What is the Effect of Antibiotic-Impregnated Bone Cement Spacers in the Healing Process of Large Bone Defects as Part of the Masquelet Technique?

Total word count: 1587

I. Rationale and Specific Aims (max 250 words)

Bone defects are one of the most challenging problems faced by orthopaedic trauma surgeons.[1,2] Large defects are often associated with soft-tissue injury and infection, which can complicate osteosynthesis. This leads to high fracture nonunion rates and significant long-term morbidity. In an attempt to address these issues, the Masquelet technique was developed to promote bone healing in the setting of complicated bone loss.[3,4]

Current practices of the Masquelet technique have adapted the original technique to include a variety of antibiotics that are mixed into the bone cement spacer.[5,6,7] Despite the widespread implementation of antibiotic-impregnated bone cement spacer into the technique, there is no study to date that has examined the effect of antibiotics on membrane formation or activity, or its overall effect on bone healing. There are some studies that suggest that antibiotics may reduce the expression and differentiation of stem cells, although this model has not been verified in musculoskeletal tissues.[8]

This proposed study seeks to investigate the effect of antibiotic-impregnated bone cement on the healing of large segmental bone defects using a rat model. Our specific aims seek to determine the effect of antibiotic-impregnated bone cement on the:

1. Formation and activity of the induced biologic membrane
2. Overall effect on bone healing

We hypothesize there is a negative dose-response relationship between antibiotic impregnated cement and the biologic membrane as well as bone healing. Our null hypothesis is that antibiotic impregnated bone cement will not have an impact on the membrane or bone healing.

II. Background and Significance (max 350 words)

Autogenous bone grafting remains the gold standard for the treatment of bone defects. However, the use autogenous bone in large defects has historically had poor results due to limitations in vascularity, capacity of the soft tissues to contain the graft material, and resorption of the graft

In an attempt to overcome these issues, Masquelet et al., used a block of methyl-methacryate cement at the bone defect site for 6 weeks, which promoted the formation of a biological membrane around the defect site.[4,5] The spacer was then surgically removed and replaced with autogenous bone graft. Encasing the bone graft within the membrane not only provided protection against autograft resorption and tissue interposition, it has subsequently been shown to provide high concentrations of

osteoprogenitor cells and osteoinductive/vasculogenic growth factors (such as bone morphogenetic protein-2 [BMP-2], vascular endothelial growth factor [VEGF], and transforming growth factor beta [TGFβ]) .[9,10] Masquelet and Bergue used this approach to successfully bridge large bone defects (up to 25cm) caused by a variety of pathologies, including infected non-unions.[5]

Current practices of the Masquelet technique have adapted the original technique to include a variety of antibiotics that are mixed into the bone cement spacer. [5,6,7] The use of antibiotic-impregnated cement spacers has been shown to achieve antibiotic concentration levels many times greater than the bacterial minimum inhibitory concentration,.[15,16] This is desirable as bone defects are often complicated by contamination or overt infection. However, the effects of the addition of antibiotics to the cement spacer on the biological membrane and bone healing have not been investigated. All of the basic science investigations conducted on the properties and effects of the induced membrane to date have been conducted without antibiotics. Currently, there is no data to support whether the presence of antibiotics of differing concentrations and chemical makeup have any effect on membrane formation/activity or bone healing.

The addition of antibiotics to the bone cement spacer in the Masquelet technique is a common practice despite the lack of evidence regarding the effects on the biological membrane and bone healing. The proposed study represents a potentially significant advance in understanding the treatment of traumatic bone-defects using the Masquelet technique.

III. Research Design and Methods (max 1000 words) –

i. Research Design

Rat Fracture Model

All animal protocols will be performed in accordance to the regulations set forth by our institution's Animal Care Committee in accordance with the law. The proposed study will use Fisher 344 rats; a syngeneic strain of inbred rats that will allow for allogeneic cancellous bone grafting. Forty animals, 8-12 weeks old and weighing between 300-350g, will be equally divided into five groups (n=8).

