TISSUE ENGINEERING: Part A
Volume 26, Numbers 23 and 24, 2020

DOI: 10.1089/ten.tea.2020.0186

termis
Tissue Engineering
& Regenerative Medicine
International Society

ORIGINAL ARTICLE

Three-Dimensional Printing for Craniofacial Bone Tissue Engineering

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The basic concepts from the fields of biology and engineering are integrated into tissue engineering to develop constructs for the repair of damaged and/or absent tissues, respectively. The field has grown substantially over the past two decades, with particular interest in bone tissue engineering (BTE). Clinically, there are circumstances in which the quantity of bone that is necessary to restore form and function either exceeds the patient's healing capacity or bone's intrinsic regenerative capabilities. Vascularized osseous or osteocutaneous free flaps are the standard of care with autologous bone remaining the gold standard, but is commonly associated with donor site morbidity, graft resorption, increased operating time, and cost. Regardless of the size of a craniofacial defect, from trauma, pathology, and osteonecrosis, surgeons and engineers involved with reconstruction need to consider the complex three-dimensional (3D) geometry of the defect and its relationship to local structures. Three-dimensional printing has garnered significant attention and presents opportunities to use craniofacial BTE as a technology that offers a personalized approach to bony reconstruction. Clinicians and engineers are able to work together to produce patient-specific space-maintaining scaffolds tailored to site-specific defects, which are osteogenic, osseoconductive, osseoinductive, encourage angiogenesis/vasculogenesis, and mechanically stable upon implantation to prevent immediate failure. In this work, we review biological and engineering principles important in applying 3D printing technology to BTE for craniofacial reconstruction as well as present recent translational advancements in 3D printed bioactive ceramic scaffold technology.

Keywords: bone tissue engineering, 3D printing, scaffold, biomaterials

Impact Statement

Surgical reconstruction for extensive bone defects has evolved over the last 20 years toward a more customized treatment approach which fulfill functional outcomes. Additionally, the merger of surgical and microvascular principles has given rise to custom tailored patient-specific free tissue flaps which reconstruct bony maxillofacial defects while rebuilding lining, soft tissue mass, and facial subunits—all of which are key to achieving outcomes that approach normalcy. The contemporary techniques for complex boney defect reconstruction remain constrained: autologous bone transfer is complicated by limited bone stock and shape, donor site morbidity, surgical site infection, delayed healing, long operative times, and cost. Due to the shortcomings associated with autologous bone, advances in bone tissue engineering (BTE), such as 3D printing for patient and site-specific devices, have sought to restore bone deficiencies using customizable devices (scaffolds).

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Introduction

Three-dimensional printing in scaffold design

THE ABILITY TO create personalized scaffolds based on 3D imaging data coupled with three-dimensional printing (3DP) has the capacity to offer a useful approach in fabrication of patient-specific fit-and-fill scaffolds for repair of bony defects.[1] Before printing, there is an image acquisition step involving technologies such as cone beam computed tomography and computed tomography (CT), which is digitally processed to fabricate scaffolds directly or employ computer-aided design and mathematical modeling methods to produce a high-fidelity template of the defect.[2–4] Within this workflow, not only is selection of the appropriate scanning system and printing technology important but also selection of an osteogenic scaffold material necessary for optimal outcome application.

Three-dimensional printing can be categorized into four primary technologies: fused deposition modeling (FDM), stereolithography (SLA), selective laser sintering (SLS), and direct ink writing (DIW). FDM (e.g., Ultimaker [The Netherlands], MakerBot [USA], and Zortrax [Poland]) is an extrusion-based platform that utilizes a thermoplastic polymer/filament dispensed from a spool through a heated extrusion nozzle and deposited onto a build platform.[5] FDM printers have minimal maintenance costs, compact size, and flexibility in printing temperature, but are constrained to a narrow range of printable polymeric materials.[6] SLA is a polymerization technology whereby a platform is placed in a vat of curable resin equipped with a photopolymerizing ultraviolet (UV) laser beam, creating each subsequent layer of the composite scaffold as the platform travels through the image dataset representing the osseous defect.[7] SLS technology is similar to SLA, but utilizes a high-powered CO_2 laser beam to fuse layers of particles, often ceramic, to form the overall structure.[8] Laser-assisted bioprinting, while less common than other printing techniques, provides increased spatial resolution along with the capacity of printing an assortment of biological materials preserving cell viability.[9] DIW, commonly referred to as robocasting, utilizes a computer-controlled needle-syringe-type system (Fig. 1a) to deposit biomaterials, biologics, or cells in a layer-by-layer approach (Fig. 1b).[10] It is feasible to obtain customized modifications to the scaffold design through the use of different bioinks in multicompartment-type nozzles (Fig. 1a.1), microfluidic strategies,[11] and tailored colloidal gels.[12]

