for downstream analyses. All procedures were carried out with the approval of the Medical University of South Carolina IACUC (AR #3452), in an Association for Assessment and Accreditation of Laboratory Animal Care International accredited facility where all husbandry and related services were provided by the Division of Laboratory Animal Resources. The animals were housed 5 to a cage which contained enrichment material and were allowed access to food and water *ad libitum*. All procedures and the reporting thereof are in compliance with the Animal Research: Reporting in Vivo Experiments (ARRIVE) guidelines (Kilkenny, Browne, Cuthill, Emerson, & Altman, 2012).

2.4 Micro-Computed Tomography (μ CT) image analysis of bone regeneration

All soft tissue was removed from the collected skulls which were then bisected from the occipital protuberance to nasale in the transaxial plane. Micro-Computed Tomography (μ CT) images of the defect were obtained via *ex vivo* Skyscan 1176 (Skyscan, Aartlesaar, Belgium) with a 0.5 mm thick aluminum filter at a voltage of 50 kV and current of 500 μ A. Data were acquired at an isotropic resolution of 18 μ m, rotation step of 0.5°, and 180° rotation. Reconstruction and analyses were performed on the blinded samples using NRecon and CTAn SkyScan software. A global threshold of 100–255 was used. Standard 3D morphometric parameters (Bouxsein et al., 2010) were determined for the region of interest (ROI; 5.0 mm circle, 51 cuts equaling 918 μ m), positioned along the initial surgical margins for all samples. Representative 3D images were created using CTvol software. Directly after testing, samples were randomized into either μ -indentation testing (n=6/experimental group) or histological experimentation (n=4/experimental group) groups.

2.5 μ -indentation analysis of the mechanical strength of the regenerate

The mechanical properties of the bone within the calvarial defect were evaluated by using the BioDent (ActiveLife Scientific, Santa Barbara, CA) system. Skulls were placed on the lower support of the apparatus so that the center of the defect was directly under the testing probe. To prevent shifting of the skull during testing, a small stabilizing preload (260–310 g) was applied. A 2 N force was loaded in vertical compression within the center of the defect for a total of 5 cycles. Force and displacement were continuously recorded during the procedure and total indentation distance (TID), indentation distance increase (IDI), and average unloading slope (Avg US) were analyzed. Results from 5 standardized locations within the defect were collected for each sample.

2.6 Tissue processing and staining

Blinded, representative samples (n=4) from each group were bisected in the coronal plane through the center of the surgical defect and prepared for either paraffin (posterior defect) or frozen (anterior defect) sectioning. The posterior portion of the defect was placed in 3.7% formaldehyde for two days, stored in 70% ethanol and then decalcified in 0.25 M EDTA at pH 7.4 for 21 days. Samples were then washed, dehydrated in graded ethanol (70–100%), cleared in xylene, and embedded in paraffin. Histology was performed on 3, 7 μ m sections in the coronal plane at least 40 μ m apart per sample for analysis of the defect area. Sections were stained with hematoxylin and eosin (H&E), picosirius red (PSR; non-polarized and polarized), Masson's trichrome (MTC; Thermo Scientific, Waltham, MA USA), and tartrate-resistant acid phosphatase (TRAP; Sigma, St. Louis, MO) by standard methods. Stained sections were photographed using a Motic Inverted Microscope with attached camera (Motic, British Columbia, Canada) and quantified using Visiopharm software (Visiopharm, Broomfield, Colorado). PSR sections were analyzed for total collagen area (non-polarized), thin immature (green; polarized), intermediate (yellow; polarized), and thick mature (red; polarized) collagen fibers. Total area of bone (osteoid) was analyzed for MTC and TRAP positive osteoclasts (red) were analyzed within the defect site.

