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Masquelet Technique: Effects of Spacer Material and Microtopography on Factor Expression and Bone Regeneration

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Abstract

We and others have shown that changing surface characteristics of the spacer implanted during the first Masquelet stage alters some aspects of membrane development. Previously we demonstrated that titanium spacers create membranes that are better barriers to movement of solutes >70kDa in size than polymethyl methacrylate (PMMA) induced-membranes, and roughening creates more mechanically compliant membranes. However, it is unclear if these alterations affect the membrane's biochemical environment or bone regeneration during the second stage. Ten-week-old, male Sprague-Dawley rats underwent an initial surgery to create an externally stabilized 6mm femoral defect. PMMA or titanium spacers with smooth (~1µm) or roughened (~8µm) surfaces were implanted. Four weeks later, rats were either euthanized for membrane harvest or underwent the second Masquelet surgery. Titanium spacers induced thicker membranes that were similar in structure and biochemical expression. All membranes were bilayered with the inner layer having increased factor expression (BMP2, TGFβ, IL6, and VEGF). Roughening increased overall IL6 levels. Ten-weeks post-engraftment, PMMA-smooth induced membranes better supported bone regeneration (60% union). The other groups only had 1 or 2 that united (9–22%). There were no significant differences in any microCT or dynamic histology outcome. In conclusion, this study suggests that the membrane's important function in the Masquelet technique is not simply as a barrier. There is likely a critical biochemical, cellular, or vascular component as well.

Keywords

Critical-sized defects; Animal Model; Bone Reconstruction; MicroCT; Bone Grafting

Introduction.

The Masquelet or induced membrane technique is a relatively new procedure to reconstruct segmental bone defects caused by tumor resection, trauma, or osteomyelitis.^{3,8,17,37,47,51}

The two-stage procedure relies on a vascularized foreign-body membrane created during the

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first stage to provide a permissive environment for morselized bone graft incorporation during the second stage. Without the membrane, grafts larger than 4 to 6 cm in length are typically resorbed. In contrast, with the membrane created during the Masquelet technique, grafts over 20 cm have successfully healed.^{25,36,40} However, the exact mechanisms that facilitate this phenomenon are unknown. The failure rate of the Masquelet technique is approximately 18%,⁴⁰ and there is little evidence to guide surgeons to improve patient selection, to optimize or customize the treatment regimen, or to develop tissue engineered products. It is currently theorized that the membrane's critical functions may be (i) simply to act as a barrier to prevent soft tissue invasion, concentrate positive regulators of bone formation (cells and biochemical factors), and contain the graft, (ii) provide a local source of positive regulators, and (iii) pre-establish a rich vascular network.

Clinically, the membrane is 'induced' by a spacer made of – polymethyl methacrylate (PMMA, bone cement). The spacer is implanted during an initial surgery and left in place for a minimum of 4 weeks to allow membrane formation via the foreign- body response cascade. Prior implant research has demonstrated that changing an implant's surface properties, such as material or surface topography, will affect membrane formation.^{2,15,27,44,54} Indeed, recent work from our lab and others have demonstrated that this principle holds true within the Masquelet Technique milieu using animal models. Nau *et al.* showed that different PMMA formulations (i.e. bone cement brands) provoked mild differences in thickness, elastic fiber density, and vascularity. More recently, we have tested the physical properties of membranes created by PMMA or titanium (TI) spacers with smooth (~1µm) and microroughened (~8µm) surfaces after 4 weeks of implantation. Roughening produced membranes with a lower elastic modulus (-58% on average) while TI-induced membranes provided a better barrier to solute transport (i.e. particles greater than 70kDa).¹⁴ Membrane compliancy may modulate progenitor cell differentiation and mature cell behavior,^{26,30} while improved barrier properties would directly affect cellular migration and biochemical movement between the defect and muscle compartments. Both studies suggest that the membrane can be altered by spacer characteristics, yet neither established whether this affects the second stage bone regeneration. Also, in our previous study we only assayed the membranes' physical environments and not the biochemical environments, which may be more impactful on bone regeneration and defect reconstruction. Thus, the purpose of this study was to determine if altering spacer material or topography changed the membrane biochemical environment or bone reconstruction.

