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Chapter

Cell Attachment and Osteoinductive Properties of Tissue Engineered, Demineralized Bone Fibers for Bone Void Filling Applications

Julie B. McLean, Nigeste Carter, Payal Sohoni and Mark A. Moore

Abstract

Demineralized bone matrices (DBMs) have been used in a wide variety of clinical applications involving bone repair. Ideally, DBMs should provide osteoinductive and osteoconductive properties, while offering versatile handling capabilities. With this, a novel fiber technology, LifeNet Health-Moldable Demineralized Fibers (L-MDF), was recently developed. Human cortical bone was milled and demineralized to produce L-MDF. Subsequently, the fibers were lyophilized and terminally sterilized using low-dose and low-temperature gamma irradiation. Using L929 mouse fibroblasts, L-MDF underwent cytotoxicity testing to confirm lack of a cytotoxic response. An alamarBlue assay and scanning electron microscopy demonstrated L-MDF supported the cellular function and attachment of bone-marrow mesenchymal stem cells (BM-MSCs). Using an enzyme-linked immunosorbent assay, L-MDF demonstrated BMP-2 and 7 levels similar to those reported in the literature. *In vivo* data from an athymic mouse model implanted with L-MDF demonstrated the formation of new bone elements and blood vessels. This study showed that L-MDF have the necessary characteristics of a bone void filler to treat osseous defects.

Keywords: demineralized bone matrix, osteoinductive, osteoconductive, allograft, growth factors, bone formation

1. Introduction

Bone voids may occur due to trauma, surgery, tumor resections, or other factors. For decades, surgeons have used bone grafting to treat a wide variety of bone defects. Bone grafts may contain up to three of the vital properties necessary for bone formation: osteoconductivity, osteoinductivity, and osteogenicity [1]. The property of osteoconductivity describes the way the graft acts as a scaffold on which host cells can attach and proliferate, leading to osseointegration. Osteoinductivity, on the other hand, describes the cellular signaling potential of a graft. Whether endogenous or recombinant, specific growth factors, such as bone morphogenetic

protein 2 (BMP-2), attract host cells to a graft and encourage mesenchymal stem cells to differentiate into lineage-committed bone cells. Finally, osteogenicity describes the ability of a bone graft to form bone matrix directly, which can only happen when live cells capable of producing bone matrix are contained within the graft. Bone graft options may contain varying amounts of these properties and are chosen based on the characteristics that the patient needs in order to achieve bone fusion. There are several graft options available, including autograft, synthetic bone substitutes, and allografts.

Autologous bone is harvested from the site of surgery in the patient or a second site, such as the iliac crest. It is still considered the gold standard by many surgeons because it can theoretically provide all three vital properties for bone formation, does not provoke an immune response, and has a long history of use. However, the use of autograft bone is associated with several disadvantages such as donor site morbidity, insufficient supply, and variable quality [2, 3]. Up to 30% of patients experience significant donor site morbidity as well as infection risk, increased operative time, blood loss, and the potential for arterial and nerve injury [4]. Additionally, autograft is limited, and the quality may be poor depending on the patient's health. For example, diabetes, low bone mass, and smoking can all increase the risk of fusion failure as well as intraoperative complications [5].

Synthetic bone substitutes are designed with the goal of mimicking the natural properties of human bone. They can be comprised of a variety of materials including but not limited to, ceramics, cements, and bioactive glass. These grafts are generally biocompatible, osteoconductive, and may be mechanically similar to bone [6, 7]. This category of graft has typically been manufactured to contain porosity similar to bone, but may lack other desirable surface properties, such as hydrophilicity or a rough surface on which cells can attach. Synthetic bone substitutes have gained popularity due to reduced cost and ready availability; however, they may have mismatched resorption rates compared to bone and generally lack osteogenic and osteoinductive properties [8]. Some synthetics, such as recombinant human BMP-2, depend almost solely upon osteoinductivity and often result in rapid bone formation. However, several studies indicate substantial side effects, including osteolysis, heterotopic bone formation, and swelling/edema [9–11]. While synthetics have improved over the last few decades, mimicking natural bone has proven difficult, and allografts, being natural bone, have continued to be a reliable source of grafting material.

