Synthetic Vascularized Bone Grafts

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All that I am, or hope to be, I owe to my angel mother and a real man called father.

Dedicated to my lovely parents and Annabelle, who have been there for me day and night.

Table of Contents

Abstract

Background

Bone graft procedures are commonly performed worldwide for segmental bone defects resulting from high-energy trauma, congenital deformity, infection or tumor resection. The current gold standard for repair of large long bone defects is the use of bone and blood vessels, as vascularized bone graft, harvested from another part of the body and transplanted to the area of the missing tissue. This creates significant donor site injury and is often an inadequate anatomical match. Making synthetic vascularized bone without recourse to potentially dangerous and expensive growth factors can potentially revolutionize reconstructive surgery and provide significant improvements in patients' lives. Inducing new blood vessels and engineering bones to custom geometry is the objective of many researchers but several technological hurdles remain. My thesis explores whether axial vascularization of a bioceramic affects bone generation from marrow aspirate and, to understand the impact of axial vascularization in bio-scaffolds.

Methods

We designed, cross-shaped, monetite bioceramic to fit the rat femoral vein and impregnated the scaffold with autologous bone marrow taken from the other leg. This implant was then implanted circumferentially around the vein of 16 rats and removed after 8 weeks. The structure, stability, and composition of the new bone was be assessed by micro computed tomography (micro-CT), Scanning Electron Microscope (SEM), and histopathology examinations.

Results

Scaffolds were printed with a mean deviation of +150 µm compared to their theoretical CAD model, were mainly composed of monetite (80.2%wt) and unreacted α- (1.8 %wt) and β-TCP (18 %wt) phases and displayed a micro- (> 50%vol between 1 and 10 µm) and Nano-porosity. A greater volume of new bone tissue was observed and quantified (SEM, µCT) in scaffolds perfused by a central vein compared with the non-perfused negative control (65.0 ± 5.6 % of the initial scaffold volume versus 29.0 ± 4.2%, $P < 0.0001$, N=4 or 5, triplicate representative areas per sample). Bone formation and ceramic biodegradation were (2.2 ± 0.2) and (2.9 ± 0.3) times higher for the experimental group than for the control one, respectively. In addition, implant biodegradation for both control and experimental groups were far greater than for both historical controls (implant and implant vascularized by a vein, ≈ 16.5% degradation).

Conclusion

Experiments showed promising results, we produced an axially vascularized tissue-engineered bone using marrow at a higher level than has been reported in the literature. This illustrates the intimate relationship between angiogenesis and osteogenesis, this is especially important to tackle the issues encountered for regeneration of critical-sized bone defects. In this study we showed that the presence of a vein perfusing a blood marrow soaked monetite implant allowed for generating enormous amount of bone (up to 65%) replacing the quasi totality of the implant (≈ 15% remaining). Even more, the structure adopted by this host-made engineered construct may surprisingly remain the structure of long bone, including a low vascularized and dense cortical layer surrounding a highly vascularized trabecular zone which hosts the marrow. Future work will focus on the mechanical stability of these vascularized bone flaps in load bearing sites.

Résumé

Contexte

Le manque de substance osseuse faisant suite à un traumatisme, malformation congénitale, infection ou encore à l'ablation d'une tumeur peut être compensé par une greffe autologue de substance osseuse. En cas de perte osseuse importante, la procédure privilégiée consiste à transplanter un greffon osseux vascularisé, provenant d'un site sain du patient, au sein du défaut osseux où il sera connecté aux vaisseaux sanguins environnants. Bien que très bénéfique, la forme du greffon est souvent mal adaptée au défaut osseux, et la procédure comporte des risques non-négligeables. Générer à façon un greffon synthétique vascularisé sans prélever d'os sain ou utiliser les techniques onéreuses et encore peu maitrisées d'ingénierie tissulaire représenterait une avancée significative dans le domaine de la chirurgie reconstructrice ainsi qu'une amélioration significative de la vie des patients. Induire la formation sur mesure d'un tissu osseux vascularisé a fait l'objet de nombreuses recherches, néanmoins de multiples verrous technologiques persistent. Mes recherches visent à étudier l'impact que pourrait avoir l'insertion d'une veine au sein d'un implant imprégné de moelle osseuse sur sa revascularisation et sur sa colonisation par une nouvelle matrice osseuse.

Méthodes

Des biocéramiques en monétite comportant un canal central ont été produites via fabrication additive. Un modèle murin (N=8 rats / group) a été utilisé pour cette étude. Après avoir été imprégné par de la moelle osseuse, prélevée sur un des fémurs, la biocéramique a été placée autour de la veine fémorale du site opposé. Après sacrifice (8 semaines d'implantation), la structure, organisation et composition du nouvel os formé a été évaluée par microCT, microscope électronique à balayage et histologie.

Résultats

Un écart moyen de +150 µm entre la géométrie des biocéramiques imprimées et leur modèle CAD a été observé. Après stérilisation, les implants comportaient une microporosité ente 1 et 10 µm, leur composition étant 80.2% en masse de monétite et respectivement 1.8 et 18% massique de réactifs initiaux (α- et β-TCP). Les analyses des explants (µCT, SEM, N= 4-5 x3) après 8 semaines d'implantation ont révélé que (i) la formation osseuse était (2.2 ± 0.2) fois plus importante ($P<0.0001$) lorsque la biocéramique était perfusée par la veine fémorale (65.0 ± 5.6% versus 29.0 ± 4.2%) et (ii) la biodégradation des céramiques était (2.9 ± 0.3) supérieure en présence de cette veine. En outre, notons que la présence de moelle osseuse au sein de ces biocéramiques a stimulé leur biodégradation (c.f. contrôles historiques).

Conclusion

L'approche considérée a permis le développement de volumes osseux vascularisés par des vaisseaux de taille importante (≈ 1mm) en site ectopique – les valeurs rapportées étant bien supérieures à celles trouvées dans la littérature. Nos résultats illustrent les liens intimes entre angiogenèse et ostéogenèse, et avancent des perspectives prometteuses pour la régénération des défauts osseux de taille critique. En bref, nous avons démontré que la présence d'une veine peut supporter la formation d'un os nouveaux en site ectopique (65% du volume de l'implant) qui vient se substituer à la biodégradation de sa structure d'accueil. De plus, la structure adoptée par l'os formé n'est pas sans rappeler la structure naturelle des os longs, avec une corticale dense et peu vascularisée et une zone trabéculaire largement vascularisée et accueillant la moelle osseuse. De futurs travaux se focaliseront sur la transplantation et stabilité de ces greffons synthétiques vascularisés en site porteur orthotopique (e.g. fémur).

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Contributions of Authors

This work consists of 1 manuscript in preparation for publication by the supervisor and candidate.

Aslan Baradaran (candidate): performed the experimental work, including implantations (animal surgeries, microvascular surgeries under microscope), explanting the constructs and lab works, data collection, literature review and data analysis.

Professor Jake Barralet (primary investigator): main supervisor responsible for the candidate throughout the experiment and thesis preparation. Dr. Barralet provided scientific insight and guidance, laboratory space and animal facility access, materials consumed, and supervision through the study.

Dr. Nicholas Makhoul (co-supervisor): provided surgical knowledge and helped through the study by solving surgical technique related issues.

Dr. Edward Harvey (advisor): provided scientific and surgical input through the study.

Dr. Uwe Gbureck: from the University of Wûrzburg, he supplied the 3D printed scaffolds.
Dr. Baptiste Charbonnier (post-doctorate at Professor Barralet lab): he provided scientific input during the study and helped with data analysis.

Author contributions and statements of originality

The work described here was performed by the author and is original. This is the first report that describes the use of vascularized scaffold can induce vessel growth and bone regeneration as a potential method for treating long bone defects.

Chapter 1 – Thesis Introduction

1.1 Rationale

Annually, there are approximately 2.2 million bone graft procedures performed worldwide for segmental bone defects[1,2]. Large orthopedic and maxillofacial bone defects can be secondary to high-energy trauma, congenital deformity, infection or tumor resection[3]. The result of any of these conditions can be profound, including esthetic deformities and a significant functional disability leading to negative psychological consequences and long-term socioeconomic burden[4,5].

Successful osseous reconstruction is dependent on the size of the defect. A critical-sized bone defect (CSD) is defined as a bone defect that is more than two and a half times the bone diameter[6-9], or a circumferential loss of greater than 50%, or a length over 2 cm[10]. Schmitz and Hollinger[11] originally described critical sized bone defects as "the smallest size intraosseous wound in a particular bone and species of animal that will not heal spontaneously during the lifetime of the animal". CSD is based on the size of the defect; in other terms, the nonunion occurs because the defect is too large to heal with solely bony tissue[11]. Since the introduction of CSDs, they have become the routine way in bone regeneration and reconstruction studies in many laboratories (see Mooney and Siegel[12]). Current surgical techniques to address CSD's are restricted to vascularized bone flaps that carry significant morbidity to the patient[13]. Result of a CSD attempting to heal will be formation of fibrous connective tissue instead of bone[14,15]. A CSD caused nonunion, indicates a condition of failed osteogenesis in which the normal processes of physiologic repair reach a terminal end point. This termination of growth can be due to formation of periosteal sheath, folding towards the bony segments and creating a fibroblastic barrier, preventing union. Other possibility is the formation of hematoma at the time of injury which prevents the gap to be organized and

bridged. Accordingly, blood vessels will not migrate and the necessary osteogenic elements are absent and a complete repair is highly unlikely[11].

Reconstruction for defects smaller than a CSD can be achieved using a bone graft. A bone graft is a non-vascularized tissue or substitute that can regenerate bone through a combination of osteogenic, osteoinductive or osteoconductive processes. Bone grafts can be in the form of autologous bone, donor bone (allograft or xenograft), or synthetic equivalents (alloplastic)[16,17]. Current developments in 3D printing have produced alloplastic ceramic materials which have customizable shapes, conforming to the patient's actual bony defect while being osteoinductive[18]. The current gold standard for the repair of CSD defects is the use of vascularized autologous bone flaps. Another useful and commonly used method is distraction osteogenesis, developed initially by Ilizarov[19] which is a gradual distraction force stimulating tissue growth. In this case, tissue distraction causes mechanical strain which leads to the formation of callus tissue and increased mitotic activity of the cell responsible for callus formation. Callus tissue is created due to the enhanced metabolic activity of the cells in the distracted tissue and the mechanical strain stimulus might be the primary signal for increased mitotic activity of the cells[20].

Flaps differ from bone grafts in that they have an intact vasculature pedicle containing a transplantable artery and vein for attachment at a recipient site. A transplanted vascularized bone flap therefore allows immediate blood supply to the implanted tissue and results in successful reconstruction of a CSD. Major limitations in its use include; significant morbidity to the donor site, and geometric and anatomical mismatch between donor and recipient site[21-23].

Tissue engineered (TE) bone presents a promising alternative to current reconstruction techniques[24]. Tissue engineering has not seen significant clinical implementation due to the inability to produce large vascularized tissues[25,26]. TE substrates are nourished by a random pattern

vascularization (peripheral vascular ingrowth), as opposed to axial vascularization (intrinsic vascular network) where there is a patent artery and a vein. Peripheral vascularization limits the size of the tissue being generated, whereas an axial vascularization permits the ability to create clinically relevant volumes of tissue.

1.2 Scope of The Project

There are certain reasons that make this study important with high impact on next studies in the future. This work will help to address the need for vascularized tissue-engineered bone graft substitutes which will eliminate bone tissue harvesting and reduces risk, surgical time and patient morbidity. This is an important breakthrough in reconstruction that is applicable to adult patients and pediatric patients with congenital bony defects. In addition, unlocking the ability to produce vascularized custom tissue engineering scaffolds can be extrapolated to other regenerative techniques including composite multi-tissue regeneration and synthetic organogenesis.