Based on previous research, we hypothesize that both variables, material and topography, will modulate the expression of factors commonly associated with the Masquelet Technique. Specifically, using TI as a spacer material or micro-roughening will increase expression of positive regulators and decrease negative regulators of bone formation. Further, these modulations in the membrane biochemical environment in combination with the increased barrier properties of TI induced membranes will result in superior bone regeneration.

Materials and Methods

Animals.

All procedures were done with the approval of our institution's animal care and use committee (protocol #2451). Male Sprague-Dawley rats (Charles River, Wilmington, MA) were used for all experiments. Experimental animals were 10 weeks old when they underwent the first surgical procedure. This is approximately equivalent to an 18-year-old human.¹³ This is on the younger end of 'skeletally mature'. However, since this technique is frequently used on trauma patients, which are typically males 18–35, and pediatric oncology patients, we felt this age sufficiently represented our patient populations of interest. Donor animals were cohorts of the experimental animals and 14 weeks old when euthanized for graft harvest. Animals were group housed prior to surgery and single housed post-surgery in standard husbandry conditions. All animals were euthanized via carbon dioxide asphyxiation in accordance with 2013 AVMA guidelines (10–30% volume/min gradual replacement).

Surgical Procedures.

All experimental animals underwent the first stage surgical procedure of the Masquelet Technique using aseptic technique, which has been described in detail previously.³⁹ Briefly, an external fixator was installed using a lateral approach. After assuring stable placement, a 6mm defect was created at approximately the bone mid-point, a cylindrical spacer (3.2mmØ × 6mm) was placed in the defect, and the skin was closed. The spacer was made of either PMMA (PalacosR, Zimmer/Biomet, Warsaw, IN) or grade 5 titanium (McMaster-Carr, Elmhurst, IL) with either a smooth (polished with 1µm diamond suspension, Buehler, Lake Bluff, IL) or rough (polished with 2500 grit sandpaper, ~8µm, McMaster-Carr) (Figure 1, fabrication and characterization described in detail in¹⁴). Four weeks later (28 days), animals were either euthanized for stage 1 bone formation and membrane studies or underwent the second grafting surgery (Figure 2). This timepoint was chosen based on the current clinical recommendations to perform the second surgery 4 to 8 weeks after the first.

For the second stage surgery, the membrane was incised laterally for spacer removal. Then micro-tipped forceps were used to gently scrape the exposed bone ends and open the marrow cavity. Then the membrane compartment was filled with ~48mm³ of morselized bone graft from an unoperated cohort animal. The muscle interval was closed with a resorbable suture (5–0 Polyglycolic Acid braided, Henry Schein Animal Health, Dublin, OH) and the skin closed as in the first surgery. These animals were euthanized 10 weeks later (Figure 2). This is a timepoint shown previously to clearly result in union (or non-union) for this technique in rats.^{20,39}

42 animals underwent only the first surgery. Of these, 2 were excluded because of significant radiolucency around one or more pins, which would indicate implant loosening or failure. The spacer remained in place in both. 58 animals underwent both surgeries. Of these 15 were excluded for implant loosening. In all these cases, osteolysis began sometime after the second surgery when the spacer was no longer there to provide mechanical support. Another animal was euthanized early because it lost a tooth and eating would have been

impaired. Another animal died of unknown causes two days post-op. The group numbers listed for each experiment reflect the numbers remaining after these exclusions.

Two *no* membrane groups were included to serve as negative controls for the second stage (n=3/group, Figure 2). The first group underwent the initial surgery to implant a smooth PMMA spacer identical to experimental animals. However, during the second surgery, the membrane was removed by excising it along with some surrounding muscle prior to implanting the graft. For the second group, the first surgery included only implanting the external fixator. The 6mm defect was created and the graft implanted during the second surgery. All other procedures for both groups were identical to the experimental animals.

Radiography.

Spacer migration and implant failure (i.e. pin loosening) were monitored via x-ray in all animals two weeks after the first surgery. Animals that underwent graft implantation were monitored bi-weekly post-engraftment to qualitatively track bone regeneration and monitor for implant failure.

Stage 1 - Bone Formation - MicroCT.