Allograft bone is obtained from deceased human donors and has a long history of use. It is readily available in a variety of forms, shapes, and sizes providing surgeons with several graft options suitable for various procedures [12–14]. Allografts can provide up to all three properties necessary for bone formation. For example, mineralized bone allografts have similar osteoconductive properties to autograft while avoiding complications such as donor site morbidity [15]. Some mineralized grafts have been processed to increase desirable characteristics such as increased surface area on which cells can attach as well as increased coefficient of friction to prevent the graft from shifting once implanted. Other allografts, such as demineralized bone matrix (DBM) are both osteoconductive and osteoinductive. To produce DBMs, acid demineralization is used to remove a portion of the mineral component of bone, thus exposing the active signaling proteins necessary to induce new bone formation. The ability of DBMs to facilitate bone healing was demonstrated in clinical applications as early as 1889 when Dr. Nicholas Senn reported using demineralized bone as a vehicle for antiseptics to treat patients with osteomyelitis [16]. However, it was not until 1965, when Dr. Marshall Urist characterized specific proteins trapped within the bone matrix, that it was understood that bone morphogenetic proteins (BMPs) contributed to the osteoinductive property of DBMs [17]. Since the discovery of BMPs, other proteins, such as those associated with angiogenesis, have also been found to contribute to the process of bone healing

and regeneration [18]. In addition to containing active signaling proteins, optimal surface characteristics of DBMs are essential for supporting cellular attachment and proliferation. For example, it is crucial to provide enough space for blood vessel formation and for the patient's own cells to migrate into and proliferate on the scaffold [19, 20]. Therefore, some allograft processors work to maintain ideal porosity for cell migration and angiogenesis. Other processes are designed to create a hospitable topography for cell attachment and proliferation as well as to enhance handling characteristics to facilitate implantation and mitigate migration.

DBMs are available in varying forms, including powders, putty, strips, and moldable paste. These grafts often contain carriers such as glycerol, starch, or hyaluronic acid to improve handling. Without a carrier, bone grafts may be difficult to implant in the desired area, or may drift away from the area during surgical irrigation or exposure to blood. However, despite improved handling characteristics, it has been reported that some carriers may inhibit osteoinductive potential [21]. In addition, a carrier dilutes the bone concentration and may easily elute from the surgical site, effectively reducing the implant volume. With these limitations in mind, a novel DBM with unique fiber technology was recently developed as described in Section 2. These fibers (**Figure 1**) are composed solely of demineralized cortical bone and are designed to provide surface features conducive for cellular attachment and easily moldable handling characteristics, all without the addition of a carrier. The purpose of this chapter is to present original research, detailing the composition, osteoinductive nature, cell attachment properties and endogenous bone growth factor content of these bone fibers through *in vivo* and *in vitro* test methods.

Figure 1.
Rehydrated moldable demineralized fibers.

2. Methodology

2.1 Fiber generation

The fibers described here are referred to as L-MDF (LifeNet Health-Moldable Demineralized Fibers, LifeNet Health, Virginia Beach, VA and clinically available as part of PliaFX® and OraGRAFT® Prime brands). The particular fibers studied below were prepared from human cortical long bones that were aseptically recovered from donors, debrided, and disassociated from marrow and trabecular bone. The resulting tissue was processed by a proprietary computer numerical controlled-milling method (CNC-milled) into long fibers and disinfected using a proprietary process. The fibers were then demineralized using proprietary procedures. Following demineralization, fiber samples were taken to quantify residual calcium levels (average 1.7%) using a calcium reagent kit (Eagle Diagnostics, Cedar Hill,

TX). The demineralized fibers were then freeze-dried, placed in final packaging, and treated via low-dose, low-temperature gamma irradiation, at a level necessary to achieve a sterility assurance level (SAL) of 10^{-6}.