1.3 Project Contributions to Healthcare

The proposed project is primarily concerned with the development of a customized vascularized bone flap for large facial and long bone reconstruction. Sprouting angiogenesis without surgical intervention is currently not possible. Induction of perfusable and transplantable vascular network with a connecting pedicle remains a major obstacle to regenerative medicine[27-29].

Understanding the extent to which the regenerative capacity of the vasculature can be harnessed by biomaterials can allow new treatments and surgical approaches to be developed. The ability to

vascularize 3D volumes can replace some current microsurgical procedures. Many cell therapies are limited by the inability to maintain cell viability during implant vascularization and our work will inform many avenues of regenerative medicine as well as facial reconstruction. Furthermore, this study also uses 3D printing to produce scaffolds for bone tissue engineering and will be focused on understanding the effects of altering the macro and micro architecture on the development of new tissue and vascularization. Patients affected by large maxillofacial defects must undergo several surgical procedures to achieve improvement of speaking, eating and breathing. Regrettably, many times despite several major reconstructive surgeries patients continue to have limited function, which results in a lack of being able to restore their normal quality of life and poses a major burden on society.

Chapter 2 – Overview of Free Bone Flaps

2.1 Flaps

Flap surgery is a technique in reconstructive surgery when a tissue is taken from a donor site and transferred to a recipient site, usually a bone defect, with an intact blood pedicle. Distant flaps are utilized in cases that the donor site is far from the bone defect. In a free flap the blood supply (pedicle) is cut and then anastomosed to another blood supply at the recipient site[30,31] [Figure 1].

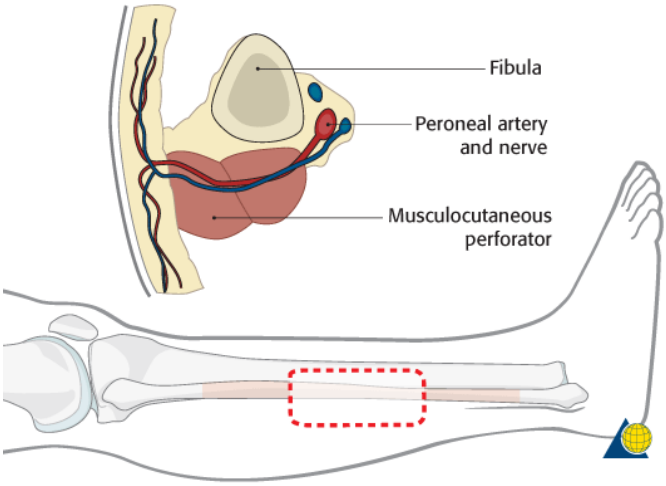

Figure 1. Donor site and basic anatomy of a fibular free graft. As shown, it is possible to transfer bone, muscle, and skin to the recipient site. (Adapted from AO Foundation, AO Surgery Reference, Harvesting of fibula osteocutaneous flaps, free access education.)

2.2 Bone Defects

Skeletal defects can be categorized according to their etiology as primary, or secondary. In primary bone defects there is usually high-energy trauma component, which causes open fractures with extensive tissue loss and bone shattering. Comminuted bone can be lost during the injury or removed as de-vascularized tissue during debridement procedure. Secondary defects are results of loss of pathologic tissue in bone diseases that can be congenital (congenital pseudarthrosis) or acquired (aseptic and septic non-unions, osteomyelitis, tumors). Massive (>5 to 6 cm) skeletal defects [Figure 2] cause considerable morbidity and functional impairment for patients, they are a true challenge for the surgeons as well[32].

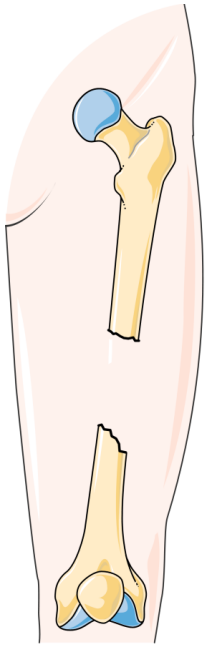

Figure 2. A massive femoral defect in human skeleton.

Options to reconstruct the defects include biological techniques, namely: bone grafts, distraction osteogenesis. Vascularized bone grafts have been used instead of avascular grafts in order to improve the outcome of reconstruction in different locations (e.g. large bone defects of the extremities or osteonecrosis of the femoral head[33]). Vascularized bone grafting was undertaken primarily in 1905 by Huntington, with transferring fibula as a pedicle graft to the same side tibia, but in 1975 Taylor et al.[34] reported a free transfer of vascularized fibular graft. Vascularized bone grafts are also possible to be taken from other donor sites, such as the iliac crest and the rib[32].

Development of free bone flaps for use in Orthopedic and Maxillofacial surgery has a substantial impact on the prognosis of patients suffering from significant loss of bone in the case of cancer patients, congenital malformation and deformities, and major traumas[35]. As discussed in the previous section, different methods exist but autologous free flap is the standard of care, and specifically when the defect is massive, the fibula flap (first used for mandibular reconstruction by Hidalgo) is the only procedure of this type allowing a bone transfer of approximately 25 cm[35,36]. Though many authors described this as the primary flap of choice for mandibular reconstruction, it is not coming without any drawbacks.

2.3 Autograft

When a bone is harvested from and implanted into the same individual then it is called an autograft. The most frequently used donor sites for harvesting in order are: the iliac crest [Figure 3], proximal tibia, distal radius, and greater trochanter[37]. A significant superiority of autografts is that they are safe from the graft-host reactions, which are initiated by one`s body immune system, since the tissue is harvested from and transplanted in the same person. However, they have their own set of

complications, namely donor site morbidity and limited tissue availability which are playing a critical part.

Autografts are the standard care of bone flaps, especially in maxillofacial surgery where significant osteoinductive and osteoconductive capacities are needed[38,39]. Cortical autografts usher significant structural resistance to the graft[2]. Ability to grow new vessels (neovascularization) and feasibility are characteristics of autografts that explain their use over allografts, and the result of all these is the capability of being osteoinductive, osteoconductive, and osteogenic. Osteogenic is defined as "relating to or derived from the tissue from which bone is developed" [40].Osteoinductivity ("act or process of stimulating osteogenesis"[41]) is the ability to stimulate pluripotent and undifferentiated cells to turn into the bone-forming cells, this is how primarily osteogenesis is induced, and osteoconductivity is a capacity in the bone surface that lets bone growth on the surface or into its pores or channels, or allowing bone passively grow and remodel on a surface[42].

During the recovery steps of the bone, vascularization is critical for the coherence of bone[43,44], and this is the same when it comes to grafts [45]. Neovascularization between the graft and the recipient site is an intricate process, there different cell types have duties[45].

There are several disadvantages to the use of autologous bone grafts, especially for vascularized flaps which are pointed out here. Leading complications are donor site pain[46,47], fracture, hematoma collection, infection, and nerve defects[48]. In addition, the limited quantity of available bone graft is a fact and harvesting of utmost volumes increases the chance of complications after harvest[49]. As the science of regenerative medicine evolves, it is the objective of many studies to replace allografts and autografts with bone grafts that can significantly improve patient outcomes.

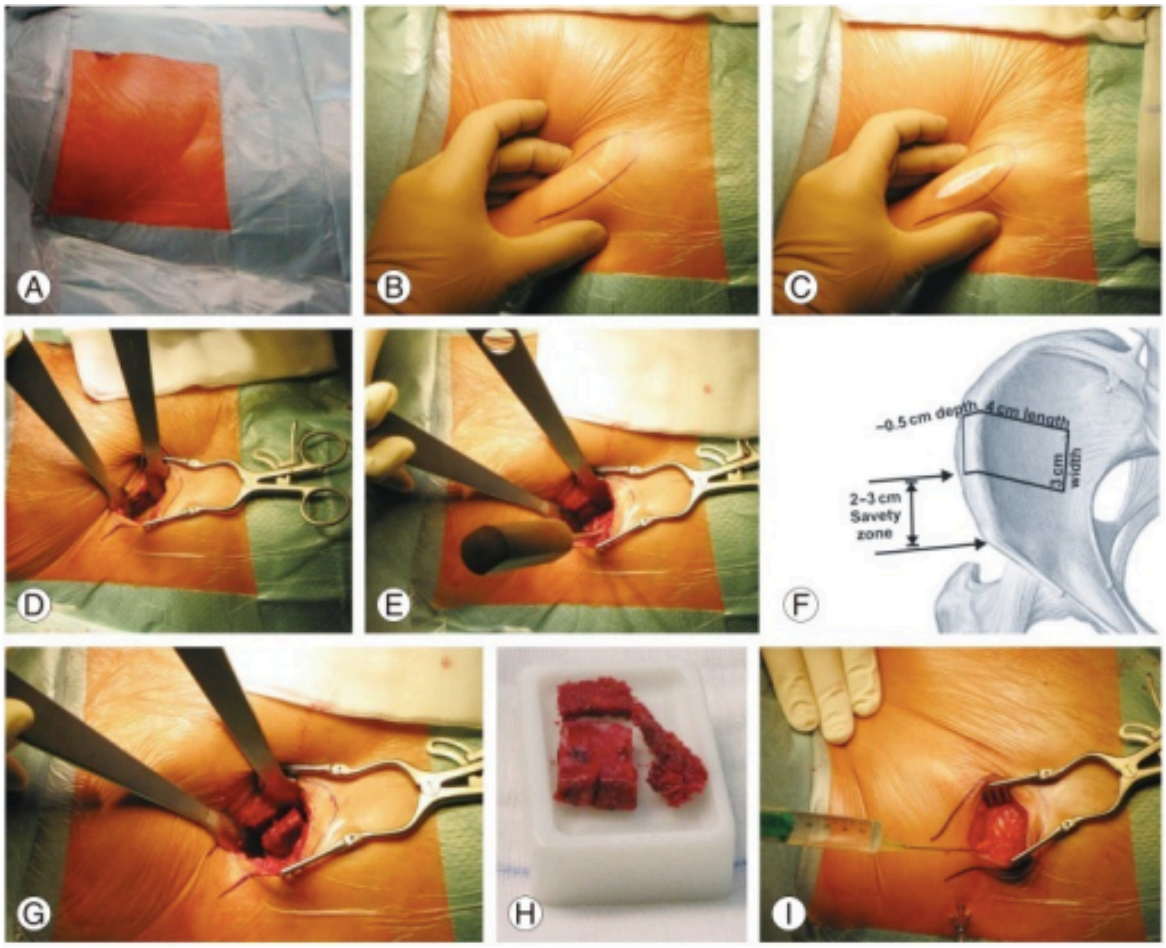

Figure 3. (A-I) Stages of surgical technique for iliac crest bone graft harvesting. Bicortical corticocancellous bone graft is harvested from the iliac crest [50].

2.4 Limitations of Free Bone Flaps

Length and shape of the bone available to harvest are playing a key role in choosing the technique of reconstruction. Fibular free flaps are among the mostly used flaps, however, the length that this bone provides cannot go above certain length to treat a massive bone defect (more than 25 cm).

The three other possible bone flaps are more restricted, namely: the iliac crest (can provide 15 cm of bone length), the parascapular flap (that can provide 10 cm of bone length), and the lateral brachialis flap (also a maximum of 10 cm) [51,52].

In the case of substantial bone reconstruction, it is difficult to reproduce a good matching shape in the transferred bone. This results in poor esthetic results, that can lead to obvious distortion of the body.

