Hypoxia Mimicking Agents for the Induction of Guided Angiogenesis in Calcium Phosphate Scaffolds for Bone Tissue Engineering of Posttraumatic Bone Defects.

I. Specific Aims (250 words maximum):

Word count: 247

The success of any scaffold-based bone regeneration approach for the treatment of long bone defects relies on the controlled induction of new blood vessels within the construct[1]. Upon scaffold implantation, vascularization occurs primarily from the periphery and is limited to tenths of micrometers per day, delaying nutrient and oxygen supply centrally[2]. Moreover, ingrowth requires that the surrounding soft tissues have a high vascularization potential – often not the case in traumatic injuries[1]. A method of rapid scaffold vascularization is implantation of an arteriovenous loop within scaffolds that results in spontaneous angiogenic development from the loop[1,3]. This technique however is not feasible in the trauma setting, requiring microvascular expertise and specific vascular and osseous anatomy. New approaches to drive angiogenesis along a graft-fracture interface are crucial to advancing bone-engineering strategies[4].

Hypoxia is a major driving force for angiogenesis and this response is under the control of the Hypoxia Inducible Factor (HIF) pathway[5]. The therapeutic manipulation of this pathway has recently proved successful. Hypoxic Mimicking Agents such as Deferoxamine (DFO) enhance HIF gene expression and sustain the activation of hypoxia-response genes creating a pro-angiogenic environment[6].

This proposal seeks to create 3D printed dicalcium phosphate scaffolds with spanning central macrochannels loaded with DFO. The grafts will be implanted into critical sized rabbit radial defects. We aim to quantify the angiogenic and osteogenic response to these scaffolds and hypothesize that DFO can induce robust vascularization within the predefined channels. We predict that promoting central vascular ingrowth with DFO, will improve osteoconductive capacity.

II. Background & Significance (350 words maximum):

Word count: 350

Dicalcium phosphates (DCP) are excellent biomaterials for bone regeneration due to their bioactivity, osteoconductivity, resorption rate and similarity in composition to bone mineral[7]. Previous work in our lab has explored 3D-printing of grafts[8]. We have shown that implantation of 4mm-thick 3D-printed microporous DCP scaffolds onto the cranium of rabbits resulted in full thickness host bone ingrowth after 8 weeks. However, bone ingrowth was heterogeneous throughout the scaffold[9]. To improve osteointegration and to translate this technology to large bone defects, rapid and functional scaffold vascularization is essential.

The use of small molecules that target the HIF pathway have recently been investigated

to induce vascular growth[5]. Under hypoxic conditions the transcription factor HIF-1α activates multiple angiogenic factors of which VEGF is a major target. The HIF-1α protein is constitutively expressed but undergoes rapid degradation under normoxic conditions. Prolyl hydroxylases are key to this process and require oxygen, iron, and 2-oxyglutarate as cofactors[10]. Small molecules termed Hypoxia Mimicking Agents interfere with these cofactors to block HIF-1a degradation. Of these, DFO, an iron chelator, has shown the most promise as a therapeutic agent and recently, encouraging results have demonstrated significant augmentation of both vascularity and bone growth in fracture models, bone defects and in distraction osteogenisis[10-14].

No study to date has used DFO in conjunction with a synthetic calcium phosphate scaffold in a mid-sized animal long bone defect. Moreover DFO has yet to be integrated within a scaffold with predefined macrochannels to guide vascular growth. DFO has been safely used in humans since 1970 for acute and chronic iron overload with side effects reported only in long-term use[15]. Therefore DFO has potential to transition into trials for bone regeneration.

For application in orthopaedic trauma, 3D-printing technology offers exciting opportunities to use spatial data collected from routine imaging to create custom implants to fill specific bone defects[16]. Moreover this technology allows for the rapid modification of scaffold geometry including porosity, pore interconnectivity and surface area. Using this technology combined with an exciting molecule, this study hopes to advance bone-engineering strategies for the management of post-traumatic bone defects that continues to represent a significant clinical challenge.

III. Research Design and Method (1,000 words maximum):

Word count: 984

The primary objective of this study is to investigate the potential of the hypoxic mimicking agent, Deferoxamine (DFO) to induce a robust angiogenic response in calcium phosphate scaffolds with spanning macrochannels implanted into a long bone fracture defect. As the overall goal of any scaffold based bone regeneration technology is to improve the healing of defects, the secondary objective is to quantify the osseous response to DFO loaded scaffolds.

A rabbit radial defect of 1.5cm will be used as a critical sized defect model. This is a well-documented model to evaluate the bone regenerative effect of bone graft substitutes. The model has several advantages, most importantly, the construct does not require internal fixation in small animals as the adjacent intact ulna provides adequate stability to the radial defect. This reduces, operating time, experimental costs and infection rates.

Scaffold architecture will first be drawn using computer aided design (CAD) and printed in calcium phosphate using a 3D printing system. Cylindrical implants of 5mm in diameter and 1.5cm in length will be printed. Implants will either be microporous (pore size ~ 20um) or microporous with square straight channels running through the long access, with openings at either end. Channel size and number will be varied as follows:

1. Four channels of 800μm x 800μm placed equidistant from the center point of the scaffold
2. Two channels 1.2mm x1.2mm placed equidistant the center point of the scaffold

Characterisation of the implant architecture and porosity will be carried out. Surface topography will be evaluated by scanning electron microscopy and micro-CT will be used to characterize and confirm uniform channel printing. Overall percentage porosity will be determined by the liquid displacement method.