Biomaterials

Three-dimensional printed scaffold constructs provide a matrix for growth of tissues (hard or soft), supporting the integration, regeneration, and repair of the native anatomical structure. Degradable biomaterials used to produce these constructs may be divided into two broad categories: bioactive ceramics and polymers. Appropriate selection of these materials provides structural support, prevents soft tissue collapse, and tailors degradation kinetics to decrease the risk of graft extrusion and soft tissue dehiscence over the implant.[13–18] The desire to balance these engineering and physiologic concerns has led to exploration of various ceramics and polymers, each with their own advantages and limitations.

Bone tissue engineering (BTE) can utilize various polymeric-based materials, either natural or synthetic.[19] Naturally derived/sourced polymeric (i.e., collagen, gelatin, and hyaluronic acid) devices/products that have received FDA approval for clinical use are commonly used in craniofacial surgery.[20] Degradation often depends on enzymes, which makes kinetics hard to predict. Synthetic polymers, on the other hand, can be tailored in controlled settings, so their physicochemical properties, (i.e., average molecular weight and size distribution) are conducive to material degradation. In addition, degradation typically relies on hydrolysis, reducing variation seen in enzyme-dependent degradation.[21] Commonly utilized synthetic polymers in the clinical environment include poly(glycolic acid) (PGA), poly(lactic acid) (PLA), and copolymers (PLGA).[22] Of note, if the degradation rate surpasses the rate of local clearance, an accumulation of degradation products may lower the pH, damaging cells and tissue. Other important polymers are poly(ε-caprolactone), which can form biocompatible blends and copolymers with other polymers,[23] and poly(propylene fumarate), whose mechanical properties can be tailored through a cross-linking agent or UV photoinitiation.[24–26] In general, these materials promote cell adhesion and proliferation, but have reduced

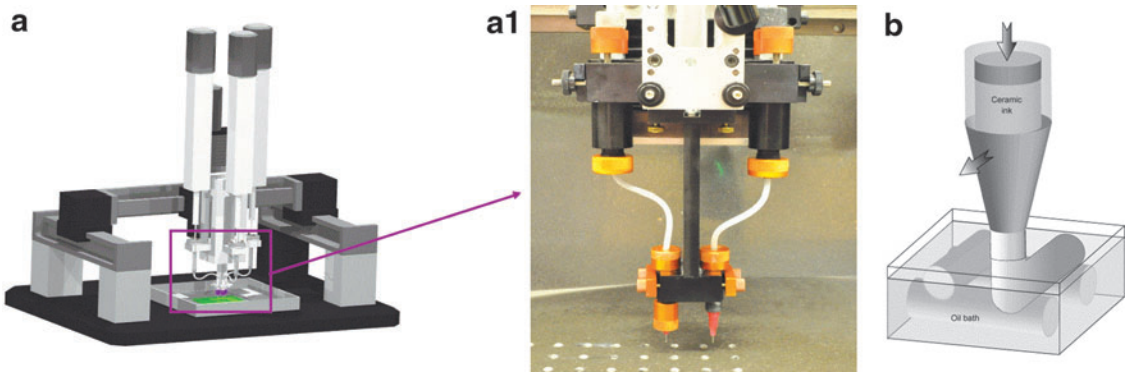

FIG. 1. **(a)** Digital CAD image of a custom-built 3D printer (3D Inks LLC, Tulsa, OK), **(a.1)** inset of the multitip nozzle attached to the 3D printer, which allows for extrusion of scaffold materials (e.g., TCP) and fugitive support material. **(b)** CAD representation of the extrusion process of the colloidal gel suspension from a Luer-Lok tip into a low-viscosity oil reservoir. CAD, computer-aided design; TCP, tricalcium phosphate.

osseoconductivity and mechanical strength compared with bioactive ceramics.[27,28]