MicroCT was used to quantify new mineralized tissue extending from the original bone ends to encase the spacer (n=9–11/group). After euthanasia, the operated limb was harvested along with the surrounding soft tissues and fixed. After carefully removing the external fixator, the area between the inner pins was scanned with microCT (MicroCT 35, ScanCo Medical, Brüttisellen, Switzerland; X-ray tube potential 70 kVp, integration time 300 ms, X-ray intensity 145 μ A, isotropic voxel size 10 μ m, frame averaging 1, projections 1000, high resolution scan). Bone extending from the original bone ends to cover the spacer was manually contoured (Figure 3). Maximal extension length and total volume for the proximal and distal bone protrusions were quantified for all groups.

Stage 1 - Histology & Immunohistochemistry (IHC).

To determine the membrane region thicknesses after 4 weeks of spacer implantation as well as semi-quantitate regenerative factor expression, histological-based methods were used. Following microCT, the limbs were decalcified, incised lengthwise using the original surgical approach to remove the spacer, and cryosectioned. One serial section was stained with picrosirius red and alcian blue and mounted with resinous media to highlight collagenous and cartilaginous tissues, respectively, as well as enhance fibril birefringence. This section was imaged under compensated and polarized light at multiple angles (15° increments from 0° to 180°, BX51, Olympus, Center Valley, PA with EC3 camera, Leica Microsystems, Buffalo Grove, IL) to illuminate the two membrane layers: the non-birefringent layer (NB) directly apposed to the spacer and the birefringent layer (BR) radially outward. The image series was analyzed with a custom MatLab program (Mathworks, Natick, MA) to determine fiber alignment in the BR layer. A single image from the series was used to measure membrane thickness and as a guide to differentiate the two layers in each animal's IHC treated sections' images. Serial sections underwent antigen retrieval and blocking (normal goat serum, 10% v/v in PBS). Experimental sections were reacted with primary antibodies against BMP2, TGF β , IL6 or VEGF (1:50 dilution in

blocking serum, rabbit polyclonal, Abcam, Cambridge, MA). These are factors commonly assayed in previous Masquelet studies. One negative control section per animal was incubated with blocking serum lacking a primary antibody under identical conditions. After washing the sections, all (experimental and negative control) were incubated with the secondary antibody (1:100 dilution in PBS, Goat polyclonal Secondary Antibody to Rabbit IgG - H&L w/ Alexa Fluor 488, Abcam). All sections were mounted with DAPI containing media (Fluoro-Gel II with DAPI, Electron Microscopy Sciences, Hatfield, PA) and imaged at the defect's medial mid-point (opposite the surgical approach) (DMI 4000B with DFC 340FX camera, Leica Microsystems). A custom MatLab program measured the average green channel fluorescent intensity in the two membrane regions (NB and BR). The values from each animal's negative control section were subtracted from the values obtained in the experimental sections to control for tissue autofluorescence and non-specific binding. The negative control's blue channel was also used to count cell nuclei in the two layers using a custom MatLab program.

Stage 2 - Bone Formation - MicroCT.

To evaluate bone regeneration 10 weeks post-engraftment, microCT scans were quantitatively and qualitatively assessed. Following euthanasia, the operated limbs were harvested and fixed (n=9–11/group). The limbs were scanned under identical conditions as in 'Stage 1 Bone Formation'. Each bone's scan was manually contoured from the original proximal bone to the original distal bone (when possible) encompassing the region that would ideally be filled with new tissue (Figure 4A – dashed line). Data on defect length, total defect volume (TV), bone volume (BV), bone volume fraction (BV/TV), bone mineral density (BMD), and tissue mineral density (TMD) were collected. To qualitatively assess union, composites of each animal's microCT scout view, anterior reconstruction, and lateral reconstruction (Figure 5) was blindly graded by an orthopaedic surgeon (DN) as united, delayed, or non-union as done previously for this animal model.³⁹

Stage 2 - Bone Formation – Dynamic Histology.

To determine the timing of regeneration, dynamic histology was used. All stage 2 animals received an injection of calcein (5 mg/kg, Sigma Aldrich, St. Louis, MO) 2 weeks post-engraftment (n=8–9/group). An injection of alizarin complexone (30 mg/kg, Sigma Aldrich, St. Louis, MO) was administered either 4 weeks (n=4–5/group) or 6 weeks (n=3–5/group) post-engraftment. Following microCT scanning, the samples were embedded in polymethyl methacrylate (Fisher Scientific, Hampton, NH), longitudinally sectioned, and polished using increasing sand paper grits until a thickness of ~30µm was reached. Section images (DMI 4000B) were analyzed using a custom MatLab program to measure the labeled area in 5 regions centered about the defect midpoint extending from the original proximal bone end to the original distal bone end (Figure 4B). Each labeled area was normalized by the total region area (bone and soft tissue) to determine the percent area labeled.