For alkaline phosphatase (ALP; ab108337 Abcam, Cambridge, MA) immunohistochemistry, samples from each group were subjected to antigen retrieval with 10 mM Sodium Citrate

Buffer. Endogenous peroxidase activity was blocked with 3% hydrogen peroxide and then washed sections were blocked in 1% goat serum with 1% bovine serum albumin. Sections were incubated with primary antibody for one hour at room temperature followed by washing and incubation with HRP conjugated secondary antibody for one hour (ab6721 AbCam). Diaminobenzidine (DAB; Vector Laboratories, Burlingame, CA) chromagen was used to identify immunoreactive structures. Sections were analyzed using Image J Software and the IHC Profiler Open Source Plugin for automated scoring of percent positivity (Varghese, Bukhari, Malhotra, & De, 2014).

2.7 Calcein analysis of bone formation rate

All surgical animals were intraperitoneally injected with 10 mg/kg Calcein 7 and 2 days before sacrifice. After μ CT imaging, the anterior portions of the defects were kept on ice until embedded in OTC Compound (Tissue-Tek, Torrance, CA) and flash frozen in liquid nitrogen chilled isopropanol. Samples were then sectioned at 10 μ m in the coronal plane and 3 sections at least 40 μ m apart were analyzed per sample. Fluorescent images were obtained using a Motic Inverted Microscope with attached camera at 20 $\times$ magnification. Images were analyzed for the mineralizing surface normalized to the bone surface (MS), the mineral apposition rate (MAR) representing the percentage of bone surface exhibiting mineralizing activity, and bone formation rate normalized to the bone surface (BFR) as defined by the area of mineralized bone formed per unit time per unit bone surface. These measures were calculated using standard methods (Kilkenny et al., 2012).

2.8 Statistical analysis

Standard t-test was used to determine differences between the release kinetics at each time point and two- or three-way ANOVA with post-hoc Bonferroni analyses were conducted for all other comparisons where appropriate. Transformations of the data were performed where needed to correct for violations and meet the assumptions of homogeneity of variance or normality. Differences were considered significant if $p < 0.05$. Data are presented as means $\pm$ SEM. A power study for experimentation was calculated based on an $\alpha = 0.05$, $\beta = 0.8$, $\eta^2 > 0.4$, necessitating 10 animals per group. Statistical differences between groups are indicated by the following schema: * different from control, # different from low dose BMP2, ^ different from medium dose BMP2, and \$ different from high dose BMP2. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$

3. Results

3.1 *In vitro* BMP2 carrier release characterization

125 I-BMP2 release was measured over a 9 day period to compare the ACS and Talymed carriers (Fig. 1). Analysis of the release of 125 I-BMP2 from the matrices showed that both matrices had an 80% overall reduction of 125 I-BMP2 after 9 days. From the 1 hour time point until day 8, Talymed showed significantly less 125 I-BMP2 release compared to ACS ($p < 0.001$). The ACS scaffold released 40% of 125 I-BMP2 after only 2 hours. From day 7 to 9 Talymed exhibited a sharp increase in the rate of release with the differences in the retention of 125 I-BMP2 by the matrices resolving by day 9.

3.2 Properties of the bone regenerate

μ CT images of bone regeneration 4 and 8 weeks after creation of the 5 mm critical-sized defect filled with either ACS or Talymed soak loaded with control, low, medium, or high dosages of BMP2 were analyzed for differences in both quantity and quality of the regenerate (Fig. 2A). 3D renderings of the μ CT images indicate that the control animals for both matrices did not spontaneously heal by 8 weeks and that the ACS matrix consistently showed greater regenerate for all BMP2 dosages compared to Talymed with the medium and high dosages of BMP2 showing the greatest bone regeneration in both matrices.