Masquelet Technique

Under sterile techniques, a 6 mm longitudinal bone defect will be created in the middle of the right femur of all rats by performing two diaphyseal osteotomies using an oscillating saw. The defect will be filled with bone cement and stabilized using a 6-hole, 1.5mm mini plate and screw fixation.

Experimental groups are divided based on the type and dosage of antibiotics (Table 1) and compared to the control group, which is treated with bone cement without added antibiotics. The cement will remain in situ for a total of 6 weeks, as this marks the threshold of membrane biological activity and common clinical practice. The cement will then be carefully removed; leaving the newly formed membrane intact, surrounding the previously created defect. The defect will then be filled with a standard volume cancellous bone graft material from a donor rat iliac crest.

Table 1:

Control group	**Treatment group**			
No antibiotics	Low dose antibiotic cement (1 g per 40 g)		High dose antibiotic cement (2.5 g per 40 g)	
	Vancomycin	Tobramycin	Vancomycin	Tobramycin
8 animals	8 animals	8 animals	8 animals	8 animals

Antibiotic Bone Cement

The liquid monomer will be added to methyl-methacrylate powder combined with the antibiotic powder of appropriate weight for the desired formulation in a sterile bowl. Mixing of the cement will then be performed as per the manufacturer's instructions, to obtain a homogeneous compound. The types of antibiotics for comparison include tobramycin and vancomycin. Premeasured dosages include 2.5 g antibiotic per 40 g of cement (*High dose group*) and 1 g antibiotic per 40 g of cement (*Low dose group*). A control group will have no antibiotics added to the cement.

Specimen Harvesting

Half of the animals in each group will be sacrificed at 6 weeks after implantation of the cement spacer for analysis of the induced membrane. The other half of the animals will be followed with serial radiographs at 2 week intervals after the bone grafting stage and then sacrificed at 10 weeks. The entire femur from the operated side will be harvested with a portion of surrounding soft tissue. Specimens sacrificed at 6 weeks after placement of the antibiotic spacer will be immediately processed for histology, cell culture and western blot analysis. Specimens sacrificed at 10 weeks after the bone grafting stage will be fixed in 4% paraformaldehyde solution for 24 hours and assessed with quantitative micro-CT and histology.

ii. Outcome Analysis

a. Membrane biologic activity

Histology:
The specimens taken from the animals at 6 weeks after placement of the antibiotic spacer will have the membrane harvested from around cement spacer. These tissue samples will then be processed, dehydrated and embedded in paraffin (without decalcification). The samples within paraffin blocks will be longitudinally cut into 5mm sections, and prepared slides will be immunostained with a monoclonal anti-CD31 antibody to allow quantification of new blood vessel formation, assessment of capillary density and cellular content of the samples, and determination of the type of tissue and vessel distribution. Haematoxylin and eosin staining of the cryosections will allow for gross histological analysis of the induced membranes and surrounding tissue.

Cell Culture:
Portions of the harvested membrane will be digested and seed in appropriate cell flasks/media to culture mesenchymal stem cell (MSC) and endothelial progenitor cell (EPC) populations to determine the relative presence of these cell types in the membrane.

Biologic Content Measurements:
BMP-2 and TGFβ content will be detected using Western blot analysis. Briefly, frozen membrane tissue will be homogenized, separated via electrophoresis and transferred to nitrocellulose membranes. We will use polyclonal goat anti-rat BMP-2 and monoclonal mouse anti-rat TGFβ antibodies and ECL detection reagents to detect protein levels. VEGF content will be measured via immunohistochemistry, using a rabbit anti-rat VEGF primary antibody and an HRP-conjugated secondary antibody.

b. Bone healing

Plain film radiographs:
Serial radiographs will be conducted at 2 week intervals until animal sacrifice to determine the features of bone healing via the antero-posterior (AP) view, using standardized tube to leg distance, kilovolts (Kv) and milliamps (mAs). The films will be reviewed and scored by a blinded assessor.