Research on bioactive ceramics has primarily concentrated on the inorganic constituent of native bone, hydroxyapatite (HA), which is both biocompatible and osseoconductive.[29–31] The material, however, has the unfavorable degradation rate of 1–2% per year *in vivo*, limiting complete bony regeneration.[32] As a result, β-tricalcium phosphate (β-TCP) ceramic was developed, with biocompatibility and osseoconductivity similar to HA, but with an increased degradation rate.[32,33] Furthermore, its degradation kinetics can be tailored with respect to the macro- and mesoscale with 3D printing through changes in lattice porosity size, strut circumference, ink formulation, and sintering, which alter the surface area and porosity.[34,35] Printing these bioactive ceramics allows for fabrication of personalized devices that fit and fill bony defects with complex geometries, promoting optimal osseoconduction between the scaffold and the defect interface for robust osseous healing.[36–41]

Bioactive molecules

Small soluble molecules such as bone morphogenetic protein-2 (BMP-2) and vascular endothelial growth factor have been utilized to stimulate angiogenesis and osteogenesis.[42–44] Simultaneous integration of these bioactive molecules facilitates the osteogenic, osteochondral, and vasculogenic/angiogenic capacity of 3D printed bone implants.

Transforming growth factor-beta (TGF-β) superfamily members, such as BMPs, of the secreted cysteine knot proteins play key roles in the development of limb, kidney, skin, hair, and neuronal aspects in addition to actively participating in bone and vascular homeostasis.[45–48] BMPs bind to transmembrane serine/threonine protein kinase and activated type I and type II receptors.[49] As one of the most investigated regenerative agents,[50–56] recombinant human BMP-2 (rhBMP-2) has successfully demonstrated osseoinduction[57] and angiogenesis[58] in preclinical models. Both rhBMP-2 and -7 are clinically approved and available as an augmentative therapy for treatment of fractures (i.e., nonunion),[45] with several groups reporting their experience with off-label use of the former agent for alveolar cleft repair.[55,59–66] Although promising data exist, the family of proteins is reported to cause ectopic bone growth, impeded bone healing, edema, and premature craniofacial suture fusion.[67–70] These concerns have motivated research in alternative osteogenic agents, such as those that activate the adenosine receptor pathway.

The purine nucleoside, adenosine, has an effect on almost every organ system through activation of different adenosine receptors: A_1, A_{2A}, A_{2B}, and A_3. These receptors, once activated, have been shown to regenerate bone in a murine model similar to BMP-2, but without the negative side effects.[71] The A_{2A} receptor, in particular, is known to attenuate osteoclast activity and population[72–74] in addition to augmenting osteoblast differentiation.[75] As an inhibitor of type 1 equilibrative nucleoside transporter (ENT1), dipyridamole indirectly activates the A_{2A} receptor.[71] The medication has a longstanding safety profile in patients, adult and pediatric, as an antithrombotic and vasodilator.[75–77] This knowledge coupled with its capacity for bone regeneration makes it an alluring candidate as a safe osteogenic agent in BTE applications.[19,67,78–81]

Other bioactive molecules that have been studied include fibroblast growth factor (FGF), insulin-like growth factor (IGF), platelet-derived growth factor (PDGF), and the TGF-β family. PDGF is a polypeptide dimer with three isoforms (PDGF-AA, PDGF-BB, and PDGF-AB) with PDGF-BB the only isoform evaluated clinically and rhPDGF having potential in craniofacial regeneration.[48,82] The TGF-β family comprises several isoforms whose activity is mediated by a transmembrane heterodimer receptor. New bone formation has been induced by TGF-β1, without ectopic bone growth in nonhuman primates, but undisputed evidence of rhTGF-β1's efficacy over rhBMP-2 is unavailable.[48,83–86] FGF belongs to a family of polypeptides with the ability to bind heparin, with FGF-2 being the most widely researched and utilized for bone regeneration, although it does not have the capacity to regenerate bone independently.[87] IGF is a polypeptide hormone with two isoforms, IGF-I and IGF-II, whose activity is mediated through the tyrosine kinase receptor, IGF-I receptor,[48] which has been studied preclinically for its bone regenerative properties alone[88] or in conjunction with PDGF.[89]

Applications of tissue engineering principles in translational preclinical models

Researchers have employed bioprinting techniques by embedding cells within an extracellular matrix, which is then 3D printed into a tissue engineering scaffold.[90,91] Adipose-derived stem cells (ADSCs)[92,93] and mesenchymal stem cells (MSCs)[94–96] are commonly utilized multipotent cell lines in craniofacial tissue engineering, both preclinically and clinically. After isolation of ADSCs and MSCs, the cells are expanded in cell culture media and seeded onto 3D printed bioresorbable scaffolds that are then implanted into bony defects.[97–101] Cell-seeded scaffolds promote the local regenerative process as pathological anomalies or clinical therapy may impede intrinsic tissue healing.