Statistics.

All data are shown as mean and standard deviation. Significance was set at p-value of 0.05. All stage 1 data and stage 2 microCT data were tested with multivariate ANOVA (factors: material – PMMA vs TI, finish: Smooth vs Roughened) with repeated measures when

appropriate. For stage 1 bone formation, the repeated measure was location (proximal vs distal). For stage 1 membrane thickness, cellularity, and IHC outcomes, the repeated measure was layer (NB vs BR). Dynamic histology data were tested in each region with multi-variate ANOVA (factors: material-PMMA vs TI, finish-Smooth vs Roughened, and time point – 2 vs 4 vs 6 weeks) with Fisher's post-hoc least square difference testing. Since union outcomes were categorical, they were compared using non-parametric Kruskal-Wallis test followed by pair-wise Mann-Whitney tests to determine which were different from each other. Radiography and dynamic histology data were first grouped by union outcome (united, delayed, non-union) to describe the healing process when it is successful and unsuccessful. This characterization may be useful for future study designs. However, this data was not statistically compared since the grouping was based on outcome, which is inherently biased and dependent.

Results.

Stage 1 Bone Formation.

As has been noted in basic science studies^{21,22} and some case studies,²⁴ mineralized tissue will sometimes extend from the original cortical bone ends to encase the spacer. There were no differences between groups in maximal bone extent length (~1.7mm) or total bone volumes. However, the proximal total volume was on average 80% larger than the distal total volume ($p = 0.002$, Figure 6).

Stage 1 Membrane Thickness & IHC.

On average the outer BR layer was twice as thick as the inner NB layer in all groups ($p < 0.001$). As a whole, TI induced membranes were approximately 35% thicker than PMMA induced membranes ($p = 0.045$, Figure 7A-B). The BR layer of all groups was similarly well-aligned (Figure 7D). There were no differences in the number of cell nuclei per unit area between groups or layers (Figure 7C). All examined factors (BMP2, TGF β , IL6 and VEGF) were more highly expressed in the NB layer than the BR layer ($p < 0.001$, all factors, Figure 8). The inflammatory factor IL6 was on average 35% higher in the roughened groups than the smooth ($p = 0.038$, Figure 8E).

Stage 2 Bone Formation.

All animals lacking a membrane went on to non-union (Figure 9, top two panels). In these animals, the graft was still present at 2 weeks, but maintained a globular morphology. The graft had clearly been partially resorbed by 4 weeks and was no longer visible on x-rays by 6 weeks. For experimental animals that united (Figure 9, third panel), the defect area had a more amorphous appearance at 2 weeks indicating robust new bone formation, although globular graft pieces were still evident. Over the following 4 weeks, there were moderate architectural and density changes suggesting additional remodeling. From 6 to 8 weeks there was a noticeable increase in radio-opacity throughout indicating increased mineralization. The 10 week microCT scan revealed trabecular bone filling the entirety of the defect (Figure 10A). Dynamic histology generally mirrored radiography. The calcein label (2 weeks) was highest at the two defect ends with decreasing label moving axially towards the defect center. At 4 and 6 weeks the bone label was more uniform (Figure 10B). For experimental

animals categorized as delayed (Figure 9, panels 4 and 5), often a radiolucent gap was evident early on (~4 weeks), and, although the bone on either side appeared to remodel and increase in density, the gap never truly bridged. However, in some animals, due to the complex gap geometry, the void was not evident until the microCT reconstructions were evaluated. Initially (2 weeks), the bone labeling was almost identical to that of united animals. However, at 4 weeks bone labeling remained non-uniform. By 6 weeks the bone labeling became evenly distributed, similar to united animals (Figure 10B). For experimental animals that went to non-union (Figure 9, bottom panel), the graft looked similar at 2 weeks to that of united or delayed animals. However, similar to negative control animals, the graft had significantly diminished by 4 weeks and was no longer visible on x-rays by 6 weeks. Bone labeling at all time points was lower than the other two categories and relatively non-uniform (Figure 10B).