In the case of pediatric reconstruction surgery, one facet that is always kept in mind before any intervention is the freedom of bone growth and that the bone length is always in jeopardy. Limb major traumas children are often linked with contraction of soft tissue, deformation, and as the result growth restriction of bone and limb length discrepnacies[53]. In the pediatric population, the fibula flap is the only option carrying a lower risk of bone growth restriction. On the other hand, the iliac crest flap leads to delayed ossification, and also harvesting a lateral brachialis flap causes significant growth defect in these bones. These can end in gait imbalance and limb-length discrepancy and all this call for another reconstruction procedure. Though all this could not happen following, what might be considered as a successful free tissue reconstruction, fibrotic changes can occur in the donor-site soft tissue and re-epithelialization about and in the surgical site. This can cause many issues related to wound healing and as a result outcome of the surgery. The similar scenario can also happen with the skin graft contraction when a transferred muscle is required[53].

Another limitation regarding pedicular free bone flap is when bone shape matching is required, and multiple osteotomies should be performed. In this scenario, there is necessarily destruction of the medullary vessels [Figure 4]. Some authors found that periosteal vascularization was ample enough as a blood supply[36,54], but other found that osteotomies could lead to ischemia and finally osteonecrosis[35].

Another restriction for the free flap procedure, as with fibular harvesting, is for patients with ischemic disease of the lower limb. Patients suffering from arteritis, the fibular artery is usually the only remaining permeable and perfusing vessel in the lower limb.

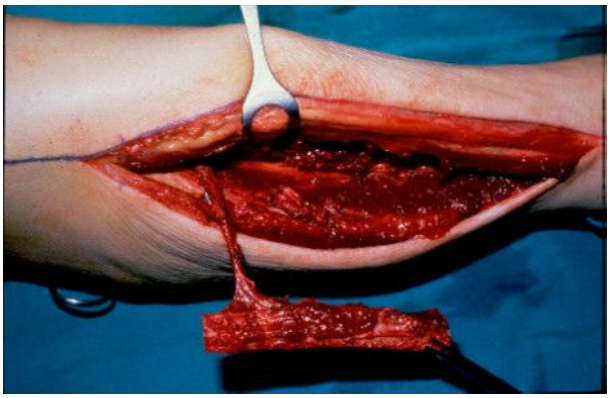

Figure 4. Demonstrating a dissected fibula ready for harvesting with a vascular pedicle, adapted from Carbiner et. Al[55].

Another high-risk case are patients with arteriosclerotic disease including the fibular artery which does not provide a permeable lumen for the flap vascularization. Although the iliac crest is perfused with the superficial iliac circumflex artery, but when it comes to arteriosclerosis, this donor site is also refused to be chosen[35].

Furthermore, when the pedicle length is short, there can be a big challenge to achieve a proper anastomosis. This might even not be foreseeable and becomes an issue during the operation. In case of fibular flaps, if there is no more than 5 cm available, then it can become extremely demanding a high level of microsurgical experience to perform the anastomosis properly.

2.5 Donor-Site Morbidity of Different Free Bone Flaps

2.5.1 Radial Forearm Free Flap

Radial forearm flap or so called "the Chinese flap" is a popular free flap built on the radial artery and its associated veins, that could bring bone to the recipient site[56]. This method can provide up to 10 cm of bone length which makes it an ideal flap for intraoral reconstructions[57]. Some of the distinct donor site complications are delayed wound healing[58], cold intolerance[59], neuroma formation[60], causing strength issues in the donor hand and increased risk of radial fractures[60], and decreased wrist range of motion[60].

2.5.2 Fibular Free Flap

Since the description, it has become one of the most popular flaps in the maxillofacial reconstruction techniques[61]. With the fibular flaps, approximately 25 cm of bone can be taken and one should preserve few centimeters of bone distally and proximally to retain the stability of the joints surrounding[62]. Fibula is perfused by some branches of the peroneal artery. Surgeon can obtain a complex flap by including the posterior shin muscles available on the bone[63]. Despite its countless advantages over other bone flaps, this method is still imposing many risks and complications. Donor site wound healing delay (or poor healing) and contracture[64] [Figure 5], peroneal nerve defects (sensory and motor disturbances)[65], significant muscular compromise and gait dysfunctions[66], and as mentioned earlier it can be more devastating in the pediatric population while impairment in lateral malleolus and valgus deformity of the ankle can happen after the fibula is removed[67].

2.5.3 Iliac Crest Free Flap

Vascularized iliac crest flap is mostly recommended for reconstruction of moderate to extensive (less than 16 cm) mandibular defects or oral composite defects[68]. This method can lead to injury to the lateral cutaneous and ilioinguinal nerves, resulting in thigh pain[62]. Contour defect after removal of the cortical bone produces notable donor-site deformity[69], and serious problems in walking can occur as a consequence[70].

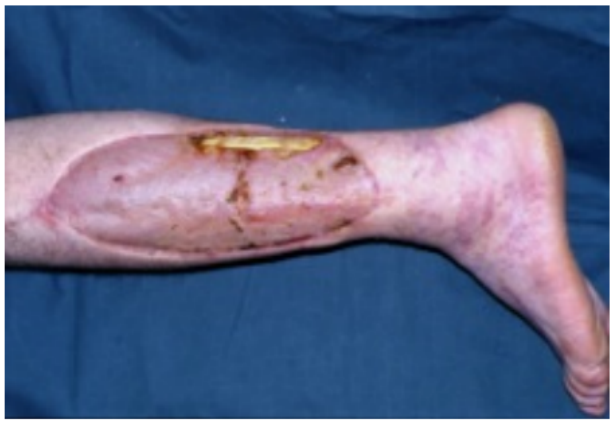

Figure 5. Fibular bone flap donor site skin contracture and delayed healing in a patient with a fibular free flap for a mandibular reconstruction, bare peroneal tendons are visible (adapted from Hartman el al. study[62]) Cosmetic issues could be negligible for some patients are can cause considerable psychological problems some individuals.

2.5.4 Scapular Free Flap

The length maximum of bone harvested from scapula is ranging from 12 cm[71] to 16 cm[72] in different studies. Evidence shows that the scapular osteocutaneous free flap is a reliable option for head and neck reconstruction surgeries[73,74], and because of the amount of soft tissue (fat and fascia) available with the flap, reconstruction of highly complex defects is possible. For this technique the donor-site morbidity consists mainly of impairment of shoulder function, which may become substantial when an ipsilateral neck opening with nerve sacrifice is performed[71].

Chapter 3 – Bone Graft Vascularization Methods - Review of the Literature

3.1 Vascularized versus Non-vascularized Bone Grafts

When managing a bone defect, autologous bone grafts can be used as the mechanical supporting structure with which to recover the main function, and they are categorized as vascularized bone graft (VBG) or non-vascularized bone grafts (NVBG). When using a VBG for a defect of long-bone, reconstruction needs a meticulous assessment of the pros and cons. The superiority of the VBG over the NVBG surround the supplying nutrients to the defect site and graft[75]. VBG is favored when there is increased rate of graft resorption and thus risk of mechanical failure, and also to prevent or lessen other types of complications (e.g. infection). On the other hand, VBG is technically more challenging for a surgeon, a lengthy surgical procedure, higher risk of donor site morbidity, and more difficult to match to the shape of the recipient site[34]. Medical practice standard revolves around not putting the patient at risk for unwanted outcome of a VBG if the defect can be fixed with a NVBG. One should be careful that the use of a NVBG where a VBG is the more fitting option, could yet result in failure of the graft.

For longer grafts, the distance over which remodeling must occur also increases and this can cause longer or incomplete recovery. In a clinical scenario we do not consider defects larger than 5-7 cm for NVBGs[76-78]. Two recent review articles about VBGs from long bones[77,78], stated that bone defects longer than 5-6 cm should be treated with VBGs. Few of these reasons are: 1) significant superiority of VBG for osteointegration rate. This is mainly due to the distribution of the blood supply which is not even through a NVBG and accordingly the osteoblastic activity, which is

crucial to bone integration, is unpredictable and variable[76]. 2) NVBG in fibular grafting provides poor healing potential, while a free vascularized fibular graft induces primary callus formation, extensive revascularization, and increased osteoinduction[79]. Based on the data in the literature VBG is generally preferred over NVBG, since the first has showed higher success rates than the latter, both in union and as pointed out earlier, the implant osteointegration. Furthermore, VBGs are suitable option for virtually any defect size, except for small defects (<3 cm). This is while NVBGs are restricted to short bone defects (<5–6 cm) or in cases with grave medical conditions to stand the added operative time needed to develop a free flap[76].

3.2 Vascularized Bone Grafts

Pedicled grafts are the first VBGs utilized, they are actually bone that is transferred with its blood supply[80]. These grafts are considered as "alive" and they have all the elements required for graft survival and osteointegration, they also provide this opportunity to avoid some of the complications coming with allografts, namely graft failure or infection. Pedicled grafts stay connected to their primary blood supply and this put a restriction on them by the need for being in the vicinity of the treatment site. These grafts are also commonly used to treat different carpal bone pathologies and to reconstruct the femoral head in case of a femoral head avascular necrosis[81,82]. Common harvest sites are: the fibula, the iliac crest, greater trochanter, and the distal radius bone. This introduces complexity to the procedure since surgeons must attain a tension free anastomoses, predict a sufficient pedicle length, and examine the graft site for blood leakage after the procedure[81].

Another technique is named free vascularized bone graft, in this method the surgeon keeps the vessels intact and connected to and from a liberated section of bone. Free vascularized grafts are picked as an alternative to pedicled grafts[82]. They carry complex delivery of the vessels and evidently need special surgical tools and are associated with higher morbidity rate at the harvest site and a relatively high rate of failure.

With these difficulties, neither of the above-mentioned techniques are widely used clinically. There is an apparent need for more advanced solutions for regenerate vascularized bone. For this aim, the field of tissue engineering shows promising results for the development of a simpler ways to generate vascularized bone graft compared to the classic complicated pedicled and free vascularized grafts.

State of the art regenerative medicine is mainly about the presence of a biomaterial boosting cell production and growth[83]. In order to re-generate the tissues, biomaterials must efficiently interact with the host tissue, at the same time send signals to the host for growing on the implanted graft and replace the biomaterial with a newly formed tissue. This process dictates the need of establishing a substantial angiogenic signaling from the beginning of implantation, leading to development of a vessels, and eventually a fully functional structure[84,85]. Most of the utilized regenerative medicine techniques are based on extrinsic vascularization, a model that the vessels are rooting from the periphery of the scaffold and accordingly it must be implanted in a heavily vascularized site, which is not feasible for many clinical scenarios (e.g. postradiotherapy)[86]. Nevertheless, if we rely on diffusion as the only mean of blood delivery, then oxygen and nutrition delivery will be reduced considerably. In fact, this is the root cause when the primary vascularization is suboptimal and as a result cell survival in the central zones of the scaffold is diminished. These obstacles of vascularization introduced the need for a new solution including

angiogenesis, and thus various in vivo models revolving around this aim to generate constructs with an exclusive vascular network[87].

3.2.1 Different Types of Construct Vascularization

By axial vascularization (refer to section 3.2.2) of a scaffold we aim to provide blood supply to the construct by a preplanned and dedicated vascular channel. As a result, the blood supply of the scaffold is guaranteed and this channel makes the implantation in areas of low vascularization, like surgery sites complicated with fibrosis or irradiated sites, possible[86].

Prelamination and prefabrication are the two well-known methods for axial vascularization. By definition, prefabrication of a tissue construct means implanting an arterio-venous loop (AVL) or a vascular pedicle inside or under the scaffold. Prefabrication leads to spontaneous sprouting of vessels from the provided channel and revascularization of the whole scaffold[88-90]. Pribaz and Fine first coined the term "prelamination" in 1944[88]. Prelamination is simply implanting a tissue construct in a vascularized territory (or flap) to tailor make a vascularized structure[83] [Figure 6 a-c].