Our group has focused on the regenerative capacity of 3D printed scaffolds comprising β-TCP, with or without a bioactive molecule coating, using critical-sized bone defects in translational animal experimental designs. A critical size bone defect (CSD) is defined as the smallest intraosseous wound that will not heal by intrinsic osteogenesis within the animal's lifetime.[102] Animals may be categorized into small animal models (i.e., mouse, rat, and rabbit) or large animal models (i.e., dog, goat, pig, and sheep),[103] and several experimental rabbit nonhealing mandibular defect models for craniofacial tissue regeneration have been described in the literature.[103–105] These animals undergo a surgical procedure in which a CSD in the mandibular bone is instrumented, which may or may not involve the alveolus. Animals are then euthanized to assess bone formation, vascular growth, and complications (infection, graft failure, and ectopic bone formation, etc.). Analysis may take the form of histology or imaging. In particular, microcomputed tomography (micro-CT) provides a robust tool to evaluate bone formation and 3D analysis of healing defects, an adjunct to conventional, two-dimensional histological methods.[106] Three-dimensional models created from imaging data may

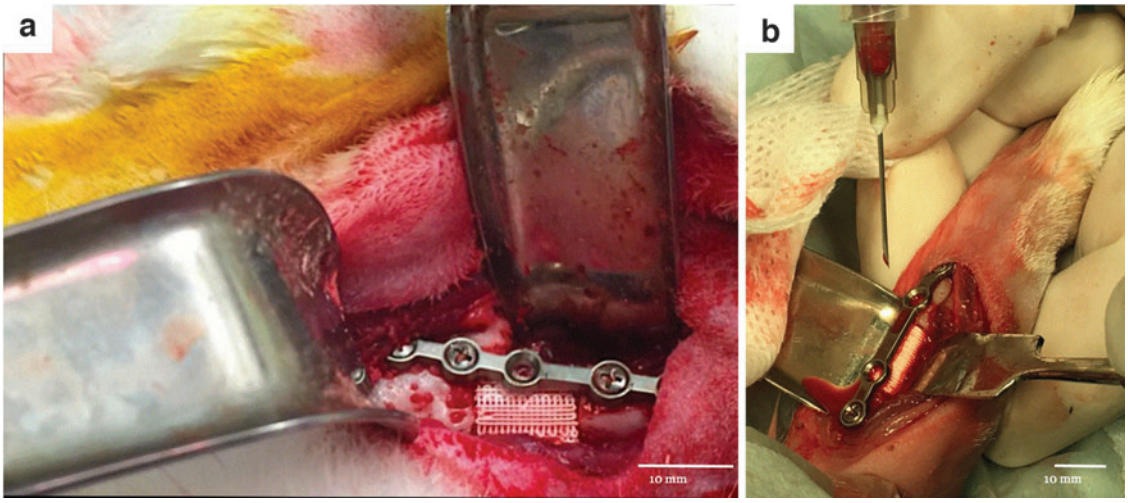

FIG. 2. Digital image of a custom, 3D printed β-TCP scaffold placed in **(a)** the mandibular body and **(b)** the radial diaphysis of New Zealand white rabbits with custom surgical hardware.

be used to quantify bone regeneration, trabecular size, vessel size, and more using quantitative, 3D image-processing software.[67,80]

Our group was the first to report the successful deployment of 3D printed bioactive ceramic scaffolds comprising 100% β-TCP with a methodical design at various length scales in a skeletally mature rabbit model for full-thickness segmental mandible (Fig. 2a)[79] and radius defects (Fig. 2b).[107,108] These studies demonstrate the utility of customized, bioactive ceramic scaffolds in a fit-and-fill model to bridge CSDs with newly formed bone *in vivo* as early as 8 weeks, with evidence of progressive scaffold degradation and further bone regeneration and scaffold resorption over a 6-month healing period.[107] Similar principles were also applied and successfully demonstrated in a larger, more clinically relevant, sheep calvarium model.[78]