When segregated into their respective groups, the PMMA smooth membranes supported bone regeneration significantly more often than the other three groups (Figures 11 and 12A). A majority of the PMMA smooth animals (60%) united by 10 weeks, while 40% were deemed non-union. No animals were considered delayed. In contrast, other groups only yielded one to two unions each. The remaining animals were split roughly evenly between delayed and non-union. PMMA smooth's dynamic histology resembled that of the united group. Initially the label was greatest at both ends and decreased moving towards the defect middle. At 4 and 6 weeks the label was predominately uniform. For the other three groups, the labels were non-uniform at both 2 and 4 weeks. At 6 weeks the label in all groups was greatest in the proximal-middle and distal-middle regions with roughly equivalent signal in the proximal, middle, and distal regions (Figures 11 & 12B). However, there were no statistically significant differences between groups in either microCT (Table 1) or dynamic histology outcomes.

Discussion.

The Masquelet technique has been gaining popularity in oncology and trauma since its introduction over 30 years ago.^{37,40,47,52} However, we do not currently understand why the procedure works, nor have we adequately characterized healing dynamics. In order to perturb first stage membrane development, two spacer variables, material and surface microroughness, were selected based on decades of implant research.^{15,19,44,46} Previously, we have shown that roughening creates more compliant membranes, which could affect cellular behavior via perceived mechanical signals, while material did not affect membrane mechanical properties. On the other hand, TI created membranes that were better barriers with no effects due to surface roughness.¹⁴

In this study we found that TI spacers created somewhat thicker membranes, which is contrary to previous research. However, this is a different situation than most previous experiments. Most general foreign-body response studies implant objects subcutaneously.^{2,11,50} Orthopaedic implant studies specifically examine the region between the implant surface and bone, and do not assess implant surface and muscle interface, which is most of the induced membrane. It is widely known that membrane formation is contextual.⁴⁷ In rabbit models, PMMA-induced membranes differ greatly if created in an intramuscular site,

subcutaneous site, or radial defect.³² The radial defects' membranes were of intermediate thickness compared with the other two locations but had much higher vascularity. Taken together with our results, which run contrary to previous implant research, this emphasizes the need to conduct experiments meant to apply to the Masquelet technique specifically in bone defects, as subcutaneous studies do not seem to adequately represent the Masquelet milieu.

Despite spacer material effects on overall membrane thickness and barrier properties, the bi-layered architecture and biochemical expression was relatively unaffected. The inner NB layer was much thinner and had higher expression of all the traditionally examined biochemical factors (BMP2, TGF β , VEGF, and IL6). Interestingly, the same layer retained the highest amount of solutes greater than 70kDa in our previous study.¹⁴ As such, the NB layer may not only create, but also trap and store, factors for release during stage 2 membrane degradation. Roughening did increase expression of the inflammatory factor, IL6. It's difficult to put this data into context since few other studies have altered the spacer in any way. Nau et al. demonstrated that different formulations of antibiotic-loaded PMMA (i.e. different brands) affected membrane thickness, elastic fiber density, and vascularity.⁴³ Although, these differences were usually only at one of the three time points assayed rather than a consistent trend. Shah et al. found that antibiotic-loaded spacers had no effect on gene expression, while infection itself did.⁴⁹ Neither study examined the second stage to determine if bone reconstruction would have been affected.

In fracture repair, the role of pro-inflammatory factors, like IL6, is complex. An early inflammatory phase is necessary to recruit the proper mix of progenitor cells and initiate angiogenesis as well as other mechanisms of the repair cascade.^{1,31,33} There is another increase in IL6 expression during the later remodeling phase which suggests it regulates osteoblast and osteoclast behavior.³³ In deed, IL6 transgenic knockout or pharmacological inhibition detrimentally affects fracture repair.^{45,57} Although, dysregulated or chronically high IL6 levels, as seen in poly trauma patients or systemic inflammation conditions like osteoarthritis or aging, are also correlated to poor fracture repair.^{4,5,9,18,23,33} It is possible that the increased IL6 levels in the roughened groups' membranes could indirectly enhance repair by supporting positive healing conditions like increased angiogenesis. On the other hand, that IL6 is still elevated 4 weeks after the initial surgery suggests that a more chronic situation has been created by using roughened spacers. When the second surgery is performed, the additional inflammation could push to local environment above some threshold and negatively affect bone regeneration. Our current information supports the latter hypothesis. However, more work on the subject is needed to tease apart these conflicting mechanisms.