2.2 Cytotoxicity testing of L-MDF using L929 mouse fibroblasts

The cytotoxic potential of L-MDF were quantitatively evaluated by the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay using L929 mouse fibroblasts. Three samples of 2.5 cc from each of the six donors (n = 18) were rehydrated with 5 mL sterile saline (0.99% w/v sodium chloride in water). Sample extracts were prepared by incubating 0.2 g of each sample with 1 mL of extraction medium (Minimum Essential Medium supplemented with 10% v/v fetal bovine serum, 100 U mL^{-1} penicillin, 100 μg mL^{-1} streptomycin, and 2 mM L-glutamine) for 24 ± 2 h at 37 ± 1°C. Negative and positive controls were prepared similarly. Extraction medium alone was used as an untreated control "extract" for quantitative comparison of results. L929 mouse fibroblasts were cultured in 96-well microplates to half-confluency and subsequently exposed to 100 μL of sample or control extracts for 24–26 h at 37 ± 1°C. Following extract exposure, cell viability of each well was measured using a MTT assay. The average results for each group were normalized to the untreated control to determine a percent viability. Per ISO 10993-5:2009, percent viability less than 70% indicates a cytotoxic effect.

2.3 *In vitro* metabolic activity of seeded bone marrow-mesenchymal stem cells

Human bone marrow-derived mesenchymal stem cells (BM-MSCs) seeded on L-MDF were measured for metabolic activity using an alamarBlue® assay (Bio-Rad, Raleigh, NC) over the course of 7 days. L-MDF from six donors were placed in triplicate in low-attachment 24-well cell culture plates at a density of 13.1 mg of fiber per cm^2 and seeded with BM-MSCs at 62,500 cells per well on day 0. BM-MSCs without fibers served as the control. After 2–4 h in culture, 1 mL of complete media was added to each well, followed by incubation at 37°C. Samples remained in the incubator until specific time points designated for analysis, at which point media was replaced. The metabolic activity of cells adhered to the fibers was measured after 1, 4 and 7 days in culture. At each time point media was aspirated and replaced with 1 mL of 10% alamarBlue reagent and incubated for an average of 2 h at 37°C. The solution was collected from each sample, centrifuged to pellet any debris, and measured in a 96-well plate at 544 nm excitation/592 nm emission. Fluorescence was recorded using relative fluorescence units (RFUs), and values were normalized to its time-matched control. A one-way ANOVA in conjunction with a Tukey post-hoc was used to determine differences in metabolic activity over time.

2.4 *In vitro* cellular attachment of seeded bone marrow-mesenchymal cells

Scanning electron microscopy (SEM) was used to qualitatively evaluate the attachment and morphology of cells seeded on four L-MDF samples (25 ± 1 mg) at 0.5–1 h, 1 and 7 days in culture. The fibers were placed in separate glass scintillation vials with 1 mL of complete media and incubated at 37°C. Following incubation, excess media was aspirated and BM-MSCs were seeded at 100,000 per cells per vial. At each time point, corresponding vials were removed from the incubator, excess media was removed, and 3 mL of 2.5% glutaraldehyde in cacodylate buffer was added to fix the samples. Cell-seeded samples were rinsed in 0.1 M cacodylate buffer, incubated in 1% osmium tetroxide for 60 min, and then dehydrated in a series of ethanol solutions increasing in concentration up to 100%. Samples were then dried via evaporation of a chemical drying agent, hexamethyldisilazane (HMDS). Prior to imaging, all samples

were sputter coated in gold palladium for 200 s at 60 mA, then secured to a holder that was placed inside a vacuum-sealed imaging chamber. Samples were then imaged at a magnification 3000× using a Zeiss Gemini HD Scanning Electron Microscope.

2.5 *In vitro* growth factor analysis

L-MDF were analyzed for the presence of BMP-2 and BMP-7 using an enzyme-linked immunosorbent assay (ELISA) (R&D Systems, Minneapolis MN). L-MDF from six donors were weighed (30 ± 10 mg per donor) and placed in microcentrifuge tubes. Samples were then rehydrated with 5 μL of Dulbecco's Modified Eagle Medium (DMEM) per milligram of fiber, followed by the addition of purified collagenase (14.47 Units/mg of fiber). The samples were digested at 37°C with constant mixing for 16–18 h. Digestion solutions were centrifuged to remove remaining undigested components and the supernatants were collected for testing. The resulting solutions were analyzed for BMP content in triplicate using an ELISA assay. The measured BMP content was averaged across all six donors and results were reported in ng protein/g of demineralized fibers.