Both methods will result in an axially vascularized construct that is supplied by a specified vascular axis. In this context we are introducing 'intrinsic' and 'extrinsic' vascularization modes. In the extrinsic mode the construct is supplied from the periphery towards the center and in the intrinsic mode the core zone of the scaffold is being vascularized primarily[91]. Likewise, prefabrication is an intrinsic method of vascularization, while prelamination is an extrinsic mode[83]. The end result of both techniques is an axially vascularized scaffold with the ability for transferring to a remote site as either pedicled or free flap.

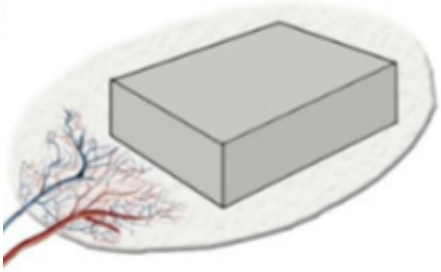

Figure 6a

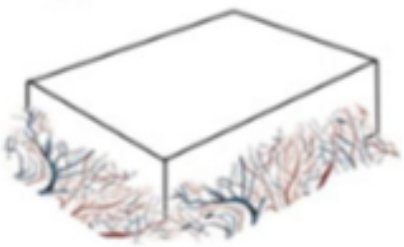

Figure 6b

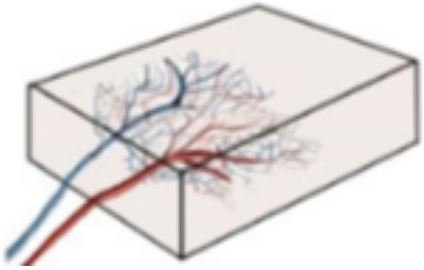

Figure 6c

Figure 6. Types of scaffold vascularization: (a) prelamination, (b) extrinsic, (c) intrinsic axial vascularization (prefabrication). (figures adapted from Eweida et al.[83])

3.2.2 Axial vascularization

Axial vascularization is one of the intrinsic vascularization methods of providing blood supple, which is based on the concept that an artery or vein can act as a source of vessels for tissue generation and transplantation.

Pre-fabrication here means to vascularize the construct by providing a vascular pedicle or an arterio-venous loop (AVL) around or inside the graft, which leads to spontaneous vessel formation from the provided loop or pedicle and eventually vascularization of the scaffold[88,89].

Arterio-Venous Loop (AV Loop), is a more common and evaluated method for vascularization [Figure 7]. In this technique, a graft construct is implanted, a vein is formed as a circle or "looped" through the graft and anastomosed to an artery. Once vascularization is maintained, the scaffold is ready for transplantation and be used as a vascularized bone substitute[92]. AVL has shown to be a promising method as an intrinsic axial vascularization. The AVL has the ability to develop a fairly good capillary network consisted of arterioles, venules, and post-capillary venules[93].

There are three main mechanisms recognized responsible for AVL to branch and generate a capillary network: 1. focal inflammation caused by surgical trauma on the vessel, 2. mechanical stress on the vasculature walls, also called shear stress, of the graft and the vein, 3. And oxygenation gradients throughout the construct. Focal inflammation secondary to the trauma due to the surgery, induces a surge in release of angiogenic substances (pro-inflammatory chemokines are responsible for inducing the upregulation of VEGF coming from platelets and endothelial cells)[94,95]. Another known factor stimulating neo-vascularization is the rise in pulsatile pressure. Adding a vascular graft into the primary arterial circulation can lead to a rise in VEGF production from the endothelial cells, this is mainly occurring as a result of mechanical stimulation[95-98]. Furthermore, a major activator of endothelial cells is the combined effect of shear stress and turbulent flow taking place at the anastomoses site[99]. Another proposed mechanism mentioned above, is the gradients in partial pressure of oxygen or the "hypoxia" of the construct that can induce considerable angiogenesis[100].

There are several limitations accounted for the AV loop technique, few important drawbacks are: considerably time consuming, surgically difficult to establish, and increased risk of having a thrombosis. Furthermore, the bone tissue produced using this technique has to be limited to an isolated chamber and this significantly restricts the bone volume generated[102]. Our preliminary data has shown that biomaterials alone can induce luminal branching in veins without an AV loop. This way one of the major disadvantages of the AV loop is tackled, without sacrificing the graft vascularization[103].

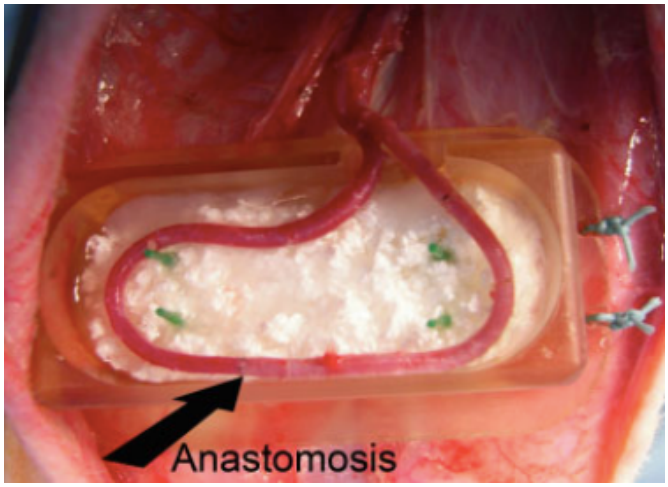

Figure 7. Intraoperative view of an isolation chamber containing the HA/β-TCP matrix, inside seats a microsurgically created AV loop, arrow shows the anastomosis site. Adapted from Beier et al. work[101].

Chapter 4 – Subcutaneous Bone Formation: A Synthetic Vascularized Bone Graft

4.1 Objective and Hypothesis

There are multiple techniques of bone repair, from the "gold- standard" method of autologous bone grafting, historical method like distraction osteogenesis, and to specialized and such as guided bone regeneration. There have been encouraging results reported in the literature with some of the more recent experimental approaches to vertical bone augmentation[104-106]. Yet a deficiency still remains that needs to be addressed: no single technique has been proven to sufficiently repair large long bone defects in a reliable way, which explains why autologous bone grafting and distraction osteogenesis remain the preferred clinical approaches, despite their limitations[107,108]. This study used monetite[105,109,110], a calcium phosphate bioceramic, to induce vascularization and bone growth with the aim of creating a vascularized bone tube, a method that has not been reported in the literature to date.

We hypothesized that our method of vascularization can be used to create an effective vascularized bone flap with the capacity of being translated to the clinic. The objective of this work is to incorporate bone marrow on our axially vascularized cross-shaped bioceramic, with the aim of producing and optimizing an isolated synthetic free flap.

4.2 Introduction

Although bone has the innate ability to self-repair, large defects that exceed a few cubic centimeters may require clinical intervention (e.g. filling) to reach a complete regeneration.

Vascularized bone grafts (VBG) or flaps are preferred for the treatment of large and critical-sized segmental bone defects[13,111,112]. In short, VBG are viable sections of bone removed with an intact vascular network and feeding artery, which are reshaped, transplanted in the defect and finally micro-surgically anastomosed to the host vasculature. Compared with avascular bone grafts, VBGs demonstrated improved survival and healing rates, mechanical properties and integration[113,114], the existing vascularization of the transplant limiting the avascular necrosis of the tissues. However, they share common drawbacks with non-vascularized autografts[111] including additional injury to the patient, limited harvest stock (often fibula, radius or iliac crest), poor fit with the patient defect leading to possible deformities (e.g. mandibula), risk of infections, paresthesia, acute and chronic pain, native bone resorption at the donor site, and non-union or morbidity[115,116].

Autologous bone marrow aspirate is source of bone progenitor cells (e.g. endothelial stem cells, mesenchymal stem cells MSCs) and is well known to be osteogenic[117,118]: transplantation of marrow aspirate was proved to induce bone formation even in ectopic sites. Clinical use of autologous bone marrow aspirate precedes an understanding of stem cell biology but today its osteogenic effect is attributed to its stem cells population. It has been shown that adding marrow aspirate to an AVL repair can lead to an even better outcome[119].

In a previous study, we demonstrated that venous angiogenesis could be induced by a bioceramics material simply by placing it in proximity to a vein. We tried to determine if this angiogenesis could have any effect on the amount of bone formation when marrow aspirate was added to the bioceramic. Here we report on the differences that vein placement made and characterize bone volume formation and vascularization.

4.3 Methods

4.3.1 Experimental design

In this study, we used 16 male Wistar rats weighing 450–500 gr, based on the preliminary studies to achieve an acceptable study power. Using the means of the two independent study arms, the common standard deviation, and keeping α as low as 0.05, we reached a fairly high power for the study (>95%)[120]. All experiments were approved by the Facility Animal Care Committee of the McGill University (#7662). After rats arrived at the Montreal General Hospital animal facility, they were given a 7-day period to adapt to the new environment, animals were then randomly assigned to the study groups (control versus experiment). All operations were performed by the same microsurgeon (A.B.). Autologous marrow was isolated from the rats` femur, other than the leg being implanted, bone marrow harvested was kept with 1% Heparin solution on ice during the operation, seeded onto β-TCP/HA scaffolds and then surgically implanted into the subcutaneous sites of rats. All scaffolds in the experiment group were surgically supplied with an axial perfusion system. For the control group, freshly isolated marrow from the same animal was loaded onto β-TCP/HA ceramics and implanted in the same fashion without axial vascularization.

4.3.2 Implant Design and Manufacture

The implants were design using Alibre design Xpress 10.0 CAD software, aiming in facilitating the surgical procedure and reaching the highest reproducibility of the animal assays. Indeed, as illustrated Figure 8A, the implant was designed in 2 halves (12 mm high) that, when assembled, created a 1.4 mm diameter channel where a vein could be hosted. The cross-shape designed of the

implant was intended to retain in its concave zones viscous. bone marrow. To maintain in place the 2 implant halves, a macroporous (12 mm pore diameter) sheath was devised (Figure 8B), also limiting the runoff of viscous fluids towards the outside.

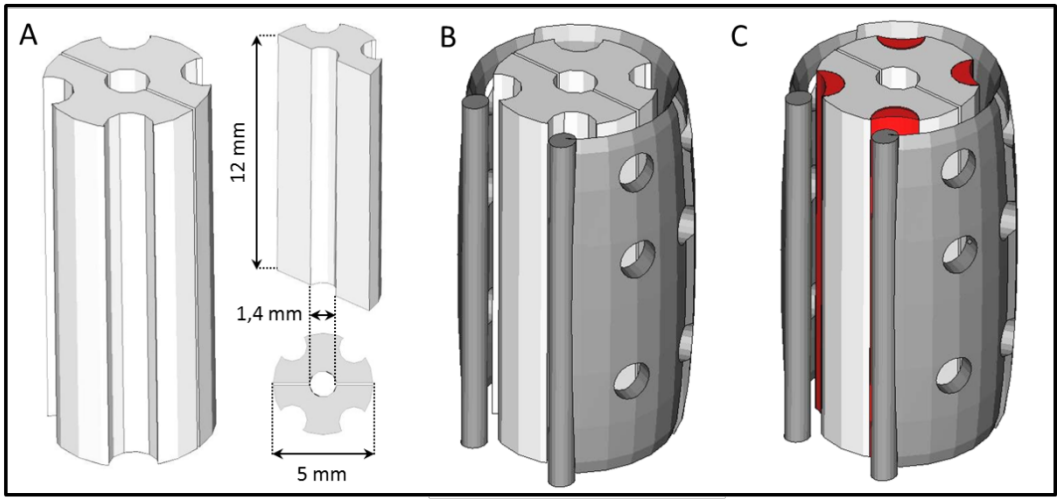

Figure 8. A) CAD design of the cross-shaped calcium phosphate implant, B) CAD representation of the calcium phosphate implant maintained by the ABS sheath and C) CAD representation of the calcium phosphate implant impregnated by bone marrow (red) and maintained by the ABS sheath.