To our knowledge, this is the first study to attempt bone formation dynamics characterization during Masquelet's second stage. Our results suggest that, similar to fracture healing,^{1,6,9,16,42,55} new tissue formation occurs in a wave extending from the proximal and distal edges toward the defect center rather than extending from the membrane edges radially inward. Also like fracture healing, there is significant activity in the first few weeks. Two weeks post-fracture, the callus has formed and is mostly mineralized with some residual cartilage at the mid-point.^{1,16,38} In animals that were united or delayed, the

two-week bone label was primarily in the most proximal and distal defect regions and declined towards the center. In the central regions, the 4 and 6 week labels were similar to that of the 2-week label at the defect ends. However, it is possible that bone formation occurred uniformly throughout the defect early on, but this early bone in the central region was remodeled during the subsequent weeks and replaced with even newer matrix. Supporting this theory, x-rays at the 2-week time point seemed to show new matrix around the graft pieces. Similarly, in traditional bone grafting (i.e. small enough defect for graft incorporation without a membrane) it is thought that host and some surviving graft cells begin laying down new matrix on all bone surfaces within the first 2 to 4 weeks.^{7,10,28} This tissue is then remodeled over the following months to years as necrotic graft pieces are resorbed. Although, it should be noted that in our study significant graft resorption (i.e. non-union) typically was not obvious radiographically until 4 to 6 weeks. In the future, cellular migration, protein expression, gene expression, and bone regeneration (microCT) should be explored at both early (days to weeks) and late (weeks to months) periods following engraftment. This will help definitively characterize successful regeneration and may illuminate early ‘check points’ which divide successful and unsuccessful repair pathways.

Our results agree with previous studies demonstrating the membrane to be necessary for bone reconstruction at this length-scale.^{20,29} Without the membrane, whether it was never created or resected, grafts were resorbed in weeks. It is theorized that the membrane’s main function is to provide a barrier from soft tissue invasion and other negative regulators of bone formation like inflammatory cytokines. However, our data does not support this hypothesis. If this were true, the two PMMA groups, which have almost identical barrier properties and thicknesses, should have had similar union outcomes. The PMMA smooth group united in 60% of animals while the PMMA rough group had only one union (9%). Further, TI induced membranes, which were better barriers, had significantly fewer unions as well (22% and 10%). Also, Luangphakdy et al. recently demonstrated that scraping induced membrane’s inner surface, which probably removed the NB layer and increased membrane permeability, lead to increased bone volume in a goat segmental defect after 12 weeks.³⁴ Together, these findings suggest that the induced membrane is not simply acting as a barrier. While the membrane’s barrier qualities may be important, it is likely that environmental factors such as protein expression and regulation, cell populations, and vascularity are equally, if not more, influential. Our theory is further supported by the results of research into “guided bone regeneration.” The concept is that large grafts (>6cm) can be protected by synthetic barrier membranes which have little to no biological activity. Yet despite many years of research, no segmental bone reconstruction products have been disseminated clinically.¹² Additional investigation into identifying the induced membrane’s critical qualities may be what is needed to design tissue engineered membranes that can achieve clinical success. It may be worth conducting subtractive comparisons between the PMMA and TI groups with more in depth techniques even though TI has repeatedly performed poorly.³⁹

There is an additional limitation that should be considered when interpreting our results. Only one time point was explored in each stage. Both stages are very dynamic. We attempted to address this in the second stage by including outcomes that relayed some timing information (i.e. longitudinal radiography and dynamic histology). It is possible that,

had we used another stage 1 duration, our results would have differed. Current clinical recommendation is to perform the second surgery between 4 and 8 weeks post-spacer implantation, when membrane thickness, cellularity, vascularity, and regenerative factor expression peak.⁵¹ However, the second surgery is often delayed much longer.^{40,41} The few clinical studies that have looked at stage 1 duration as a factor have found no correlation between the timing of the second surgery and reconstructive outcomes.⁵² This topic has not been broached a pre-clinical setting (i.e. highly controlled animal studies). Thus, it's plausible that our results would have been the same. Although, we feel strongly that definitively determining if the first stage duration affects the second stage is an important research endeavor.