2.6 *In vivo* osteoinductive potential (OI)

The osteoinductive potential of L-MDF was evaluated using an *in vivo* athymic mouse model at NAMSA (Northwood, Ohio) following American Society for Testing and Materials (ASTM) F-2529 guidelines. Four 20–25 mg replicates of L-MDF were rehydrated with 100–150 μL of sterile 0.9% w/v sodium chloride and loaded into 0.3 cc sterile syringes. Samples were then compressed to remove excess solution, and implanted bi-laterally between the biceps femoris and superficial gluteal muscle of athymic mice. All mice were euthanized 5 weeks post-implantation by carbon dioxide inhalation. Explants were fixed with 10% formalin and bisected along the long axis. Bisects of each explant were paraffin embedded, and three slides were generated each with 4–6 μm-thick tissue sections. Once stained with hematoxylin and eosin (H&E), slides were evaluated by a blinded pathologist. The presence of cartilage, chondroblasts, chondrocytes, osteoblasts, osteocytes, osteoid, newly formed lamellar bone, and bone marrow were evaluated as new bone elements as they are indicators of endochondral bone formation process.

3. Results

3.1 Cytotoxicity

Cytotoxicity assay results showed that negative and positive controls behaved as expected (i.e., percent viability ≥70% for the negative control groups and <70% for the positive control groups). The average percent viability for negative and positive controls were 94 and 4%, respectively. The average percent viability of L-MDF (91%) was above the 70% threshold, and thus, based on the criteria of the protocol and ISO 10993-5 guidelines, L-MDF are considered to be non-cytotoxic.

3.2 L-MDF supports attachment and sustained metabolic activity of bone marrow-mesenchymal stem cells

Overall, the cellular activity of the BM-MSCs was shown to significantly increase over the course of the 7 day investigation. The results indicated that cells seeded on L-MDF showed a significant increase in proliferation between days 4 (51.3 ± 1.2 RFU) and 7 (59.5 ± 1.5 RFU) compared to day 1 (21.3 ± 0.8 RFU) (**Figure 2**).

SEM images confirmed BM-MSC attachment to L-MDF within 30 min of seeding. Cells appeared rounded with numerous folds and ridges and minimal surface contact (**Figure 3A**). After 1 h in culture, BM-MSCs became elongated and began spreading and increasing surface contact with the fibers (**Figure 3B**). After 1 day, imaging showed flattened cells with multiple adhesion points and cellular extensions as well as extracellular matrix (ECM) secretion (**Figure 3C**). By day 7 in culture, BM-MSCs infiltrated between fibers and demonstrated cell-to-cell interactions (**Figure 3D**).