Measurements of the defect volumes show that groups utilizing the ACS matrix had significantly more bone volume/tissue volume (BV/TV; Fig. 2B) than Talymed across all BMP2 treatments at both the 4 ($p<0.048$) and 8 week ($p<0.03$) time points. A significant increase in the BV/TV was found in the medium and high BMP2 dosage groups compared to control ($p<0.01$) with the medium dose of BMP2 showing the largest volume of regenerate within the defect site. Further analysis of the quality of bone in each surgical group indicates an increased trabecular number (Tb.N; Fig. 2C) in all ACS groups at 4 weeks ($p<0.002$) and the medium BMP2 dose ($p<0.001$) at both time points. Interestingly, while the ACS groups showed increased BV/TV and Tb.N, Talymed was found to produce bone with increased trabecular thickness (Tb.Th; Fig. 2D) when paired with BMP2 compared to ACS at the 8 week time point ($p=0.034$). Congruent with the Tb.N results, trabecular separation (Tb.Sp; Fig. 2E) was shown to be significantly lower in the ACS groups at 4 weeks ($p=0.02$) with the medium and high BMP2 dosages displaying significantly decreased Tb.Sp compared to control at both the 4 and 8 week time points ($p<0.001$).

H&E representative images of control and medium BMP2 dosage for both ACS and Talymed matrices are shown in Fig. 2F. Representative images from only the control and medium dose BMP2 groups were shown to highlight the most dramatic differences found. These images illustrate the lack of bone regeneration within the control groups for both matrices at 4 and 8 weeks. ACS medium BMP2 samples contained regenerate with the typical morphology of bone stimulated by exogenous BMP2: large spaces between bone spicules spanning across the majority of the endocranial portion of the defect site. Talymed was found to result in an idiopathic decellularized area between the periosteum and the endosteum centrally within the defect which was maintained at the 8 week time point. BMP2 was not found to eliminate this decellularized space; instead the increased bone found within these samples was shown to originate from the surgical margins only.

The quality of bone regenerate within defect sites were evaluated through μ -indentation biomechanical testing. TID indicating the total displacement of the probe into the bone after 5 cycles of 2 N is inversely related to the ability for bone to resist crack initiation and propagation, and is an indicator of the degree of failure within a sample. ACS groups were found to have significantly greater TID than Talymed at 4 weeks ($p<0.001$; Fig. 2G). However, these differences were resolved by the 8 week time point. In line with those results, IDI, which is inversely correlated with toughness of the bone, was also greatest for the ACS samples only at the 4 week time point ($p=0.004$; Fig. 2H). The ability for the regenerate to recover from the deformation caused by the 2 N loading force was measured

through the Avg US (Fig. 2I). Talymed had an increased resistance to indentation at 4 weeks ($p=0.002$) but no differences were found between matrices by 8 weeks. Some differences in these measurements were found between the different dosages of BMP2 at 4 weeks but most were resolved by 8 weeks with no one dosage producing an overall higher quality of bone regenerate.

3.3 Collagen matrix structure

Due to Talymed having lower bone volume but improved biomechanical properties of the regenerate at 4 weeks, coupled with the observation of a decellularized space within the Talymed samples, collagen fibers within the defect were investigated to determine if the endogenous collagen matrix structure was altered due to the use of the Talymed matrix. The total collagen makeup within the defect site was analyzed utilizing non-polarized PSR stain (Fig 3A). Representative images of ACS and Talymed control and medium BMP2 exposed groups showed samples with the ACS matrix to have collagen matrix formation along the endocranial portion of the defect in each group. The ACS control samples show accumulation of the fiber below the ACS matrix at week 4 which leads to the formation of a mature matrix within the center of the defect by 8 weeks. ACS medium BMP2 samples show thick collagen structures spanning across the majority of the endocranial portion of the defect site. In contrast, Talymed samples show very little collagen matrix formation in comparison either in control or medium BMP2 exposed groups. Investigation of the decellularized spaces shows collagen fibers bordering the empty space but very few fibers penetrating the area unless noticeable breakdown of the material filling the space was observed. While the collagen fibers were unable to infiltrate that area, it was observed that a dense collagen matrix was formed along the surgical margins in Talymed samples, indicating that the defect was healing from the surgical margins towards the center of the defect. Analysis of the total collagen area within the defect sites showed significantly higher amounts in the ACS groups at both 4 and 8 weeks ($p<0.001$ and $p=0.007$, respectively; Fig 3B). Medium and high BMP2 exposures were found to increase the total collagen area compared to control at 4 weeks. Investigation of the maturity of the collagen matrix through polarized PSR indicated a decrease in thin immature fibers (green; Fig. 3D) with a subsequent increase in intermediate collagen fiber (yellow; Fig. 3E) area from 4 to 8 weeks, indicating that the resulting collagen content is not evidence of a delay in healing but represents a mature structure. ACS was found to have a more mature collagen content with more intermediate and thick, mature (red; Fig. 3F) fibers compared to Talymed ($p=0.001$ for both). At 8 weeks BMP2 exposed groups were found to have significantly decreased immature fibers and increased intermediate and mature collagen fibers as compared to control.