In conclusion, we have shown that compared to PMMA induced membranes, TI induced membranes are thicker but relatively similar in architecture and expression of BMP2, TGF β , VEGF, IL6. Both have an inner NB layer that is thinner and has higher expression of all 4 factors than the outer BR layer. Roughing the spacers causes a slight increase in the inflammatory factor IL6. Despite the relative similarities between groups, smooth PMMA induced membranes had better union rates 10 weeks post- implantation. This indicates that surgeons should be aware of the surface they are creating when implanting PMMA spacers, and that titanium spacers are likely not a viable option for clinical application in the Masquelet procedure. Surface finish could be somewhat controlled by vacuum mixing the PMMA and using molds to create most of the spacer's structure. This should create a significantly smoother surface with few pores.^{35,53,56} Although, increased porosity may be beneficial as this tends to correlate with increased vascularity.^{44,48} It's also possible that a patient's local and systemic inflammatory status may significantly affect bone outcomes as IL6 was elevated in the micro-roughed groups and lead to fewer unions. There are very many possible avenues for future research on the Masquelet Technique that are directly translatable to clinical application.

In sum, this work suggests there is a biological component to the membrane that influences bone reconstruction more than its ability to act as a simple barrier. This indicates that the first stage treatment regimen could be customized to each patient to optimize second stage outcomes. Although, what those customization goals should be are still unknown. Future research should thoroughly analyze the membrane's cellular populations, vascularity, and gene/protein expression so that the critical features can be elucidated.

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Abbreviations:

PMMA	Polymethyl Methacrylate – Traditional Spacer Material, also known as bone cement
TI	Titanium – Experimental Spacer Material
PBS	Phosphate Buffered Saline – Wash Solution
DAPI	4',6-diamidino-2-phenylindole – Nuclear Stain
TGFβ	Transforming Growth Factor Beta – Positive Regenerative Protein
BMP2	Bone Morphogenetic Protein 2 – Positive Regenerative Protein – promotes Osteogenic differentiation
VEGF	Vascular Endothelial Growth Factor – Positive Regenerative Protein - Promotes Angiogenesis
IL6	Interleukin 6 – Negative Regenerative Protein – Proinflammatory factor microCT – Micro computed tomography
BV/TV	Bone Volume/Total Volume Fraction – fraction of volume of interest filled with bone
TV	total volume – total volume of interest
BV	bone volume – bone within the total volume of interest
BMD	Bone mineral density – average mineral density of both bone and space within the volume of interest
TMD	Tissue Mineral Density – average mineral density of only bone within the volume of interest

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Figure 1. Example Spacers.

(A) PMMA (top) and TI (bottom) spacers are 6mm in length and ~3.2mm in diameter with ~1mm TI pegs on each end that fit into the intramedullary cavity to prevent spacer migration. (B) SEM images of the spacer surfaces (images acquired for ¹⁴)

Figure 2. Design of Experiments.

(Top Panel) All experimental animals underwent the first surgery to install an external fixator and create a 6mm femoral defect. The defect was filled with a spacer made of smooth PMMA (PS), roughened PMMA (PR), smooth titanium (TS), or roughened titanium (TR). Four weeks later, the animals were either euthanized for stage 1 studies or underwent the second surgery to remove the spacer and implant a morselized graft for stage 2 studies. The latter animals were euthanized 10 weeks later. (Bottom Panel) Two no membrane negative control groups were included. The first was given PS spacers and went on to the second

surgery. However, during the second surgery the membrane was resected before the graft was implanted. The second control group did not have a defect created or spacer implanted during the first surgery. The defect was created and directly filled with graft during the second surgery.

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Figure 3. Stage 1 MicroCT Scanning & Contouring Example.

(A) MicroCT scoutview of an animal with a PMMA smooth spacer implanted. The white dashed line indicates the region scanned for each sample. (B) 3D reconstructions of the microCT scan for the same sample. The white dotted lines on the right panel indicate the regions that were contoured to determine the total volume.

Figure 4. Stage 2 MicroCT & Dynamic Histology Example.
(A) MicroCT 3D reconstruction of the entire scanned area in a PMMA smooth animal. The white dashed line indicates the volume that was contoured for analysis. (B) The dynamic histology slice from the same animal. Here the white dotted lines indicate the line between each analysis region.

Figure 5. Stage 2 Union Composite Example.