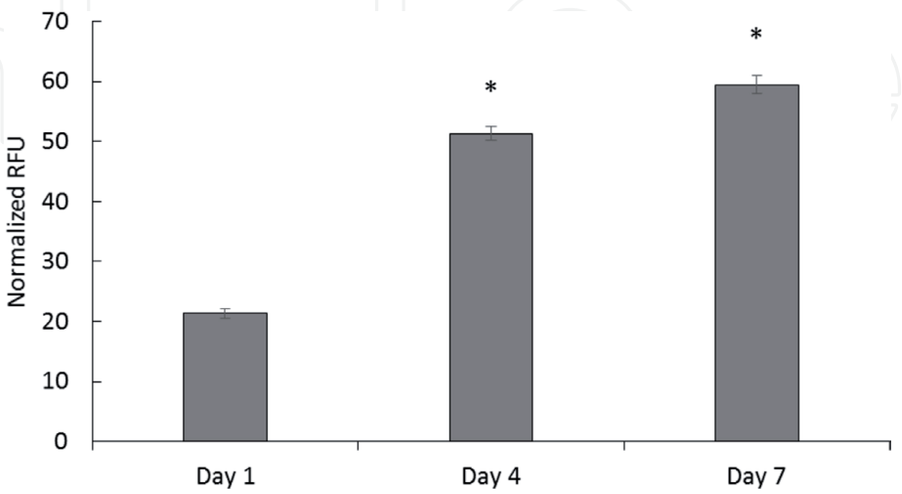

Figure 2.
Proliferation of BM-MSCs attached to L-MDF over 7 days. The average relative fluorescence unit (RFU) values for each set of triplicate test samples were normalized to the average RFU of the corresponding control group (fibers of the respective donor cultured without cells) for all six donors. Asterisks represent statistically significant differences from day 1 proliferation activity.

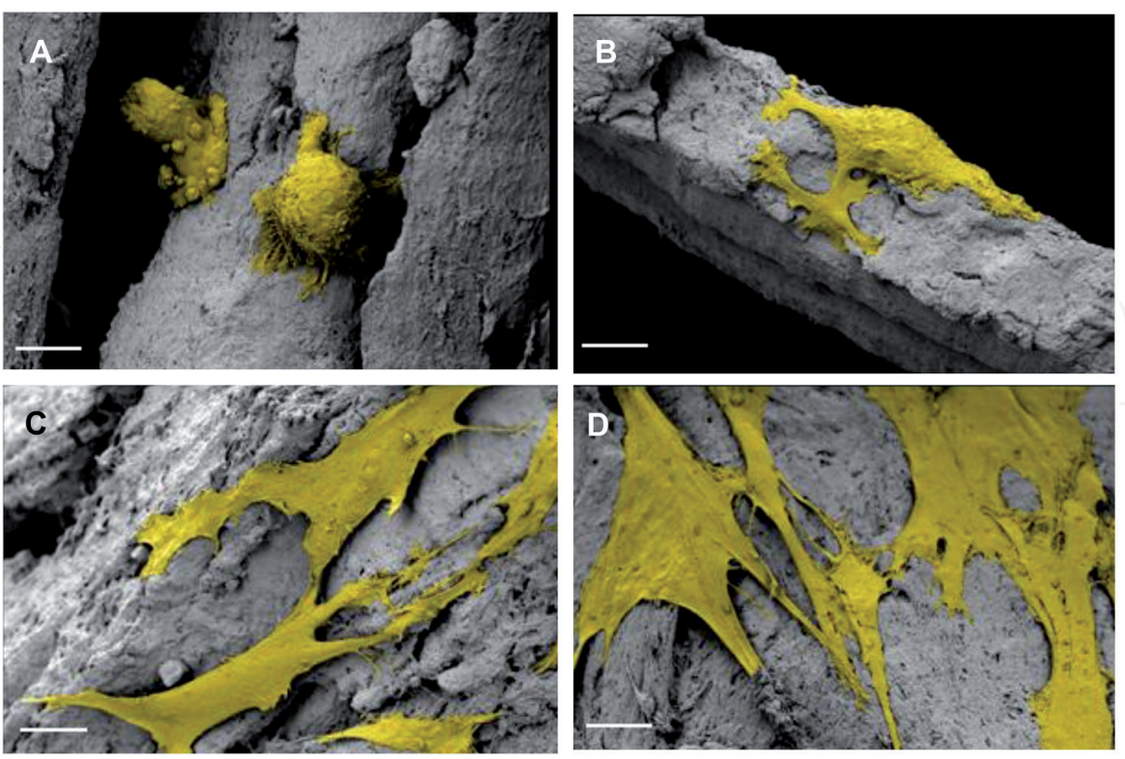

Figure 3.
Representative SEM images illustrating the morphology of cells attached to L-MDF. Following culture for 30 min (A), 1 h (B), 1 day (C) or 7 days (D), respectively, the samples were fixed in 2.5% glutaraldehyde and processed for scanning electron microscopy. Images are representative of all samples evaluated and were taken at 3000× magnification. Scale bar represents 10 μm. Images were pseudo-colored in Adobe Photoshop to distinguish the cells (in yellow) from the fibers.