3.4 Bone formation analysis

Disruption or disorganization of normal collagen matrices negatively affects the deposition of calcium and the subsequent formation of bone. To determine if the observed effects of Talymed on the collagen matrix prevented osteoprogenitor and osteoblast cell ALP activity, immunohistochemistry for ALP levels was performed (Fig. 4A). ALP activity was found to be significantly impaired in Talymed groups at 4 weeks ($p<0.001$) but differences between matrices were resolved by 8 weeks (Fig. 4B). Medium and high BMP2 exposures were

found to significantly increase ALP production within the defect site at 4 weeks compared to control ($p<0.001$) with most of the increase in ALP activity occurring within the ACS group.

Subsequent analysis of the osteoid formation within the defect site showed ACS control groups to have osteoid along the surgical margins at both 4 and 8 weeks (Fig. 4C). The addition of BMP2 to the ACS matrix resulted in osteoid formation not only along the surgical margin but along the endocranial portion of the defect around the established collagen matrix. In contrast, Talymed showed little to no osteoid formation in control animals and the addition of BMP2 slightly increased the amount of osteoid, but only at the surgical margins. At both the 4 and 8 week time points, ACS groups had significantly increased osteoid ($p<0.001$; Fig. 4D). Again, medium and high BMP2 exposures caused higher deposition of osteoid compared to control at 4 weeks ($p<0.001$).

Despite decreased ALP and osteoid levels in Talymed samples, there was no significant difference found for the MS, MAR, or BFR values analyzed via calcein injections between matrices. However, the high dose of BMP2 was shown to have significantly increased MAR and BFR than control at 4 weeks ($p<0.05$). By 8 weeks all significant differences in the MS and BFR were resolved. Interestingly, it was found that the MAR increased from 4 to 8 week ($p<0.001$) while both the MS and BFR decreased ($p<0.001$).

3.5 Analysis of bone resorption

To determine if the remodeling process was also affected by the different matrices, osteoclast activity was evaluated via TRAP staining (Fig. 5A). Osteoclast activity within the Talymed groups was primarily contained within the diploic spaces adjacent to the surgical margins. In contrast, ACS groups showed high levels of TRAP positivity along the entire defect site. This was especially true within the BMP2 exposed groups as the bone spicules were seen to have large TRAP positive areas along their edges. ACS groups were seen to have significantly increased TRAP positive areas compared to Talymed at 4 and 8 weeks ($p<0.001$ and $p=0.003$, respectively; Fig. 5B). BMP2 exposure also significantly increased TRAP staining compared to control at 4 and 8 weeks, with medium BMP2 exposed groups having the highest TRAP positive area. Interestingly, osteoclast activity was seen to increase from 4 to 8 weeks.