In our large, preclinical sheep model, each calvaria received two ipsilateral 11-mm-diameter defects created using a trephine along with a second set of trephine-induced defects on the contralateral side at 3 weeks, creating a two-time point study (Fig. 3). The custom 3D printed scaffolds were coated with a bovine type I collagen carrier that was bound to the scaffold through cross-linking. The experimental groups constituted either the (i) collagen carrier or (ii) collagen augmented with 100 μM dipyridamole. Each defect received a scaffold. Animals were euthanized at 6 weeks following the initial surgery, and scaffolds were subjected to microcomputed tomography (μCT) and non-decalcified histology for further evaluation. Independent of the treatment group, uncoated and dipyridamole-coated scaffolds yielded new bone formation, but dipyridamole significantly enhanced healing at both time points (i.e., 3 and 6 weeks). As has been previously observed and reported, $A_{2A}R$ activation by dipyridamole enhanced the intrinsic osteogenic capacity of the local dura mater.[109]

Following the success of skeletally mature models, the work further expanded our model to include skeletally immature rabbits to study a novel tissue engineering strategy in a pediatric craniofacial model by investigating both alveolar and calvarial surgical defects.[67,80,81] To investigate the bone regenerative capacity of these bioceramic scaffolds through the time of facial (skeletal) maturity, immature New Zealand white rabbits were subjected to surgical procedures to create a unilateral 10-mm calvarial defect (Fig. 4a) with an ipsilateral 3.5×3.5-mm full-thickness alveolar defect (Fig. 4b).[81] Each defect received either a 3D printed β-TCP scaffold coated with 1000 μM dipyridamole or clinical standard of care autogenous bone grafting (harvested from the

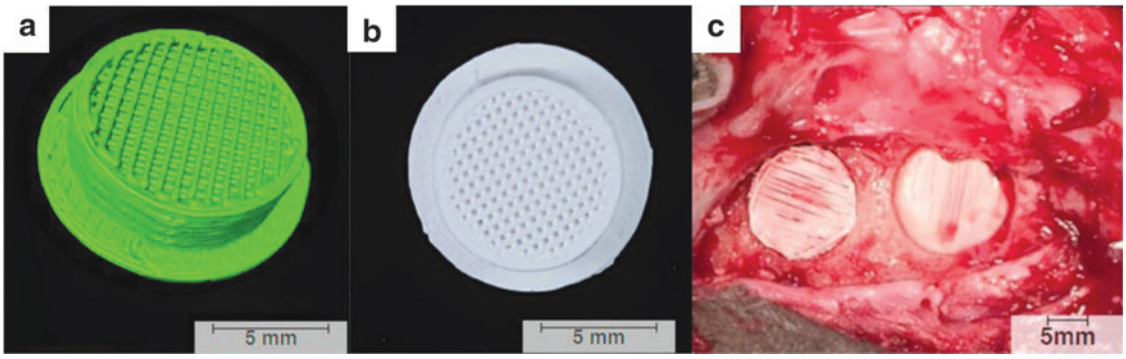

FIG. 3. **(a)** Three-dimensional reconstruction of the scaffold created using Amira 6.1 software (Visage Imaging GmbH, Berlin, Germany). **(b)** Inferior surface of the scaffold showcasing the porous core with central lattice. **(c)** Intraoperative photograph showing scaffold placement in anterior and posterior calvarial defects.