3.3 L-MDF contains important growth factors and demonstrates new bone formation *in vivo*

The ELISA results indicated the presence of growth factors in L-MDF. The average BMP-2 and 7 concentrations in the samples fibers were 11.24 ± 1.49 and 85.78 ± 6.84 ng/g, respectively (**Figure 4**).

Additionally, in the athymic mouse muscle pouch model, histological analysis revealed new bone elements around and within the implanted scaffold at time of sacrifice (5 weeks; **Figure 5**). Panel A shows a set of merged images that illustrate

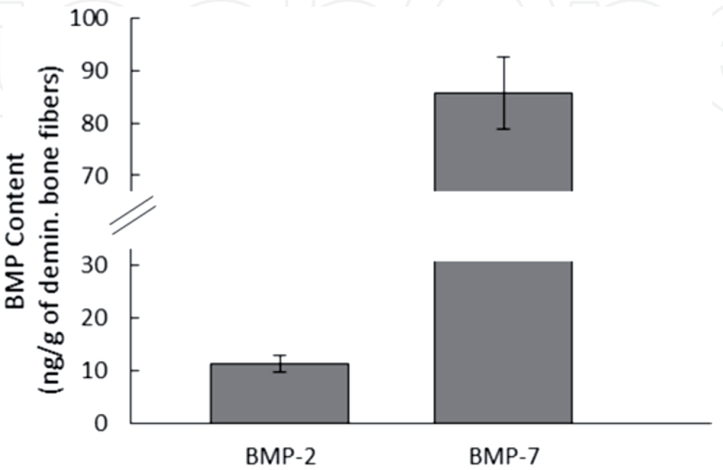

Figure 4.
BMP-2 and BMP-7 content in L-MDF. L-MDF produced from six different donors were digested in collagenase for 16–18 h. Using ELISAs, the resulting digestion solutions were tested for BMP-2 and BMP-7 content in triplicate (mean ∓ SE).