4. Discussion

Clinical approaches applying alternative methods for bone replacements that overcome deficiencies in the reconstruction of osseous defects have led to robust investigations into the use of various tissue engineered scaffolds within fracture sites. While the clinical use of bioengineered scaffolds has increased due to their ability to augment surgical interventions using various types of grafting techniques, certain limitations persist. A lack in the optimization of the biocompatibility of the constructs and the use of supraphysiologic dosages of growth factors, such as BMP2, has led to severe side effects that impede fracture healing (Hussein et al., 2012; Kaneko et al., 2000; Langenfeld, Kong, & Langenfeld, 2006; Lee et al., 2012; Li et al., 2009; Smucker, Rhee, Singh, Yoon, & Heller, 2006). The presented work investigated healing of murine calvarial critical sized defects with a pGlcNAc nanofiber scaffold, Talymed, compared to the clinical standard ACS in

conjunction with exogenous BMP2 treatment. Our data showed that while Talymed had better binding properties for BMP2 compared to ACS, the resulting increase in the bioavailability of the BMP2 did not lead to increased bone regeneration within the defect site. Talymed was found to result in significantly less BV/TV at both 4 and 8 weeks compared to ACS. μ CT analysis of the regenerate showed that while Talymed increased the thickness of the trabeculae, it reduced the number of trabeculae significantly. These results indicate that the osteoconductive nature of ACS allows for bone to regenerate not only along the surgical borders but also within the center of the defect. Conversely, Talymed is not as osteoconductive. This is evidenced by regenerate within the Talymed samples forming only along the surgical borders with thicker but less numerous trabeculae. This thicker bone regenerate was found to have improved biomechanical properties at 4 weeks compared to ACS. However, by 8 weeks all significant differences between the biomechanical properties of the regenerate were resolved.

Histological investigation of the collagen structure within the defect site led to the observation of a decellularized space within Talymed samples devoid of any collagen content. This unique feature was maintained even with the use of BMP2. Analysis of the maturity of the collagen fibers showed that the formed matrix had increased yellow and red mature fibers at 8 weeks while the immature green fibers decreased from 4 to 8 weeks. This indicated that the collagen matrix was mature and any inhibition of regenerate formation was not due to a delay in healing. The decellularized space associated with the Talymed was maintained during the healing process. Downstream osteogenic factors such as ALP and osteoid levels were found to be similarly impaired in the Talymed samples with the amount of osteoid being significantly less than ACS at both 4 and 8 weeks. The use of BMP2 did not have any impact on the ability for Talymed samples to deposit osteoid on the newly formed collagen matrix. In contrast with the lack of ALP and osteoid formation, calcein results showed no difference between matrices at either 4 or 8 weeks. This could be due to the fact that Talymed, while having no bone regeneration within the center of the defect site, was shown to have robust bone regeneration along the surgical margins by 4 weeks and by 8 weeks the defect site has reached a steady state with little bone regeneration occurring. It could be that an intermediate time point would show differences between the matrices. Interestingly, the increase in the mineral apposition rate from 4 to 8 weeks could indicate an increase in the remodeling process. To determine if the lack of bone formation was tied to an altered remodeling homeostasis, osteoclast function was also investigated and found to be severely impaired with the use of the Talymed matrix at both 4 and 8 weeks. The reduction in both bone formation and resorption denotes that the inhibition of the formation of bone regenerate is not likely due to the effect of Talymed on a single cell type but through the inhibition of the entire remodeling process.

Despite Talymed's proven ability to augment healing in cutaneous wounds via enhanced cellular activity, anti-inflammatory and non-immunogenic properties (Lindner et al., 2015; Pietramaggiori et al., 2008), and tightly bind BMP2, our findings showed the use of Talymed was contraindicated in fracture repair as compared to the standard ACS scaffold. As previous studies have established, the crucial role of microarchitecture and the physical properties of scaffolds are important for the ability of bone tissue to regenerate (El Sayed et al., 2013; Tong, Xu, Liu, & Wang, 2015; Wang et al., 2012). However, these findings point