A composite of each animal's microCT scoutview, anterior view, and lateral view was assessed by an orthopaedic surgeon under blinded conditions. Each animal was categorized as united, delayed, or non-union. Both delayed and non-union would have a void space traversing the entire defect.

Figure 6. Stage 1 Bone Formation Results.

There were no differences between groups for either (A) maximal extension length or (B) total volume. However, the tissue extending from the proximal bone end was significantly more voluminous than that from the distal bone end.

Figure 7. Stage 1 Membrane Histology.

(A) Picrosirius Red and Alcian Blue stained sections showed similar over all architecture in all groups. Each had a non-birefringent layer (NB) directly apposed to the spacer that was not visible under polarized light. Radially outward, there was a birefringent layer (BR). (B) Contrary to previous research, TI spacers induced significantly thicker membranes. Although in all groups the NB layer was significantly thinner than the BR layer. (C) Both layers had similar cell nuclei density and was similar between groups. (D) The fibers of the BR layer in all groups was very well aligned.

Figure 8. Stage 1 Membrane Factor Expression.

(A-E) The NB layer was more enriched than the BR layer for all examined factors. (E) The inflammatory protein IL6 was the only factor significantly different between groups. On average, it was 35% in the roughened groups.

Figure 9. Stage 2 Bone Formation – Radiography – Healers vs Non-Healers.

Our data suggests that the divide between final outcomes is made relatively early after engraftment surgery although it may be difficult to determine from radiography alone. (Top two panels) Animals lacking a membrane, whether it was never created or was removed, all went on to non-union. Complete graft absorption was clearly evident by 6 weeks. (Third Panel) Animals that united showed subtle remodeling throughout the observation period. (Fourth & Fifth Panel) Delayed animals often had a visible gap from 4 weeks on, however, if the geometry was complex, it may not have become evident until the final microCT measurements. (Sixth Panel) Non-union animals had similar dynamics to that of animals lacking a membrane. The graft had resorbed by 6 weeks.

Figure 10. Stage 2 Bone Formation – MicroCT & Dynamic Histology – Healers vs Non-Healers. (A) MicroCT reconstructions revealed bone with a highly trabecular structure in all united and delayed animals. Delayed animals had a gap transversing the defect entirety and bone with a ruffled border on either side of the gap. Non-unions typically had a much larger gap and the bone on either side was smooth indicating little bone formation. (B) All groups had little bone label at the defect center at 2 weeks. Animals that united had more uniform labeling at 4 and 6 weeks. Delayed animals' labeling remained non-uniform at 4 weeks, but looked similar to that of united animals at 6 weeks. Non-union animals' labeling was lower than the other two groups at all time points.

Figure 11. Stage 2 Bone Formation – MicroCT and Dynamic Histology Examples.

Examples of each spacer group. The left panels are animals given the calcein label at 2 weeks and the alizarin label at 4 weeks. The right panels are animals given the calcein label at the same time but the alizarin label was given later at 6 weeks.

Figure 12. Stage 2 Bone Formation – Union & Dynamic Histology Results.

(A) As a group, the PMMA smooth animals had better outcomes than in the other three groups. 60% of these animals united while only 1–2 animals in the other groups united. (B) All groups had similar distribution of the 2 week bone label with more at the distal and proximal ends than in the middle region. At 4 weeks, the PMMA smooth group had a more uniform bone label distribution while the other three groups remained bimodal. At 6 weeks,

most groups have increased bone label, but with more label in the regions adjacent to the middle.

Table 1

– MicroCT Results

Group	Defect Length (mm)	Defect Total Volume (mm ³)	Bone Volume (mm ³)	Bone Volume Fraction (BV/TV)	BMD (mgHA/cm ³)	TMD (mgHA/cm ³)
PMMA Smooth	5.87 ± 0.60	82.1 ± 42.7	36.6 ± 27.6	0.398 ± 0.154	467 ± 167	1067 ± 23
PMMA Roughened	5.44 ± 0.75	58.5 ± 14.1	16.0 ± 6.9	0.291 ± 0.147	346 ± 168	1071 ± 32
TI Smooth	6.00 ± 0.89	83.5 ± 29.1	32.7 ± 19.8	0.385 ± 0.179	459 ± 201	1083 ± 33
TI Roughened	5.85 ± 0.45	78.2 ± 26.4	33.6 ± 18.6	0.412 ± 0.135	490 ± 149	1082 ± 20