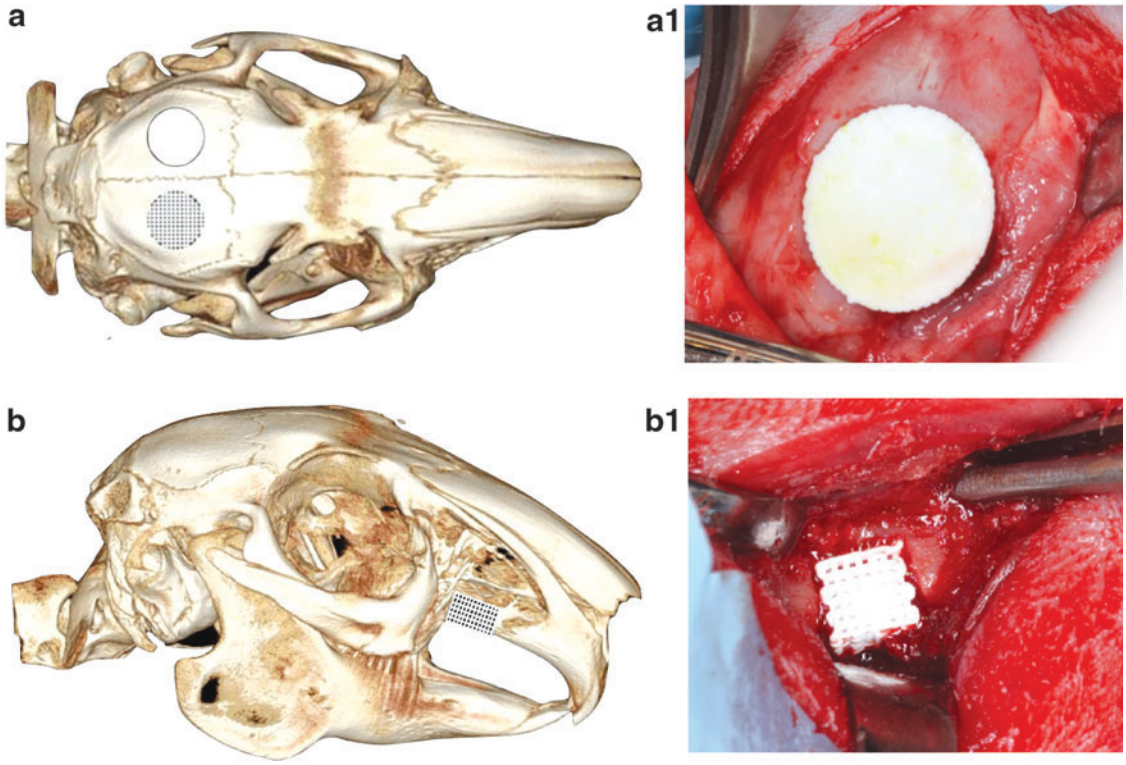

FIG. 4. micro-CT image of rabbit skull, schematic depicting **(a)** calvarial defect and **(b)** alveolar cleft model, respectively. The skeletally immature rabbits underwent surgical resection of alveolar calvaria and alveolar cleft, each site receiving custom-designed and 3D printed β-TCP scaffolds. Intraoperative placement of scaffolds in **(a.1)** calvaria and **(b.1)** alveolar ridge defects using a fit-and-fill process.