Quantitative Micro-CT:
Micro-computed tomography (micro-CT) analysis will be completed on specimens sacrificed at 10 weeks after the bone grafting stage. All trimmed femur samples will be placed in a poly-ethyl-imid (PEI) holder with 70% ethanol and scanned at 70kVp and 114mA using a MicroCT40 system (Scanco Medical). After scanning and reconstruction, a region of interest (ROI) including the osteotomy site will be

determined to quantify bone volumes and morphometry. After samples have been processed they will be further examined via histology.

iii. Statistical analysis

One-way and multivariate ANOVA will be used to analyze the statistical differences among the treatment and control groups, with a p value of ≤0.05 considered statistically significant.

IV. Role of the Resident (max 200 words)

As the resident involved in this project I will be functioning as the principal investigator. I have played an integral role in the development and design of the proposed study as well as the literature review and preparation of the current research proposal. I will be performing all of the surgeries, following the animals, and preparing samples for analysis. With assistance from other members of the lab, I will be performing the radiological, histological and molecular analyses. Technicians are available to assist with these analyses. I will perform all data collection and statistical analysis. I will also be responsible for preparation and submission of abstracts and manuscripts.

Total word count: 1587

References

1. United States Bone and Joint Decade: The Burden of Musculoskeletal Diseases in the United States. Rosemont, IL: American Academy of Orthopaedic Surgeons; 2008.
2. Praemer A FS, Rice D. Musculoskeletal Conditions in the United States. Chicago: American Academy of Orthopaedic Surgeons; 1992:85-24.
3. Masquelet AC, Begue T, Muller GP. Long bone reconstruction using an induced membrane and cancellous autograft: a series of 35 cases. J. Bone Joint Surg Br. 2001; 83 Supplements 1: 41.
4. Masquelet AC, Fitoussi F, Begue T, Muller GP: Reconstruction of the long bones by the induced membrane and spongy autograft [French]. *Ann Chir Plast Esthet* 2000;45(3):346-353.
5. Fitoussi F, Masquelet AC, Rigal S, Poichotte A, Bauer T, Fabre A; French Society of Orthopedic and Traumatologic Surgery (SoFCOT). Inter-tibiofibular graft for traumatic segmental bone defect of the tibia. Orthop Traumatol Surg Res. 2012 Apr;98(2):214-9.
6. Donegan DJ, Scolaro J, Matuszewski PE, Mehta S. Staged bone grafting following placement of an antibiotic spacer block for the management of segmental long bone defects. Orthopedics. 2011 Nov 9;34(11):e730-5.
7. Gouron R, Deroussen F, Juvet M, Ursu C, Plancq MC, Collet LM. Early resection of congenital pseudarthrosis of the tibia and successful reconstruction using the Masquelet technique. J Bone Joint Surg Br. 2011 Apr;93(4):552-4.
8. Cohen S, Samadikuchaksaraei A, Polak JM, Bishop AE. Antibiotics reduce the growth rate and differentiation of embryonic stem cell cultures. Tissue Eng. 2006 Jul;12(7):2025-30
9. Aho OM, Lehenkari P, Ristiniemi J, Lehtonen S, Risteli J, Leskela HV. The mechanism of action of induced membranes in bone repair. J Bone Joint Surg Am. 2013 Apr 3;95(7):597-604.
10. Pelissier P, Masquelet AC, Bareille R, Pelissier SM, Amedee J: Induced membranes secrete growth factors including vascular and osteoinductive factors and could stimulate bone regeneration. *J Orthop Res* 2004;22(1): 73-79.
11. Viateau V, Bensidhoum M, Guillemin G, et al: Use of the induced membrane technique for bone tissue engineering purposes: Animal studies. *Orthop Clin North Am* 2010;41(1):49-56.
12. Masquelet AC, Begue T: The concept of induced membrane for reconstruction of long bone defects. *Orthop Clin North Am* 2010;41(1):27-37.
13. Viateau V, Guillemin G, Calando Y: Induction of a barrier membrane to facilitate reconstruction of massive segmental diaphyseal bone defects: An ovine model. *Vet Surg* 2006;35(5):445- 452.
14. Henrich, D., Seebach, C., Nau, C., Basan, S., Relja, B., Wilhelm, K., Schaible, A., Frank, J., Barker, J. and Marzi, I. Establishment and characterization of the Masquelet induced membrane technique in a rat femur critical-sized defect model. J Tissue Eng Regen Med; 2013 Nov 8.
15. Cui Q, Mihalko WM, Shields JS, Ries M, Saleh KJ: Antibiotic-impregnated cement spacers for the treatment of infection associated with total hip orvknee arthroplasty. *J Bone Joint Surg Am* 2007;89(4):871-882.
16. Joseph TN, Chen AL, Di Cesare PE: Use of antibiotic-impregnated cement in total joint arthroplasty. *J Am Acad Orthop Surg* 2003;11(1):38-47.
17. Anagnostakos K, Kelm J, Regitz T, Schmitt E, Jung W: In vitro evaluation of antibiotic release from and bacteria growth inhibition by antibiotic-loaded acrylic bone cement spacers. *J Biomed Mater Res B Appl Biomater* 2005;72(2): 373-378.
18. Hanssen AD, Spangehl MJ: Practical applications of antibiotic-loaded bone cement for treatment of infected joint replacements. *Clin Orthop Relat Res* 2004;(427):79-85.
19. Jaeblon T: Polymethylmethacrylate: Properties and contemporary uses in orthopaedics. *J Am Acad Orthop Surg* 2010;18(5):297-305.
20. Hsieh PH, Chen LH, Chen CH, Lee MS, Yang WE, Shih CH: Two-stage revision hip arthroplasty for infection with a custom-made, antibiotic-loaded, cement prosthesis as an interim spacer. *J Trauma* 2004;56(6):1247-1252
21. Hanssen AD. Local antibiotic delivery vehicles in the treat- ment of musculoskeletal infection. *Clin Orthop Relat Res* 2005 ; 437 : 91-96.
22. Hanssen AD. Prophylactic use of antibiotic bone cement : an emerging standard-in opposition. *J Arthroplasty* 2004 ; 19, Suppl 1 : 73-77.