After sterilization, scaffolds will be loaded with DFO ensuring 2mM of DFO are present in each construct[14]. A micro-syringe will be used to inject 1mM of DFO solution into each of the 1.2mm channel scaffolds or 0.5mM into the 800μm channels. Scaffolds without channels will be soaked in a 2mM solution of DFO. All control non-DFO loaded scaffolds will be created in identical fashion, substituting sterile water for the DFO solution. Scaffolds will be frozen until implantation as previously described[14]. Release kinetic profiles of DFO from each different scaffold type will be tested using a solution leakage method and high performance liquid chromatography.

Eighteen 5-month-old New Zealand rabbits will be used in this study in compliance with the ethical committee of the study institution. Rabbits will be randomly assigned to three groups with five rabbits in each. Three rabbits in each group will be used for micro-CT and histological analysis and three will be used for microangiography. Each rabbit will have two implants placed, one in each radii. Both implants will be of identical geometrical composition, one loaded with DFO and one control.

Group 1: 6 rabbits – 1.2mm channel scaffold

Group 2: 6 rabbits – 800μm channel scaffold

Group 3: 6 rabbits – Solid scaffold

Surgical technique: A longitudinal skin incision will be made over the radial bone at the mid one-third of the front leg. The periosteum will be separated from the surrounding muscle and a 15-mm defect will be created at about 2-2.5 cm proximal to the radiocarpal joint. The periosteum will be resected with the bone segment and the area washed prior to scaffold implantation. No internal fixation will be used and the animals will be allowed unrestricted weight bearing. Tetracycline (30 mg/kg) will be injected intramuscularly at 13 days and 3 days before sacrifice into all rabbits that will be used for osseous histology.

At 8 weeks the animals will be sacrificed and the grafts explanted. Angiogenesis and osteointegration will be quantified using the parameters in the methodology below. Due to varying channel size, the total volume of each scaffold type is different therefore each scaffold loaded with DFO will be compared to its geometrical control. Mean differences in measured parameters will then be used to compare the two macrochannel scaffolds to each other and to the solid scaffold.

Histology and histomorphometry:
Histological analysis will be performed on fixed, dehydrated and embedded explants.

Sagittal sections will be cut with a microtome and stained with methylene blue/basic fuchsine. The optical images of sagittal sections will used to perform the histomorphometrical analysis to quantify new bone and the remaining unresorbed DCP. Non-implanted scaffolds will be used as a control comparison to determine the amount of implant material resorbed. Histologic sections of the implant area will be divided into smaller areas to performed localized histomorphometrical analysis. Interpolation of these values will be used to determine the distribution of bone within the implants. Several sections on each implant will also be examined under florescent microscopy to calculate new bone formation using the double tetracycline labeling method.

Micro-CT:
Prior to histological analysis the explants will be scanned using a bench top micro-CT. Several studies have been successful in using micro-CT to distinguish among three phases - newly formed bone, remaining calcium phosphate biomaterial and soft tissues[17]. A constant volume of interest will be selected for each scaffold design and 3D images will be created based on this. The bone volume fraction, trabecular thickness and trabecular number will be calculated.

Micro-CT-based Microangiography and decalcified histomorphometry:

Three animals in each group will be subjected to injection of contrast and fixative agents prior to sacrifice for microangiography and decalcified histology. We will base this methodology on a described protocol for microangiography in rabbit long bone[18]. Briefly, the axillary arteries of the rabbit are canulated and sequentially injected with, heparinized saline, formalin and then a radiopaque silicone lead contrast agent (Microfil). After sacrifice, the forelimbs will be harvested, fixed and subsequently decalcified. With help from experienced technicians, we will use a micro-CT scanning protocol to separate the radiopaque vessels from background bone/scaffold to create 3D angiograms. Quantification of vessel number and volume will be performed. Additionally the number of vessels filled with microfilm will be calculated on select histological cuts of these fixed explants.

IV. Resident role (200 words maximum):

Word count: 85
The candidate will be performing the study over the course of a dedicated full time 12-month period. The candidate in consultation with two supervisors, one clinician and one biomaterial engineer, was responsible for the formulation and writing of this proposal. The candidate will be responsible, in consultation with appropriate experts, for:
1. Design of the implants
2. Surgical implantation and animal upkeep
3. Surgical explanation
4. Histological preparation and interpretation
5. Micro-CT and Microangiography image construction and analysis.
6. Literature review and production of manuscript

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OTA Resident Research Grant Budget Sheet – No 96

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 associate x 1 weeks of work total over the course of 12 months @ $1346/week	%	$1346
	%	
	%	
Fringe Benefits _____% of Salaries and Wages Salaries and Wages plus Fringe Benefits	TOTAL	**1346**

PERMANENT EQUIPMENT (Justification to be appended)	
Subtotal	

CONSUMABLE SUPPLIES (Exclude animals and animal care)	
1KG of calcium phosphate printing material @ $500 /KG	500
Micro-CT / microangiography 30 samples @ $80/ sample	2400
Deferoxamine mesylate (sigma)	250
Microfil contrast agent for 3D angiography (Flow Tech Inc)	125
Subtotal	**3275**

ANIMALS AND ANIMAL CARE	
15 RABBITS @ $1000 PER RABBIT (includes all associated costs)	15000
Subtotal	**15000**

ALL OTHER EXPENSES	
Subtotal	

TOTAL DIRECT COSTS $19621