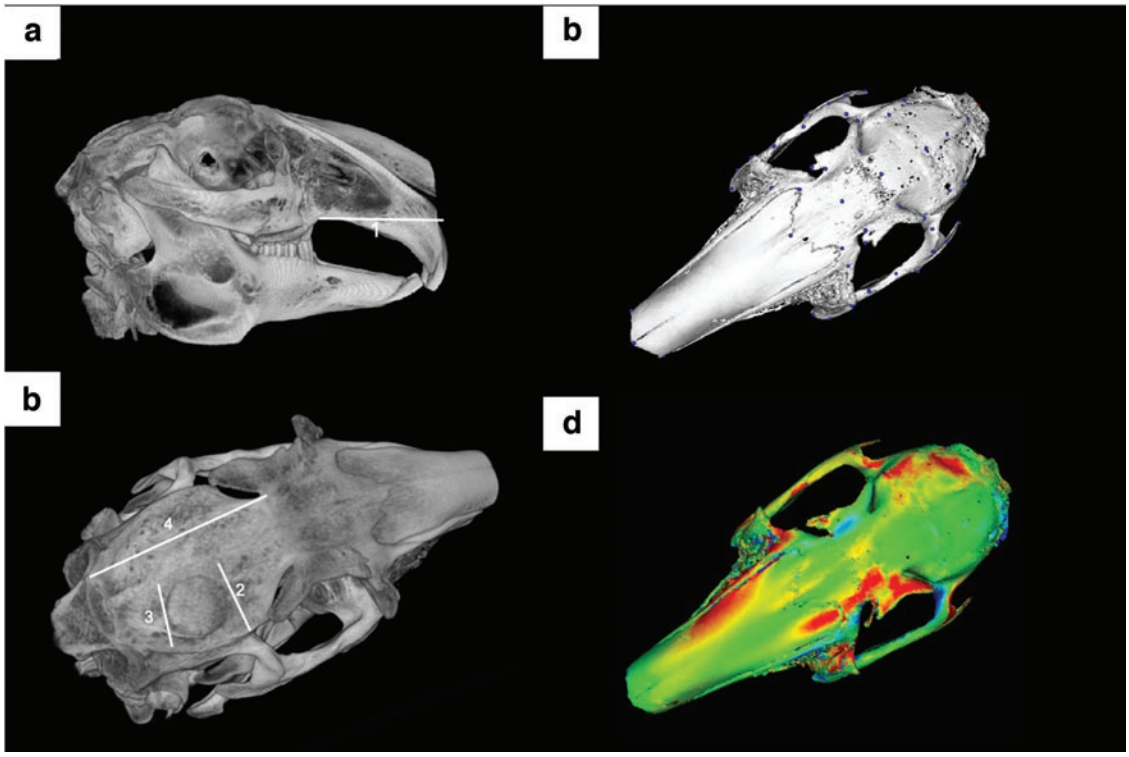

FIG. 5. micro-CT rendition of rabbit calvaria for cephalometric measurements, **(a)** alveolar cleft and **(b)** calvaria. **(c)** Example of model landmarking for rabbit craniofacial surface for building of a global facial model and **(d)** resultant heat map model when overlaying the unoperated base mesh with that of the mesh of an operated animal treated with a custom scaffold for defect repair.

respective animal's radius). Rabbits were euthanized at 8 weeks and 6 months. Bone regeneration, scaffold degradation, trabecular thickness, and trabecular spacing were calculated using micro-CT reconstruction. Vessel density, vessel diameter, osteoblast density, osteoclast density, and osteoblast-to-osteoclast ratio were calculated using histology. Elastic modulus and hardness were calculated using nanoindentation. Facial development and symmetry at facial maturity were evaluated using cephalometric measurements and analysis (Fig. 5). The 3D printed scaffolds yielded significant osteogenic regeneration, volumetrically, in both alveolar and calvarial defects in comparison with autologous bone graft and without any detriment to normal craniofacial growth, ectopic bone growth, or premature suture fusion. Similarly, newly organized and vascularized bone showed histological and mechanical properties similar to native bone, reinforcing the potential to repair the defective site and to regenerate the native form.

Adenosine receptor ligation's bone regenerative capacity was unequivocally demonstrated through our skeletally mature and immature animal models. Using 3D printed ceramic scaffolds as carriers of dipyridamole, we leveraged localized drug delivery to the injury sites to promote bone regeneration, while avoiding unintended systemic effects. The osteogenic agent amplified the osseoconductive properties of β-TCP scaffolds without damage to craniofacial sutures or ectopic bone growth, which are documented complications of rhBMP-2,[69,110,111] even with doses logarithmically increased up to 2× more than necessary to increase bone regeneration.[67,80]

Conclusions and Future Directions

The clinical repair of tissues, hard or soft, is constantly evolving based on emerging technology and surgical approach. The recent progress in 3D printing technology and tissue-engineered devices will not only assist in healing but also aid in the craniofacial surgeon's approach. Therefore, a strong understanding of the biology, materials science, pharmacology, and engineering concepts involved in BTE and the growing field of regenerative medicine is paramount for researchers and clinicians alike.

Although still exploratory in the clinical setting, tissue engineering is gaining popularity among surgeons and translational researchers. Of importance is our continued exploration of the physiological microenvironment as we continue to manipulate native tissue physiology. Infection, wound dehiscence, graft failure, and premature resorption are fundamental adverse events that we must overcome. Expanding our understanding of fabrication modalities, biomaterials, degradation kinetics, biopharmaceuticals, bone remodeling, and cell harvesting will bring 3D printed bioactive scaffolds to patients as safe and efficacious devices for craniofacial skeletal reconstruction.

Disclosure Statement

No competing financial interests exist.

Funding Information

This work was supported by the National Institutes of Health [R21/R33 HD090664-01 ''Use of 3D Printing for Creation of Implantable Pediatric Devices''], and Dr. Coelho was supported by grants NIH [R01 AR068593] and NYU-H+H Clinical and Translational Science Institute Grant (1UL1TR001445).

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Received: July 2, 2020
Accepted: August 6, 2020
Online Publication Date: October 1, 2020