Calcium phosphate scaffolds were produced by additive manufacturing according to a reactive 3D-printing technique co-developed by the authors[121,122]. In short, a reaction between tricalcium phosphate powders (α- and β-TCP, $Ca_3(PO_4)_2$) and diluted phosphoric acid (H_3PO_4) allowed for the area selective binding of the powder grains. After printing, samples were soaked in 20% phosphoric acid for 60 s, washed and sterilized by autoclaving[121-123]. A Fortus 400mc 3D-printer (Stratasys, USA) allowed for the production the sheath, using food-grade, sterilizable and certified biocompatible (ISO 10993 USP Class VI) acrylonitrile butadiene styrene (ABS, ABSM30i).

4.3.3 Characterization

X-ray diffraction pattern of the printed implant were recorded with a Siemens D5005 diffractometer (Siemens, Karlsruhe, Germany). A step size of 0.02° was used to measure from 20 to 40° 2θ range with a total measuring time of 3s per step. Phases were identified and quantified (Rietveld Refinment analysis) using TOPAS 2.0 software (Bruker AXS, Karlsruhe, Germany) combined with the International Centre for Diffraction Data patterns serving as reference for alpha-TCP, beta-TCP, brushite and monetite. Scaffold architecture was investigated by micro-tomography X (SkyScan 1172; SkyScan Kontich, Belgium) equipped with a 0.5 mm aluminum filter at a resolution of 12 μm. The microstructure of the implant was investigated using scanning electron microscopy (SEM, Hitachi S-4700 FE-SEM; Tokyo, Japan) at an accelerating voltage of 20 kV. The porosity and pore-size distribution of the 3D printed implants was determined by Hg porosimetry (PASCAL 140/440, Porotec GmbH, Hofheim, Germany).

4.3.4 Animal Surgery Procedure

All animal handling and surgical procedures were conducted according to McGill University Animal Care Committee (UACC) guidelines for the care and use of laboratory animals and approved by the Facility Animal Care Committee (FACC). All surgical procedures were performed under general anesthesia, Rats received combination of Carprofen and Buprenorphine administered 30 minutes prior to the surgery for analgesia and anesthetized with Isoflurane. Isotonic fluids administered subcutaneously (0.2 to 0.5ml/10g body weight) to maintain proper hydration.

Drops of Lidocaine/Bupivacaine were administered to the surgical site prior to the closure with

sutures. The incision was closed with absorbable (Vicryl\ 5-0, Johnson & Johnson Intl.) sutures using an inverted suture pattern. Rats received Carprofen (5-10 mg/kg SC) up to 72 hours post-operatively. The rats were allowed to recover from the anesthesia without further manipulation. The rat is monitored once daily for 3 days immediately after surgery, then 3-4 times per week for the general well-being of the animals. At 8 weeks, rats were euthanized with anesthesia gas and CO_2 and the implants were retrieved.

4.3.5 Bone Marrow Harvest

In a preliminary work, we developed a technique to efficiently access the rats` femur medullary canal and obtain proper amount of marrow. In this technique the animal is laid flat on their back (supine) close to the edge of the surgical table with the aimed leg hanging from the table and skin decontamination being carried out. This position allows the operator to accurately flex the knee and angle towards the medullary canal without disrupting the anterior cruciate ligament or damaging the femur shaft without performing an extra surgery. After flexion, distal femoral articular surface is exposed, with an 18-gauge needle that was flushed with Heparin, anterior intercondylar notch was felt and gently the needle was inserted through the skin. When the needle was inserted, with proper pull on the leg, the femoral shaft axis was aligned with the needle and aimed towards the greater trochanter slid the needle into the medullary canal [Figure 9]. 1 cc of marrow was aspirated, transferred into a tube, and placed on ice to be placed on the scaffold. Finally, a 2% lidocaine hydrochloride (without vasoconstrictor) local anesthetic injection was administered.

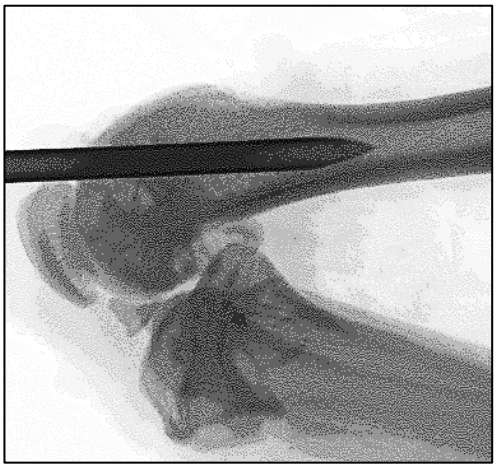

Figure 9. X-ray showing needle bone marrow aspiration from one of our animals in the control group.

4.3.6 Microscopic Surgery & Vessel Preparation

All rats were placed on their supine position while in contact with a heat source, maintaining body temperature in anticipation of surgical time exceeding 30 minutes, the medial side of the left leg was exposed and positioned for prep & drape and the surgical approach [Figure 10]. Beginning from the medial side of the knee, a vertical incision of skin was performed without violating the subcutaneous tissue. After blunt penetration and dissection of soft fat layers, left femoral vessels were exposed and dissected between the inguinal ligaments proximally and the bifurcation of the saphenous and popliteal vessels distally. Left femoral vein was gently isolated from artery and nerve with Ophthalmic Microscopic Surgical Instruments under microscope (Leica DI C800). Vessel branches were stripped-off using either electrocoagulation or suture ligation. At this point the scaffold was impregnated by the previously harvested bone marrow from the other leg in a

sterile dish and transferred gently to the surgical site and placed around the femoral vein under microscope view. Special attention was given to an uncompromised blood flow within the scaffold, whose continuity was left untouched. During preparation and after implantation, pulse-synchronic movements of the main trunk were continuously present, and During the procedure the surgical site is flushed periodically with heparinized saline (4 ml of 10,000 IU in 500 ml saline), while this allows the site to be cleaned of blood and debris but also preventing any clots forming which could complicate the microsurgery. Perfusion was double checked using common microvascular surgery techniques, such as smoothing out of vessels against the direction of blood flow.

Finally, the scaffold halves are sutured together and superficially to the underlying muscle with 5-0 Prolene® non-absorbable sutures. To further secure the scaffold the overlying facia was placed over it and this was secured with a single Monocryl® absorbable suture and the skin was closed as explained before.

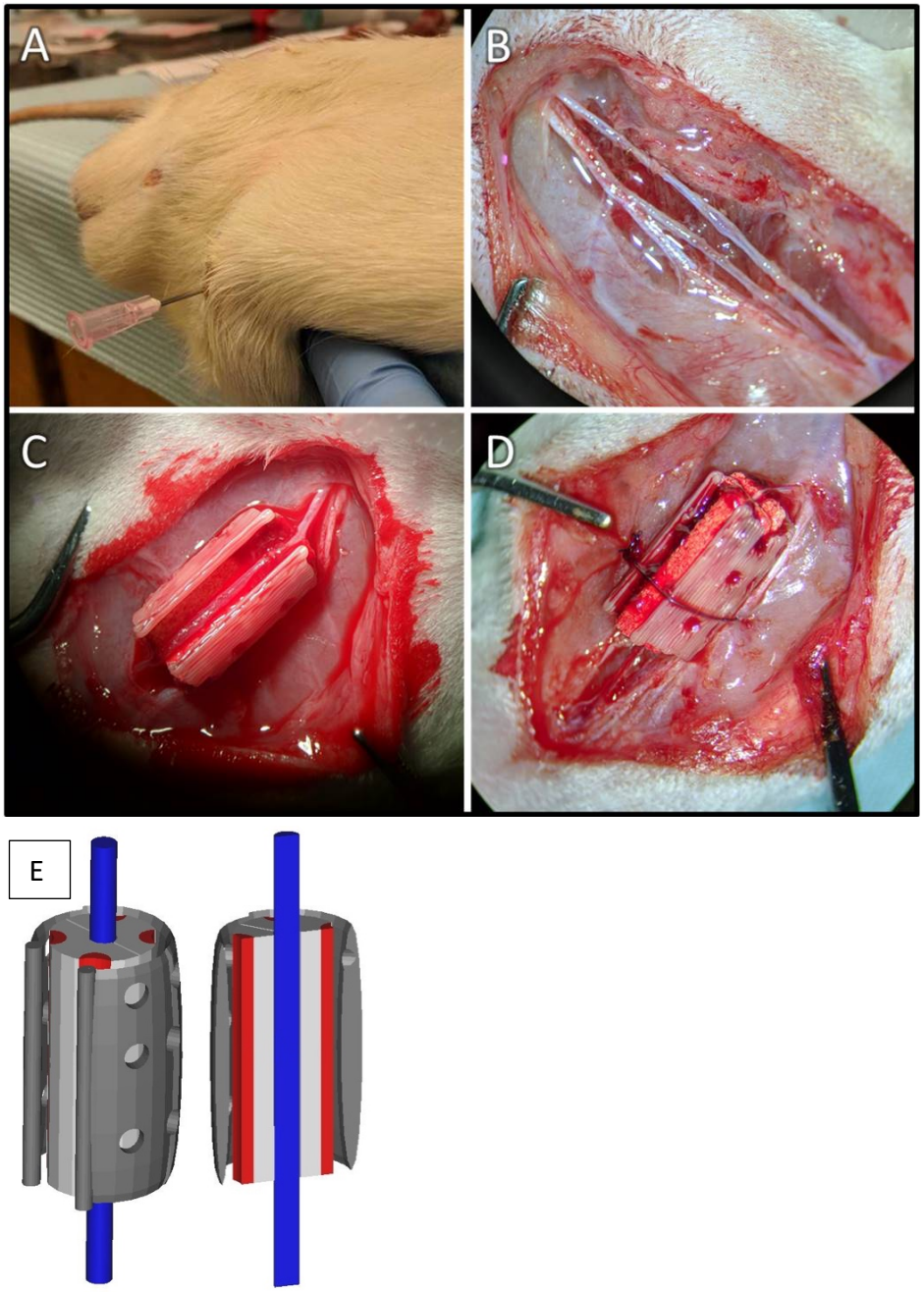

Figure 10. Illustration of the surgical procedure A) transcutaneous aspiration of bone marrow, B) B) the dissection of femoral blood vessels and nerve using ophthalmic microscopy, D) the positioning of the vein inside the bioceramic channel. E) schematic figure of vein passing through the ceramic and fixed with the plastic clip.

4.3.7 Microcomputed Tomography Imaging

After skin incision, 8 weeks implants were harvested with their surrounding soft tissue fixed in 4% neutral buffered formalin solution for 24 h. After fixation, samples were analyzed micro-computed tomography (SkyScan 1072, Belgium) machine operated by an X-ray source at a voltage of 40 kV with Aluminum filter, rotated through 180- with a rotation step of 0.45-, an acquisition time of 5.6 s per scan and a pixel size of 10.8 Am. Three-dimensional reconstructions were then performed with the software 3D Creator SkyScan.

4.3.8 Perfusion Study

Eight weeks after the implantation Microfil® perfusion was performed to determine vascularization of the vascularized scaffolds. First, thoracic cage was cracked and elevated, left ventricle was rapidly cannulated and flushed with PBS-heparin (100 IU/mL) solution until clear fluid leaking out of the punctured right atrium. 3 animals in the experiment group were perfused with 20mL of Microfil® solution (Microfil MV-122, containing 0.6mL of curing agent; Flow Tech). Finally, the aorta and inferior cava were ligated, and the rats were placed at 4°C overnight. Afterwards, constructs were explanted, fixed in 4% formalin solution.