to Talymed itself having certain properties that, contrary to our hypothesis, inhibit bone healing. This begs the question which properties are most important for osseous bone wound healing. The repair of critical-sized bone defects requires scaffolds that allow the transportation of nutrients and a passive or active drive toward osteogenesis (Li et al., 2009). It is well established that the dura mater that underlies calvarial bone and the surgical margins of a wound provide osteoprogenitors and nutrients required for the mineralized content within the defect site (Opperman, Chhabra, Nolen, Bao, & Ogle, 1998; Rentsch et al., 2014). The formation of a decellularized region within the Talymed samples could indicate that the tightly woven nanofibers of the Talymed matrix form a barrier that both cells and nutrients are unable to penetrate. The reduction of this cellular infiltrate would certainly inhibit the initiation of the healing process as well as the specific functions of cells involved in the remodeling process. Further, the greater retention and slower release of growth factor, although attractive in tissue engineering strategies (Agrawal & Sinha, 2017), may be less important with peptides such as rhBMP2 which has been shown to induce a feedback loop increasing local and circulating endogenous BMP2 also important for sustained healing (Hussein et al., 2012).

This study highlights the obstacles of optimizing biocompatibility and function in bioengineered scaffolds. While Talymed was found to be overall inferior to the standard ACS matrix, causing lesser BV/TV and a unique decellularized space within the defect site that limited ALP, osteoid, and TRAP activity, these results showed that Talymed contained important features, including the ability to bind BMP2. These features in particular may be used in a clinical setting in combination with another scaffold, potentially ACS, to optimally augment bone wound healing. Future studies will need to address whether the antimicrobial and anti-inflammatory effects of Talymed in conjunction with the increased retention of BMP2 could lead to a synergetic effect when used with ACS. With the socioeconomic burden of the 1 million fracture repair surgeries performed annually in the United States (Shekaran et al., 2014), there is a clear, unmet clinical need for improved growth factor delivery scaffolds that promote bone healing. Though Talymed was unsuccessful at precipitating optimal bone regenerate, it still possesses the potential to be an important clinical tool in bone wound healing if used in conjunction with other osteoconductive and osteoinductive factors.

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^{125}I -BMP2 Carrier Release Characterization **Fig. 1:**

In vitro ^{125}I -BMP2 carrier release characterization. Talymed retains 20% more ^{125}I -BMP2 than ACS over an 8 day time course. Both carriers exhibited an 80% reduction in ^{125}I -BMP2 from the initial time point at day 9. Differences between matrices and BMP2 exposures analyzed by two-way ANOVA (matrix by time; $p<0.001$). Differences by time point analyzed via students t-test *** = $p<0.001$.

Fig. 2: Bone regenerate analysis. A) Representative μ CT images of bone regeneration 4 and 8 weeks after creation of 5 mm critically-sized calvarial defect. The black ring indicates the original surgical margins of the defect. B) BV/TV of each regenerate with ACS generating higher bone volumes at both 4 and 8 weeks ($p < 0.001$ and $p = 0.03$, respectively). C) Trabecular Number (Tb.N) significantly increased in ACS groups at 4 weeks ($p < 0.001$). D) Trabecular Thickness (Tb.Th) was higher in 8 week Talymed samples ($p = 0.034$). E) Trabecular Separation (Tb.Sp) increased in Talymed samples at 4 weeks ($p = 0.001$). F) Representative H&E images of ACS and Talymed control and medium exposure groups illustrate the large decellularized space observed within the Talymed groups at both time points. G) Total Indentation Distance (TID) illustrating that Talymed has a lesser degree of failure at 4 weeks compared to ACS groups ($p < 0.001$). H) Indentation Distance Increase (IDI) shows that Talymed has a higher degree of toughness at 4 weeks ($p = 0.004$). I) Elevated

Average Unloading Slope (Avg US) in Talymed groups indicates an increased resistance to indentation at 4 weeks ($p=0.002$). Scale bar = 400 μm . Differences between matrices and BMP2 exposures analyzed by three-way ANOVA (matrix by dose by time), $p<0.05$ being significant; * different from control, # different from low dose BMP2, ^ different from medium dose BMP2, and \$ different from high dose BMP2. * $p \leq 0.05$, ** $p \leq 0.01$, and *** $p \leq 0.001$.