OTA Resident Research Grant Budget Sheet

Budget cannot exceed $20,000

Submitting a budget over this amount disqualifies your application for consideration

- Salaries and Wages: Enter name, percentage of time on project and salary requested as well as fringe benefits charged to the grant. Please also state what each person will be doing.
- Permanent Equipment: Justification to be appended.
- Consumable Supplies: Excludes animals and animal care.
- Animals and Animal Care: Justify all requests where need is not apparent.
- All Other Expenses**:** Charges for overhead are not covered by OTA Grants. No indirect costs will be funded.

SALARIES AND WAGES (List all personnel for whom money is requested)	% Of Time on this project	Requested from OTA Funds (Omit Cents)
Research Technician (Annual Salary $50,000)	10%	$5,000
– Assisting with cell culture work and non-surgical animal procedures	%	
	%	
	%	
Fringe Benefits 22% of Salaries and Wages		$1,100
Salaries and Wages plus Fringe Benefits	**TOTAL**	**$6,100**

PERMANENT EQUIPMENT (Justification to be appended)		
	Subtotal	**$0**

CONSUMABLE SUPPLIES (Exclude animals and animal care)		
Histology Supplies – As detailed in attached budget		$710
Biologic Content Measurement Supplies – As detailed in attached budget		$1,965
	Subtotal	**$2,675**

ANIMALS AND ANIMAL CARE		
Procurement and Boarding – As detailed in attached budget		$5,595
Surgical Supplies and Procedures – As detailed in attached budget		$4,130
	Subtotal	**$9,725**

ALL OTHER EXPENSES		
Micro-CT Scanning and Analysis ($50/hr for approx. 10hrs)		$500
Conference Presentation and Related Travel Expenses		$1,000
	Subtotal	**$1,500**