4.3.9 Histological Analysis of Implants

After this, samples were dehydrated in ascending graded ethanol series (24 hours for each concentration) and then in pure Xylene for 24 hours. The samples were finally left for 6 days in the pre-infiltration solution (Technovit® 9100, Heraeus Kulzer, Wehrheim, Germany) and

embedded in Methyl Methacrylate (PMMA) resin. Blocks were cut with a chainsaw in order to trim and eliminate excess PMMA. Blocks were cut in multiple levels axially, with a circular diamond saw (saw microtome sp1600, Leica, Germany). Cross sections were stained with hematoxylin and eosin (H&E) for histology while the other block was used for histomorphometrical measurements. Sections of 10 micron were made and observed with a polarized light microscope. All cross sections were photographed using a Leica microscope (Carl Zeiss) and a digital camera under 10x magnification and subsequently merged to one image. One sample from each group was decalcified in ethylenediaminetetraacetic acid (EDTA; Sigma-Aldrich Chemie GmbH) for 3 weeks at 4°C.

4.3.10 Tartrate-Resistant Acid Phosphatase (TRAP) staining

Samples were embedded in cryomatrix (Thermo Shandon, Pittsburgh, USA) and frozen by immersion in cold isopentane. Sections of 5μm were cut with a microtome (Leica sp1600), placed on polylysine-coated glass slides and dried. TRAP activity was analyzed using the TRAP Kit 386A (Sigma–Aldrich, France) according to the manufacturer's instructions. TRAP positive cells appeared in red with nuclei in blue.

4.3.11 Image Analysis

We used NRECON and CTAn (Bruker microCT) software to accordingly reconstruct the micro-CT images and semi-quantitively calculate the total bone/mineralized tissue volume (BV) and tissue mineral density (TMD) in each implant. The Bone Analysis feature of the software was used and bone voxels were defined for all specimens.

To measure the porosity, surface area, and bone volume, we also utilized scanning electron microscope (SEM). Samples were cut at 100 μm thickness and coated with 20 nm of platinum and examined by SEM at specific locations. Images captured at 100x magnification as normal and backscatter, afterwards images were analyzed by ImageJ software to measure the densitometric quantification of all images. The dense material within the implant identified as new ectopic bone formation since its density was within the density spectrum of bone.

4.3.12 Statistical Analysis

Data are reported as mean, standard deviation, and percentage. Statistical analysis was performed using StatPage calculator (http://statpages.info/anova1sm.html) with one-way analysis of variance ANOVA (turkey post hoc test) and a P-value smaller than 0.05 was considered significant.

4.4 Results

Implant characterization

3D powder printing allowed for the reproducible production of cross shaped implants with similar characteristics than in previous studies[103]. In short, scaffolds were printed with a mean deviation of +150 µm compared to their theoretical CAD model, were mainly composed of monetite (80.2%wt) and unreacted α- (1.8 %wt) and β-TCP (18 %wt) phases and displayed a micro- (> 50%vol between 1 and 10 µm) and nano-porosity.

Explant analyses

The calcified [Figure 11] and decalcified [Figure 12] histological analyses revealed at first glance that the biodegradation of the ceramic seemed to be higher and that bone formation occurred ectopically when bone marrow was soaked in the scaffold prior implantation (no bone formation was observed in the historical controls).

H&E staining of decalcified samples showed that an important collagen matrix (pale pink) invaded the bioceramic in all the conditions, however the latter seemed to have a totally different maturity, density and organization in presence of bone marrow [Figure 12 A1/B1 vs C1/D1]. Even more, the matrix seems to have colonized and replaced an important fraction of the ceramic with bone marrow and axial vein perfusion [Figure 12 C1 vs D1] which indicated that bone formation might have been highly stimulated in these conditions. TRAP staining, which was negative in the absence of bone marrow [Figure 12 A3/B3], unveiled the presence of an osteoclastic activity within the

ceramic scaffolds soaked with bone marrow and perfused by a vein, proving that the higher biodegradation of scaffolds compared to the historical control was due to cellular activity.

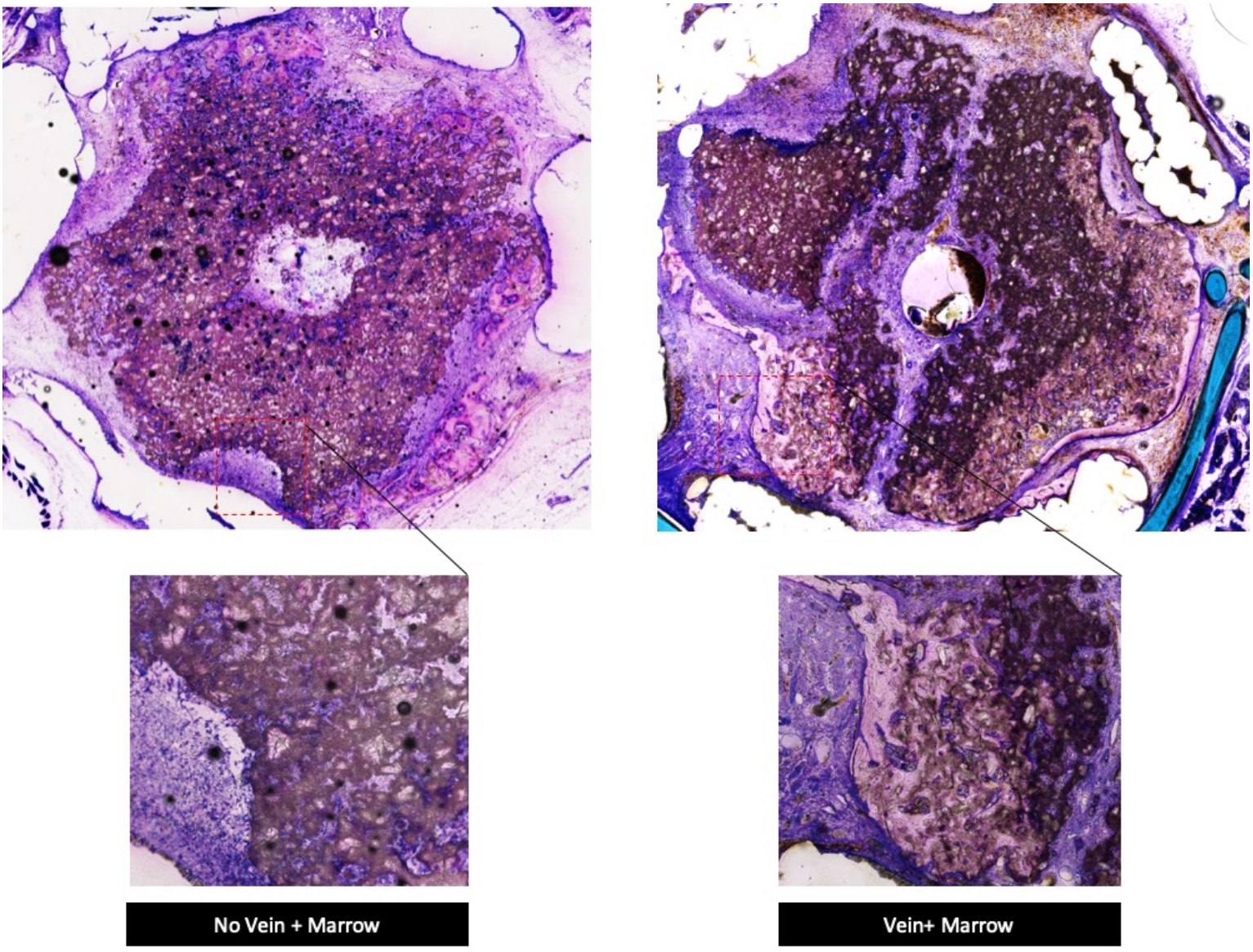

Figure 11. Basic staining of methylene blue and fuchsine on calcified samples of the control (left) and experimental groups (right). Higher magnification of samples showing enormously bigger amount of bone formation in the experiment group.

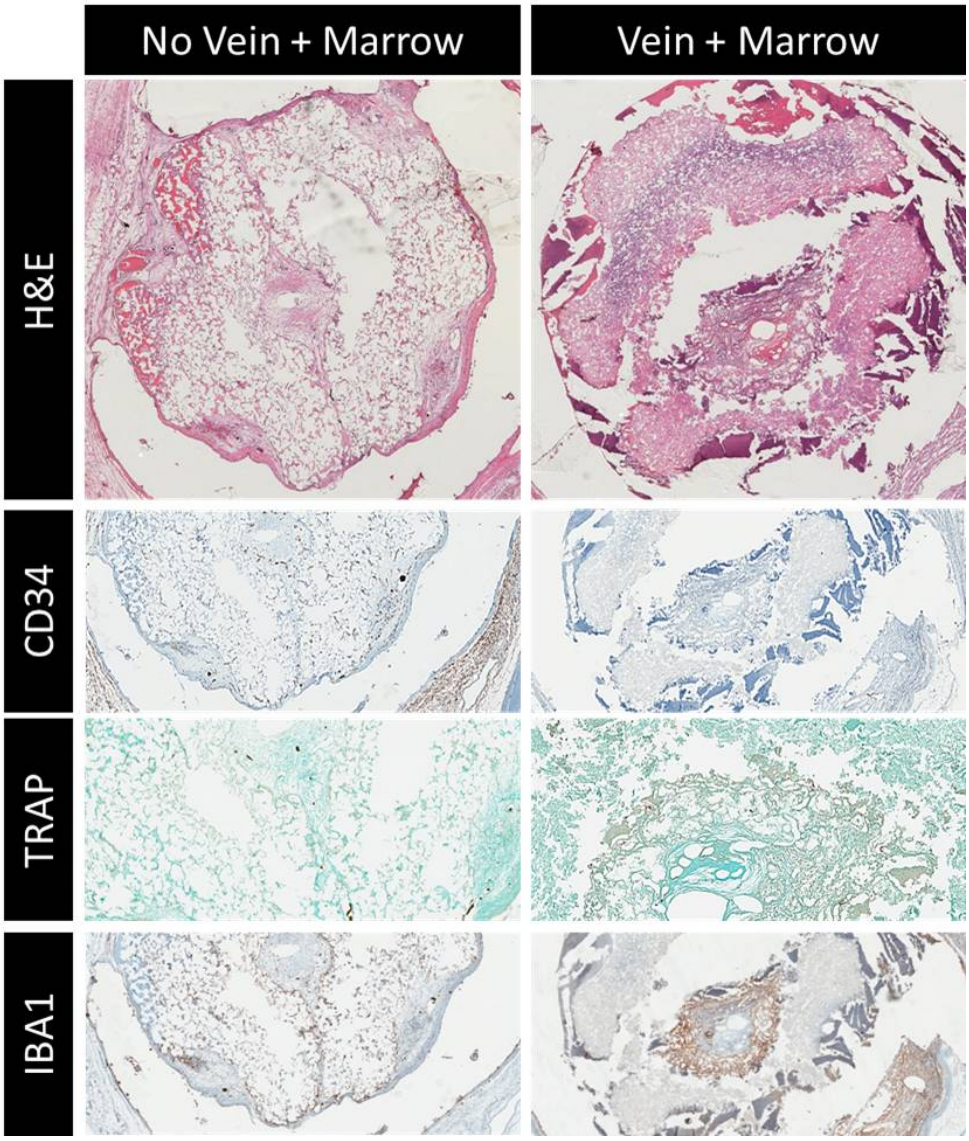

Figure 12. Optical microscopy images of the control (left) and the experimental (right) groups stained after different staining. Histological slices of the scaffolds after implantation under different experimental conditions, stained with H&E, CD34 and TRAP and imaged by optical microscopy.