Fig. 3:

Analysis of collagen matrix structure. A) Representative non-polarized PSR images of the defect site at 4 and 8 weeks post-surgery. B) Total collagen area indicating increased area in ACS groups at 4 and 8 weeks, $p < 0.001$ and $p = 0.007$, respectively. C) Representative images of polarized PSR. Green = thin immature; Yellow = intermediate; Red = thick mature collagen fibers. D) Thin, immature collagen fibers with all BMP2 exposures showed a decrease in area by 8 weeks compared to control ($p < 0.001$). E) Area of intermediate collagen fibers increased in ACS compared to Talymed at 4 and 8 weeks ($p < 0.001$). The medium and high BMP2 exposures showed the highest intermediate fiber area ($p < 0.001$). F) Thick mature collagen fiber area was greater in ACS at 4 weeks ($p < 0.001$). Scale bar = 400 μm . Differences between matrices and BMP2 exposures analyzed by three-way ANOVA (matrix by dose by time), $p < 0.05$ being significant; * different from control, # different from low dose BMP2, ^ different from medium dose BMP2, and \$ different from high dose BMP2. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Fig. 4:

Bone formation. A) Representative ALP images of the defect site at 4 and 8 weeks. B) At 4 weeks, ACS had higher ALP activity levels ($p < 0.001$). Medium and high BMP2 exposures increased ALP activity compared to control ($p < 0.001$). C) MTC representative images. Scale bar = 400 μm . D) ACS had larger areas of osteoid formation at 4 and 8 weeks ($p < 0.001$). Medium and high BMP2 exposures precipitated increased osteoid formation at 4 weeks ($p < 0.001$), while at 8 weeks osteoid area decreased in low and medium BMP2 groups. E) Calcein representative images for the rate of bone formation. Scale bar = 200 μm . F) A significant reduction in the mineralizing surface (MS) was seen between 4 and 8 weeks ($p < 0.001$). The medium BMP2 exposure had significantly greater MS than control ($p < 0.05$). E) The mineral apposition rate was significantly increased at 8 weeks ($p < 0.001$) with high BMP2 showing higher mineralization rates than control and low BMP2 exposures ($p < 0.05$). F) Bone formation rate significantly declined from 4 to 8 weeks ($p < 0.001$). Black arrows indicate areas of positive staining. Differences between matrices and BMP2 exposures analyzed by three-way ANOVA (matrix by dose by time) ($p < 0.05$) being significant; *

different from control, # different from low dose BMP2, ^ different from medium dose BMP2, and \$ different from high dose BMP2. * p 0.05, ** p 0.01, and *** p 0.001.

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Fig. 5: Osteoclast Activity. A) Representative TRAP images of the defect site 4 and 8 weeks post-surgery. Scale bar = 400 μ m. B) TRAP positivity was increased at 4 and 8 weeks in ACS groups compared to Talymed ($p < 0.001$ and $p = 0.003$, respectively). Medium and high BMP2 exposed groups had increased osteoclast activity at the 4 and 8 week time points ($p < 0.001$). Black arrows indicate areas of positive staining. Differences between matrices and BMP2 exposures analyzed by three-way ANOVA (matrix by dose by time), $p < 0.05$ being significant; * different from control and # different from low dose BMP2. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$.

Table 1:

Sample sizes of C57BL/6 individuals for the associated BMP2 dosages and time points utilized in this study.

Time point	ACS				Talymed				Total
	Control (sex)	Low BMP2 (sex)	Medium BMP2 (sex)	High BMP2 (sex)	Control (sex)	Low BMP2 (sex)	Medium BMP2 (sex)	High BMP2 (sex)	
4 Weeks	10	10	9	9	10	10	10	10	78
	(5M, 5F)	(5M, 5F)	(4M, 5F)	(4M, 5F)	(5M, 5F)	(5M, 5F)	(5M, 5F)	(5M, 5F)	
8 Weeks	10	10	10	10	10	7	10	10	77
	(5M, 5F)	(5M, 5F)	(5M, 5F)	(5M, 5F)	(5M, 5F)	(2M, 5F)	(5M, 5F)	(5M, 5F)	
Total	20	20	19	19	20	17	20	20	155