TOTAL DIRECT COSTS **$20,000**

Detailed Budget

Histology Supplies

Item	Description	Price Per Unit	Request Quantity	Total Amount
Paraformaldehyde	Fixative (Sigma P6148)	$50 per 500g jar	1 jar	$50
EDTA	Decalcification (Sigma ED)	$25 per 100g jar	1 jar	$25
Ethanol	Sample storage (Sigma 270741)	$90 per 2L bottle	1 bottle	$90
Paraffin Wax	Embedding samples (Sigma 327204)	$50 per 1kg jar	1 jar	$50
Hematoxylin Solution	Biological stain (Sigma HHS16)	$50 per 500mL bottle	1 bottle	$50
Eosin Y Solution	Biological stain (Sigma HT110216)	$50 per 500mL bottle	1 bottle	$50
Anti-mouse CD31	Endothelial stain (BioLegend 102502)	$225 per 500ug vial	1 vial	$225
Microscope Slides	Superfrost Plus (Fisher 12-550-15)	$140 per box of 100 slides	1/2 box	$70
Microscope Cover Glass	For microscope slides (VWR 48393-106)	$50 per box of 100 coverslips	1/2 box	$25
Permount	Mounting medium (Fisher SP15)	$75 per 500mL bottle	1 bottle	$75
Subtotal for Histology Supplies				**$710**

Biologic Content Measurement Supplies

Item	Description	Price Per Unit	Request Quantity	Total Amount
Anti-Rat VEGF	Antibody for immunohistochemistry (AbD Serotec AAR42)	$450 per 100ug vial	1 vial	$450
Anti-Rabbit HRP-Conjugated Antibody	Secondary antibody for IHC & Western (abcam ab6721)	$175 per 1mg vial	1 vial	$175
Anti TGF Antibody	Antibody for Western blot (abcam ab66043)	$400 per 100uL vial	1 vial	$400
Anti-BMP2 Antibody	Antibody for Western blot (abcam ab14933)	$400 per 100ug vial	1 vial	$400
SDS-PAGE pre-cast gel	TruPAGE 4-20% (Sigma PCG2004)	$100 per box of 10 gels	1 box	$100
Nitrocellulose Membrane	Western protein blotting (Sigma N7892)	$150 per package	1 package	$150
ECL Reagents	Detection of Western blot proteins (GE RPN2232)	$290 per kit	1 kit	$290
Subtotal for Biologic Content Measurement Supplies				**$1,965**

Procurement and Boarding for Study Animals

Item	Description	Price Per Unit	Request Quantity	Total Amount
Rat	Fisher F344 Strain (ordered from CRC)	$80 per animal	50 rats	$4,000
Transportation Fee	Charge per shipment	$55 per shipment	5 shipments	$275
Boarding Rate	Price per week, per animal	$2.50 per week	50 rats; 8-16wk	$1,320
Subtotal for Procurement and Boarding				**$5,595**

Surgical Supplies and Procedure Costs

Item	Description	Price Per Unit	Request Quantity	Total Amount
Surgical Costs	Bone Defect Surgery	$40 per day OR use	8sx per day	$200
Miniplates and Screws	Defect stabilization (Synthes)	$9 per animal	40 surgical rats	$360
Bone Cement	Simplex P Quickset (Stryker)	$200 per 40g package	10 packages	$2,000
Tobramycin	Antibiotic for bone cement (Sigma PHR1079)	$90 per 1g vial	2 vials	$180
Vancomycin	Antibiotic for bone cement (Sigma 861987)	$270 per 1g vial	2 vials	$540
Surgical Costs	Repeat Intervention Surgery	$40 per day OR use	8sx per day	$200
Surgical Costs	Radiographs	$30 per day OR use	1-5X per rat	$450
Surgical Costs	Study End	$40 per day OR use	8sx per day	$200
Subtotal for Surgical Supplies and Procedure Costs				**$4,130**