SEM analyses confirmed the qualitative observations about bone formation and ceramic biodegradation [Figures 13-15]. Indeed, bone formation and ceramic biodegradation were (2.2 ±

0.2) and (2.9 ± 0.3) times higher for the experimental group than for the control one, respectively. Bone formation increased significantly (from 29.0 ± 4.2% to 65.0 ± 5.6 %, $p < 0.0001$) when the scaffolds were axially perfused by a vein. In addition, implant biodegradation for both control and experimental groups were far greater than for both historical controls (implant and implant vascularized by a vein, ≈ 16.5% degradation). Qualitative SEM analyses demonstrated that bone formation was 143 ± 15% higher for the experimental group than for the control [Figure 13, $p < 0.0001$). Comparable trends were determined from the volumetric μCT analyses.

Bone bridges between the cross edges [Figure 13 B1/B2] were observed in both control and experimental groups, however this phenomenon was for frequent in the later. Comparable trends resulted from the volumetric μCT analyses [Figure 16,17], more representative of the samples but much less accurate.

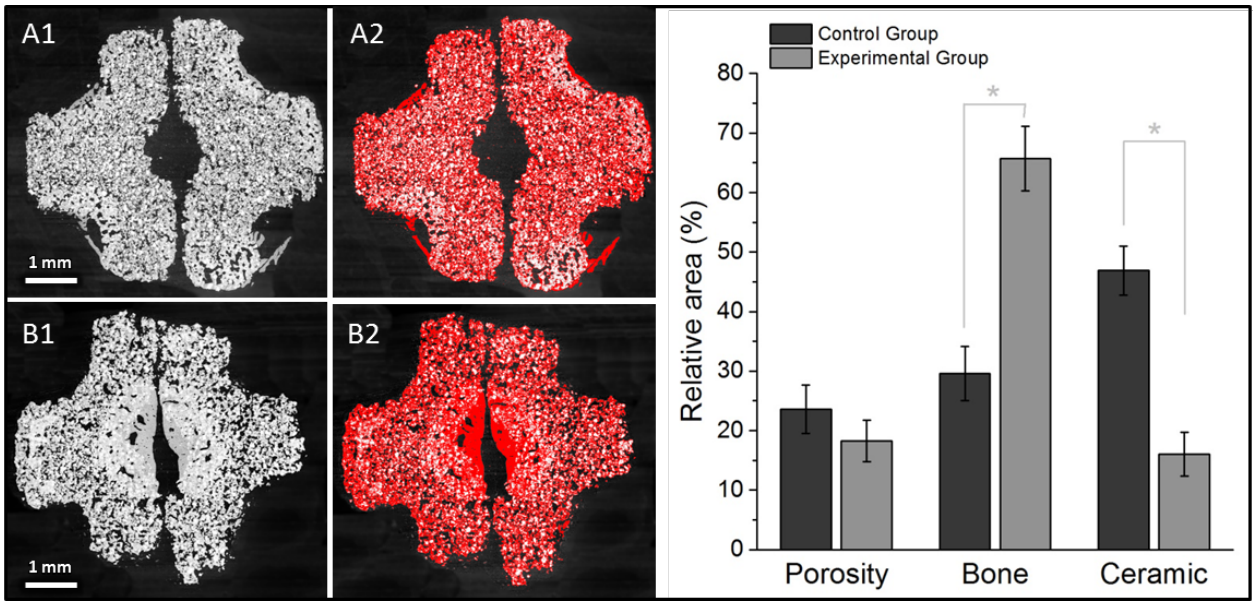

*Figure 13. Quantification of the bone formation, ceramic biodegradation and porosity for the control (top A1-A2) and experimental (bottom (B1-B2) groups using SEM images taken with back scattering electrons mode. Right graph of area occupied by bone, ceramic and pores normalized by the area of the region of interest. * ($P<0.05$). As an example, ceramic was represented in red for both top slices of samples from the control and experimental groups.*

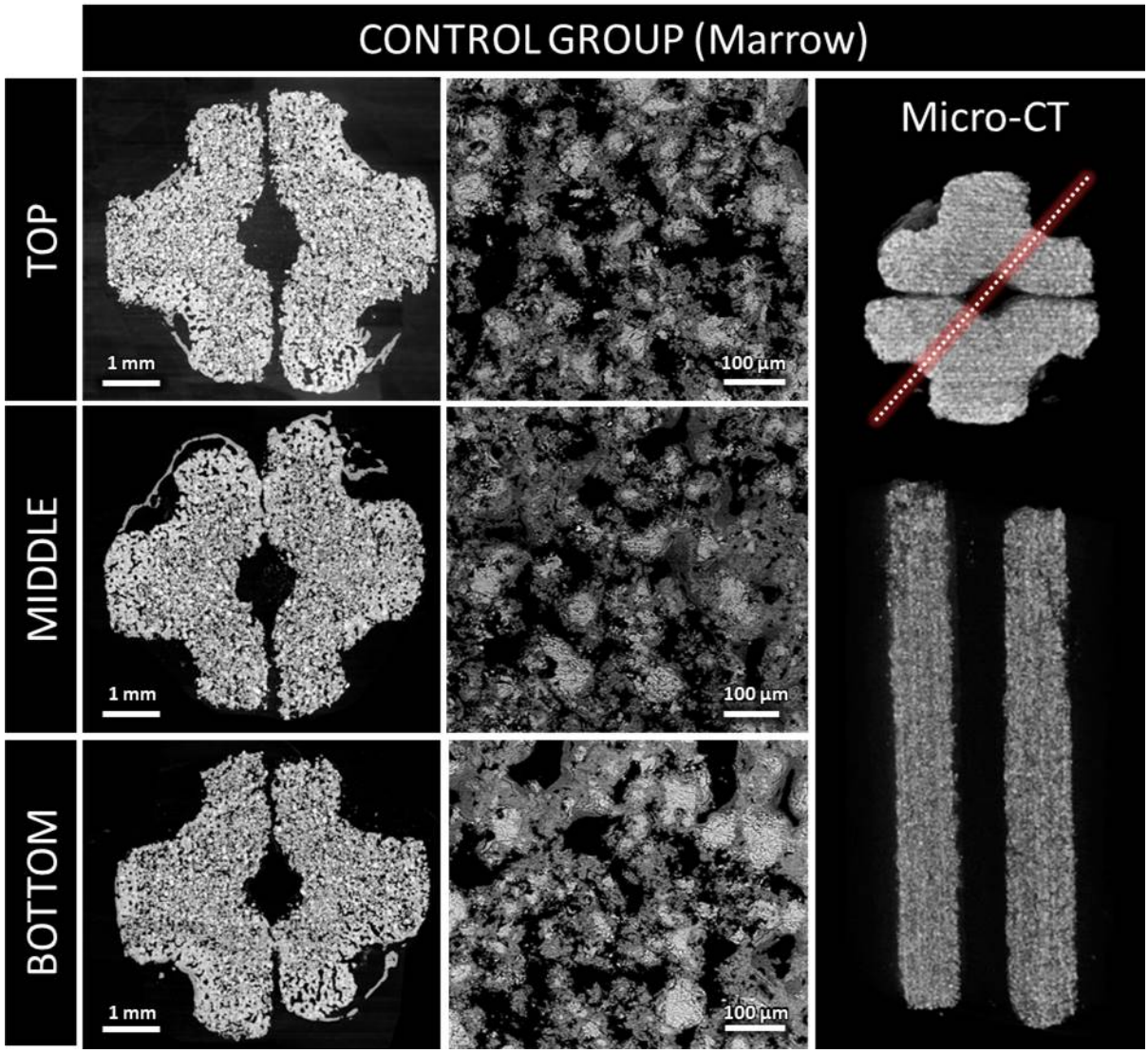

Figure 14. SEM (left) and micro-CT analyses of a cross-shaped monetite scaffold soaked in bone marrow after 8 weeks of implantation (control). SEM was performed using back scattering electrons mode on sections from the top, middle and bottom part of the sample, respectively.

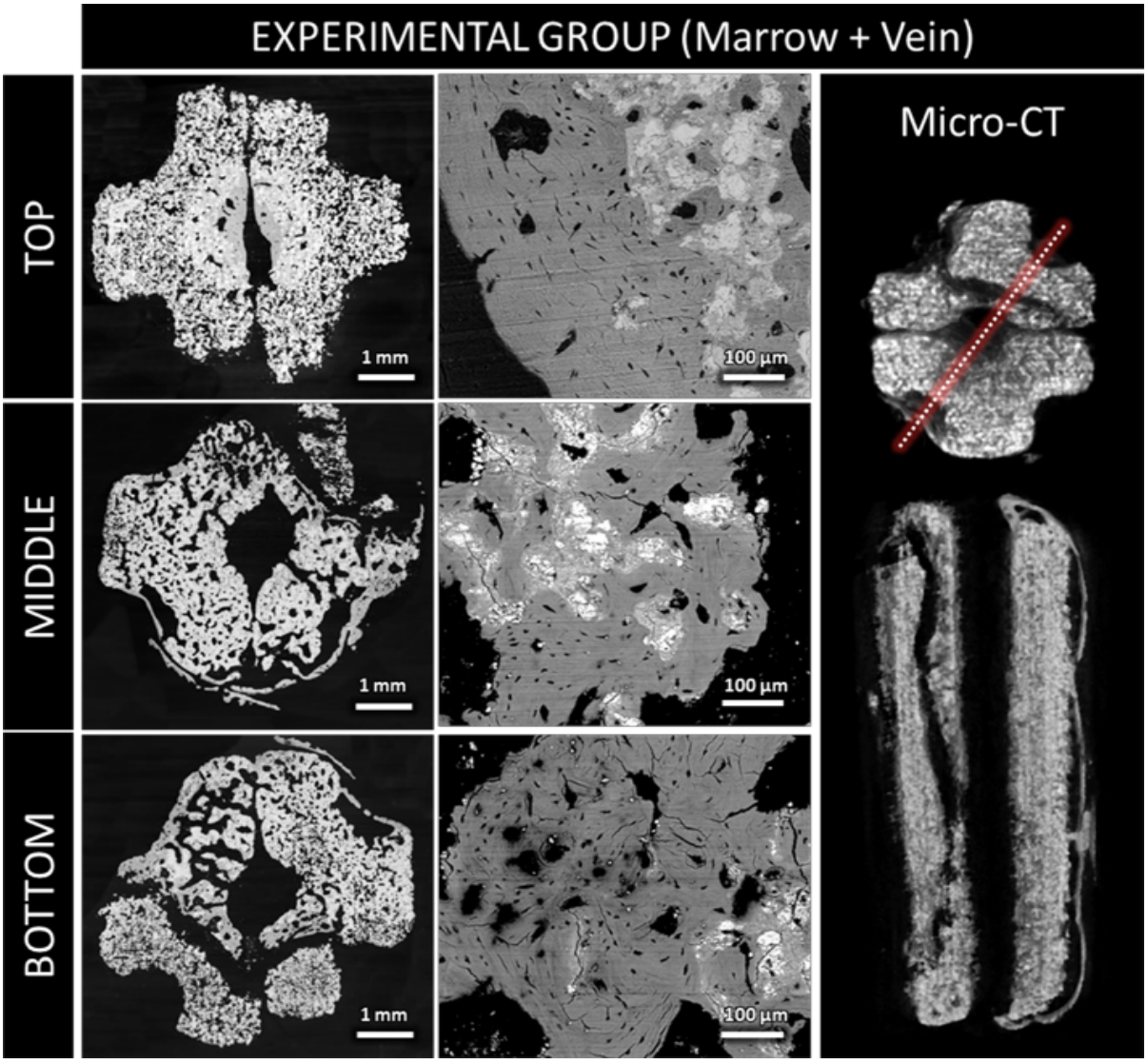

Figure 15. SEM (left) and micro-CT analyses of a cross-shaped monetite scaffold soaked in bone marrow and axially perfused by a vein after 8 weeks of implantation (experiment). SEM was performed using back scattering electrons mode on sections from the top, middle and bottom part of the sample, respectively.