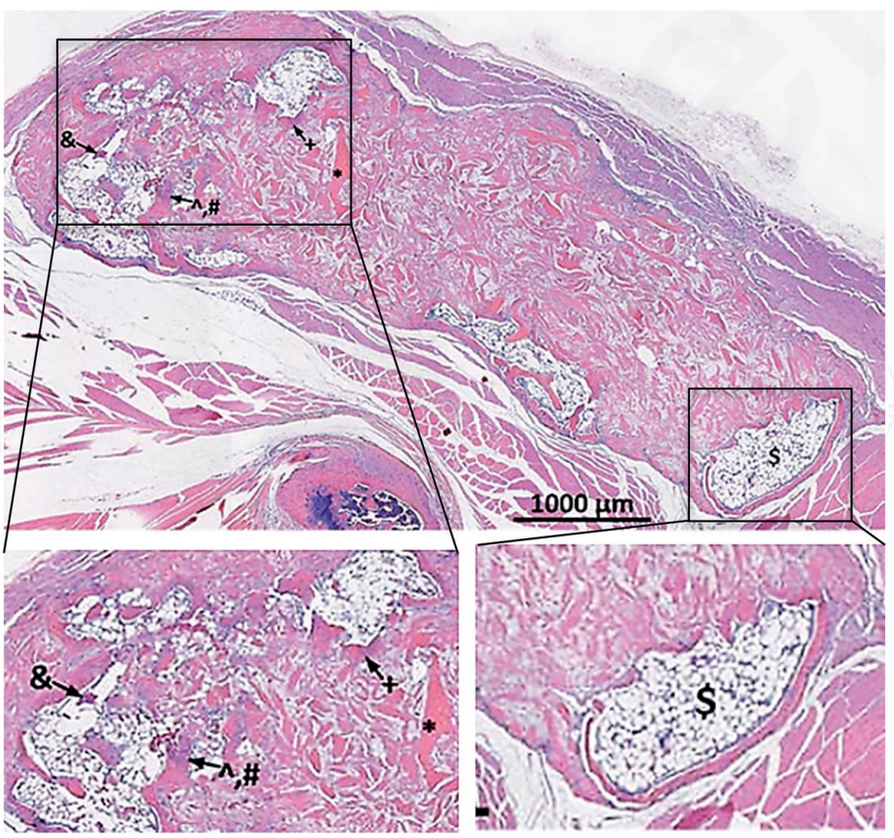

Figure 5.
H&E staining of explants from an athymic nude mouse implanted with L-MDF (*). Merged set of H&E images showing new bone elements present in the entire explant at 35 days post-implantation (4× objective). Expanded areas show the presence of new bone elements such as cartilage (^), chondroblasts/cytes (#), bone marrow ($), new blood vessels (&), and new bone (+) around L-MDF implant (*) at 35 days.

new bone elements present in the explant (4× objective). Panels B and C highlight the presence of new bone elements such as cartilage, chondroblasts/cytes, bone marrow, new blood vessels, and new bone.

4. Discussion

Demineralized bone matrices (DBMs) are widely used in spinal, orthopedic, craniomaxillofacial, and dental procedures to treat bone voids. An ideal DBM provides both osteoinductive and osteoconductive properties to promote new bone formation and provide a scaffold upon which cells can attach and proliferate. Furthermore, DBMs should be malleable and resist graft migration once impacted into a bone defect. To achieve these characteristics important for bone healing, manufacturers use a variety of techniques to process and sterilize DBMs. Despite demineralization being a well-known technique, the proportion of the osteoinductive element—the demineralized bone—of clinically available DBM-based graft materials varies widely by manufacturer. Differences in carrier material and sterilization may also contribute to variability among these grafts. The moldable demineralized fibers described here represent a recently developed allograft configuration that can function as an independent bone void filler without the need of a synthetic carrier. This study was conducted to ensure L-MDF possess the necessary qualities to function in this capacity.

An osteoinductive bone graft has the ability to induce bone growth. Factors such as residual calcium level and growth factor content play important roles in a DBM's ability to grow bone. In particular, residual calcium level can serve as an indicator for the availability of growth factors necessary for bone formation. The literature suggests that DBMs with different degrees of residual calcium show significant differences in osteoinductivity. Zhang et al. evaluated the effects of varying degrees of demineralization, particle size, donor age, and gender on the osteoinductivity of DBM *in vivo* (athymic mouse model) and *in vitro* (alkaline phosphatase assay) [22]. The authors suggested that demineralized bone with a residual calcium level of approximately 2% is "optimally osteoinductive". Similarly, Turonis et al. found that a 2% residual calcium level in human demineralized freeze-dried bone allograft appears to enhance osseous wound healing [23]. The L-MDF samples discussed in this chapter were demineralized using a proprietary and patented process targeted at achieving an optimized level of residual calcium of 1–4%. Furthermore, the presence of specific proteins in DBM is frequently associated with its osteoinductive potential as growth factors can provide signals that direct cellular behavior [18, 22, 24]. In particular, BMP-2 and 7 are important for bone growth as they are known for their "ability to stimulate differentiation of MSCs to osteochondroblastic lineage" [18]. Previous studies have reported a wide span of BMP-2 and BMP-7 levels in demineralized bone, with ranges from 6.5 to 110 and 44 to 125 ng/g demineralized bone, respectively. In this study, ELISA results indicated the presence of BMP-2 and 7 in L-MDF (11.24 ± 1.49 and 85.78 ± 6.84 ng/g) consistent with values reported in the literature. This milieu of growth factors illustrate that L-MDF contain the appropriate trophic factor profile necessary for bone formation and are consistent with expected physiological levels.

The osteoinductive and osteoconductive potential of DBMs are commonly evaluated using an *in vivo* athymic mouse intramuscular pouch model to histologically assess new bone formation [25].