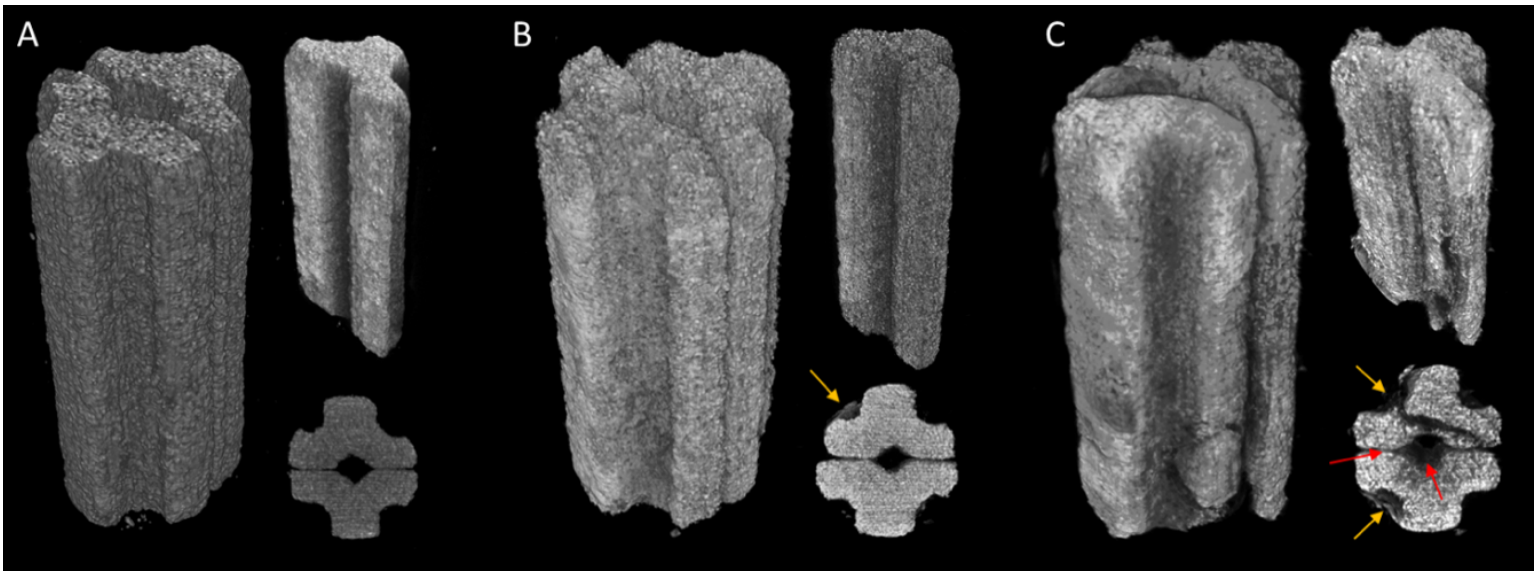

Figure 16. Comparing ceramics (A) empty, (B) with bone marrow seeded without axial vascularization, (C) bone marrow seeded and axially vascularized.

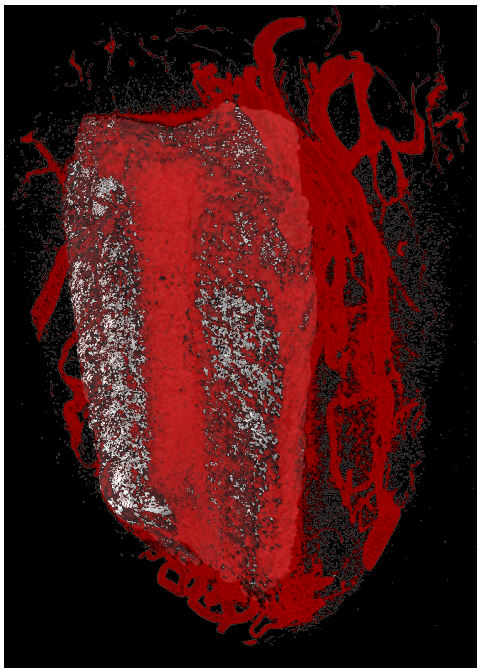

Figure 17. Microfil® perfusion showing vascularization of the vascularized scaffolds captured with micro-CT scan.

Chapter 5 – Discussion and Conclusion

Bone marrow and especially bone marrow mesenchymal stem cells (BMSCs) are known to induce the formation of bone within scaffolds when soaked in scaffolds implanted in ectopic sites (e.g., subcutaneously), and this for calcium phosphates bioceramics, metals, and natural and synthetic polymers [Table 1]. As BMSCs are thought to drive bone formation despite their extremely low number (0.0029% of the total cell population in bone marrow), thus a large majority of studies has been purifying and expanding bone marrow before seeding BMSCs in the scaffolds prior to implantation [Table 1]. However, this process is costly and highly demanding, and the efficacy of transplanted BMSCs for enhanced bone regeneration and healing in clinical cases is still debatable compared to total bone marrow. Whether it is with bone marrow or BMSCs, the values of bone formation within the scaffolds reported in the literature are between 9.0 and 26.6 % after 6 to 8 weeks of subcutaneous implantation in different animal models [Table 1], which appears to be at least comparable if not much lower than the one observed for the microporous 3D-printed monetite scaffolds soaked in bone marrow investigated in this study. This would suggest that the implant itself stimulates directly or indirectly the ectopic formation of new bony tissue, through its composition and architectural features (e.g., porosity, surface topology).

The axial perfusion of scaffolds by an AVL, combined with bone marrow or BMSCs, leads to a significantly higher bone formation than without perfusion, as reported by Spalthoff et al.[119] and Ma et al.[124] (+ 12.8 and 16.2 %, respectively). This shows the intimate relationship between angiogenesis and osteogenesis, that piques the attention of the scientific community[125,126], especially to tackle the issues encountered for regeneration of critical-sized bone defects. In a

previous study, we demonstrated that the degree of vascularization within a microporous 3D-printed monetite scaffold was higher when centrally perfused by a vein rather than an AVL. Interestingly, we showed in this study that the presence of a vein perfusing a blood marrow soaked monetite implant allowed for generating huge amount of bone (up to 65%) replacing the quasi totality of the implant (≈ 15% remaining). Even more, the structure adopted by this host-made engineered construct may surprisingly remain the structure of long bone, including a low vascularized and dense cortical layer surrounding a highly vascularized trabecular zone which hosts the marrow [Figure 11 CD34 & IBA-1and Figure 13,14].

Urist first reported bone induction via growth factors in 1965[127], since then many animal models and clinical studies on bone regeneration with BMPs have been done. rhBMP-2 and rhBMP-7 have been available for use in humans and are now in clinical use in orthopedics and spine surgery for nearly a decade. BMPs support proliferation and differentiation of mesenchymal cells into chondroblasts and osteoblasts, production and maturation of bone matrix and differentiation of osteoclast precursor cells into osteoclasts. Despite the positive effects of BMPs especially the rhBMP-2 on bone healing (e.g. elimination of the risk of autograft harvesting and osteoinduction), their application can be associated with wound complication, surgical site infection, local bone resorption, pseudarthrosis, local edema and erythema, ectopic bone formation, osteolysis, nerve injury, resistance to BMP therapy, and compartment syndrome[128-130]. It is believed that some of these drawbacks may be due to the inductive effects of rhBMP-2 on the inflammatory host reactions[130]. Another critical complication related to the application of rhBMP-2 is inflammatory vessel fibrosis and scarring that leads to vascular injury and life-threatening condition[130]. Furthermore, application of BMP is associated with significantly higher costs compared to

procedures without BMP. The hospital costs for operations associated with BMP is around $15,000 more than interventions without BMP[131]. In Nationwide Inpatient Sample (NIS) retrospective cohort examination only for lumbar pseudarthrosis between 2002-2008, they showed that utilizing BMPs added more than 900 million dollars to hospital fees. Foreseeably, they reported that introduction of BMP did not reduce the use of autograft bone harvest.

There has always been a controversy about BMP use over bone marrow. Multiple studies have shown that rhBMP-2 has similar outcomes comparing to the autologous iliac rest bone graft[132-134]. The risk and rate of adverse effect linked with rhBMP-2 has accentuated, from both ethical and legal points of view[135]. Autologous bone grafting is the gold standard for the treatment of bone defects and currently the approach of delivering osteogenic cells directly to the defect is the use of bone-marrow aspirate from the iliac crest. This procedure enhances the bone repair and the results are relatively satisfactory[136].

Table 1. Literature review of studies with the aim of subcutaneous bone generation.

Study	Implant	Cells	Substance	Animal	Vascularization	Implantation	Bone Formation
Hartman et al.[137] 2004	Titanium Mesh	BMC	None	Rat	None	6 w	9 ± 6 %
Zhang et al.[138] 2008	BCP	Rat BMSCs	None	ID Mouse	None	10 w	22 ± 3.6 %
Komlev et al.[139] 2010	BCP	Sheep BMSCs	None	ID Mouse	None	24 w	Up to 8.4 % after 8 w
Egashira et al.[140] 2018	β-TCP	Concentrated hBM	BMP2	ID Mouse	None	4 w	10.2 ± 3.3 %
Brennan et al.[141] 2014	BCP	Human BMSCs	None	ID Mouse	None	8 w	15.9 ± 4.0 %
Kjaergaard et al.[142] 2016	HA	Expanded Sheep BMSCs	None	ID Mouse	None	8 w	19.8 ± 2.5 %
Spalthoff et al.[119] 2015	β-TCP	Bone Marrow	None	Sheep	With or without AVL	6 m	Without AVL: 23.7 ± 0.8 % With AVL: 36.5 ± 2.6 %
Buehrer et al.[102] 2014	HA, Si-TCP, De-cell bone	BMSCs	BMP-2	Rat	AVL	12 w	Up to 21.6 ± 3.7 %
Ma et al.[124] 2016	β-TCP	BMSCs	None	Rabbit	With/without AVL	8 w	Without AVL: 26.6 ± 3.5 % With AVL: 42.8 ± 5.9 %
Our study	Monetite-β-TCP	Bone Marrow	None	Rat	With/without Vein	8 w	Without Vein: 29.1 ± 2.1 % With Vein 63.2 ± 3.2 %

ID: immunodeficient; w: weeks; AVL: arteriovenous loop; HA: Hydroxyapatite; TCP: tricalcium phosphate; BMSC: bone marrow stem cell; hBM: human bone marrow; BMP: bone marrow protein.

Conclusions

In this work we combined autologous bone marrow, aspirated from another femur, directly with an osteoconductive ß-TCP/HA bioceramic in the rat model. Directly auto-transplanted bone marrow can be easily obtained and are not associated with limitation such as other forms of MSC; then the femoral vein was added to this construct and fixed under the skin. Avoiding processing and expansion of the bone marrow aspirate, could reduce surgery time, and the gap between the event and the surgery, and reduce risk of infection, intoxication, and other adverse effects named before in a clinical application in the future. Furthermore, regulatory concerns for in vitro expansion would render the use of directly auto-transplanted bone marrow a more attractive approach. In this study we showed that combined effect of bone marrow and axial vascularization of the scaffold can enhance co-development of bone and supplying vessels.

Future direction: developing a synthetic axially vascularized bone flap would represent an important development and a significant contribution to clinical treatments. However, for this to occur this work would have to translated to human patients. Future direction for this work would involve implanting bone flaps into human patients followed by maxillofacial reconstruction. Another direction this work can take is to build on the vascularization strategies developed in this work to apply this to other materials such as allogenic tissue. The application of our vascularization work to donor bone would, for example, present an interesting development and we are currently planning work on this subject.

Chapter 6 – Bibliography

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