In the study described here, histological analysis revealed the presence of new bone elements demonstrating the osteoinductive potential of L-MDF. In addition, newly formed blood vessels were observed, which can also be indicative of the

osteoconductive nature of the bone graft in providing a conducive environment for new bone formation. The surface characteristics of DBMs play an important role in their ability to provide a scaffold for new bone formation [19, 20]. Bone cells need a hospitable environment in which to attach and thrive. In particular, increased surface area, a rough topography, and interconnected networks are known to promote cellular attachment and cell spreading [26]. As demonstrated by the SEM imaging presented here, the long, interconnected L-MDF create a hospitable environment for BM-MSCs to infiltrate and make cell-to-cell connections. The ability of cells not only to quickly attach to the matrix but also maintain a healthy morphology throughout the duration of culture provides evidence of the osteoconductive qualities of L-MDF.

The need for versatile handling has led to the addition of various inert carriers in commercial DBMs. However, studies have shown that carriers may negatively affect the inherent properties of a DBM. In particular, Lee et al. concluded that Poloxamer 407-based hydrogel may inhibit MSC osteoblastic differentiation by filling up spaces between DBM powders, negatively affecting the release of growth factors [21]. In a rat calvarial defect model, investigators found that the two types of DBM had significant differences in bone regeneration, which was attributed to the type of carrier [27]. Furthermore, varying the ratio of carrier to DBM can alter handling characteristics such as malleability and resistance to graft migration. Through *in vivo* and *in vitro* analyses, studies have found that increased bone content in DBMs produces larger amounts of new bone formation [25, 28, 29]. With this is mind, L-MDF were produced by proprietary CNC-milling cortical bone to create specially designed rough surfaces allowing fibers to interlock, allowing this bone void filler to be carrier-free. The roughness also provides numerous attachment points for the cells and their lamellipodia, encouraging a flattened morphology. These interlocking fibers thereby encourage malleability, graft placement in the implant site, and resistance to irrigation, all of which represent ideal handling characteristics.

Finally, terminal sterilization is a processing measure used to ensure the safety of DBMs by reducing the risk of disease transmission. This is in contrast to aseptic processing alone, which introduces no additional bioburden from the environment but alone does not guarantee sterile tissue [30, 31]. Unlike aseptic-only processed tissue, terminal sterilization can result in a graft with a defined sterility assurance level (SAL). For example, an SAL of 10^{-6} indicates a 1 out of 1,000,000 chance that a viable organism exists within any single graft [31]. Although gamma irradiation is currently the most common method for terminally sterilizing allografts, some reports suggests that gamma irradiation can negatively impact the inherent properties of DBMs. There are several factors to consider when evaluating the effects of gamma irradiation on DBMs such as dose and temperature. Irradiation performed in a high dose range or at uncontrolled temperatures can result in denaturing of the osteoinductive signaling proteins, rendering them inactive, and/or structural damage to the collagen matrix due to generation of reactive oxygen species. Weintroub and Reddi evaluated DBM samples which were irradiated on ice at varying doses [32]. Histologic analysis showed DBM irradiated at 0.5–2.5 Mrad were similar to the non-irradiated control, indicating no effect on the induction properties of the implant. In another study, investigators found that DBM irradiated on dry ice (−72°C) demonstrated new bone formation comparable to non-irradiated samples [33]. These results demonstrate DBMs irradiated at low dose and low temperatures are expected to retain properties important to clinical performance. Thus, L-MDF are terminally sterilized to an SAL of 10^{-6} using low-dose, ultra-low temperature gamma irradiation to avoid negative impacts to the osteoinductive and osteoconductive potential, as verified by the results presented here.

5. Conclusion

L-MDF were engineered with the ideal characteristics of a DBM in mind. The cortical bone fibers are demineralized to target optimal levels of residual calcium to yield tissue with osteoinductive potential, and also terminally sterilized to minimize the risk of disease transmission. The results presented here demonstrate that L-MDF exhibit the osteoinductive potential and osteoconductive properties desirable to promote bone formation while also being easy to handle for surgical procedures. These characteristics suggest that L-MDF are a suitable option to treat bone defects in a number of orthopedic, spinal, trauma, craniomaxillofacial, and dental applications.

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Conflicts of interest

JBM, NC, PS, and MM are employees of LifeNet Health, a nonprofit organization.

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