Osteoconductive and Osteoinductive Properties of Kore Fiber™

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INTRODUCTION AND BACKGROUND

Demineralized bone grafts derived from allogeneic bone are often utilized to treat bony defects. The regenerative efficacy of these materials is believed to come from the presence of endogenous growth factors such as bone morphogenetic proteins (BMPs) which give rise to the ability of the grafts to elicit and support new bone formation at the defect site. This phenomenon is a biologic response known as osteoinduction. In addition, if the allograft can provide a scaffold that supports cellular attachment, survival and osteogenic differentiation, the allograft is osteoconductive.

Kore Fiber (processed by MTF Biologics, Edison, NJ; distributed by Kolosis BIO, Salt Lake City, UT) is a 100% bone allograft that consists of demineralized cortical bone fibers. It has been processed to be moldable and putty-like, once hydrated in a fluid such as blood or saline. In order to minimize any negative impact on the biologic activity during processing, Kore Fiber is subjected only to gentle, aseptic chemical processing and does not undergo a terminal gamma irradiation sterilization process. The purpose of this study was to demonstrate that the inherent biologic properties of Kore Fiber have been preserved during processing. This study evaluated the cortical bone fibers for the presence of multiple growth factors known to be associated with new bone formation and for its osteoinductive potential in an athymic mouse model. The fibers are also evaluated for their ability to support cell attachment, proliferation and differentiation using an *in vitro* cell culture model.

MATERIALS AND METHODS

Growth Factor Characterization

In this study, demineralized cortical fibers, representative of those in Kore Fiber, were aseptically processed from the cortical bone of three distinct donors using proprietary methods. At the time of sampling, the tissue was placed in a fixative solution, and shipped to an external lab (IHC World, LLC) to be embedded, sectioned, and stained for a panel of growth factors known to be relevant to bone healing (BMP-2, BMP-7, PDGF-BB, FGF-1, FGF-2, IGF-1, TGF-β, VEGF). These growth factors all contribute to the bone repair process and the expression of these growth factors can vary at different points during the bone healing cascade. After IHC staining, imaging was done using conventional microscopy, the growth factors are visualized in the results section.

Cell Attachment Study

In this study, demineralized cortical fibers from two donors, similar to those in Kore Fiber, were aseptically processed. Cryopreserved human mesenchymal stem cells (hMSCs) provided by Lonza were cultured and seeded onto the fiber scaffolds at a density of 0.5 million cells per sample. The scaffolds were placed in Osteogenic Differentiation Media, with media changes occurring every 3-4 days. Samples were removed from the media for analysis at different time points throughout the study; cell count (performed at MTF Biologics R&D laboratories) and cell distribution (performed at the Rutgers Center for Confocal Imaging) were assessed using laser-scanning confocal microscopy at 24 hours, 7 days, 14 days, and 28 days. Cell differentiation, assessed using hematoxylin & eosin (H&E), Von Kossa, and Alizarin Red histological staining, was evaluated at 14, 28, and 42 days (performed at Yale Orthopaedic Histology and Histomorphometry Laboratory). The histological images were analyzed for matrix deposition that would indicate the hMSC's are differentiating into bone forming cells within the samples.

Athymic Mouse Study

The study design was based on testing 3 donor lots of samples of the Kore Fiber. Prior to implantation, fiber samples (N=8 for each lot) were hydrated in saline and then 25mg of wet fibers were transferred to a 1cc syringe. Next, samples were implanted bilaterally in the hamstring muscles of athymic mice at the testing lab (Apptec, St. Paul, MN). The hamstring muscle group (*biceps femoris* muscle) was used since it is a large, easily accessible muscle, which is commonly used as an implant site to evaluate heterotopic bone formation[1]. Animals were sacrificed at 28 days post-implantation. Decalcified histology was then performed on the explanted samples. Subsequently, slides were stained with hematoxylin and eosin, and tissue sections were evaluated for osteoinductivity.

The relative amount of osteoinduction was evaluated semi-quantitatively by the study investigators using the scoring system described below (Table 1), to be consistent with standard in the industry[1]. Osteoinductive scores were based on the degree to which new bone, bone cells, osteoid, calcified cartilage remnants, and marrow elements were present. The overall score for the test group was determined by averaging the scores from the samples. The results of semi-quantitative scoring are presented as a mean ± standard deviation. Images of histological slides from each test group were also captured and stored using a digital camera and computer system.

Score	Criteria
0	No evidence of new bone formation
1	1-25% of the section is covered by new bone
2	26%-50% of the section is covered by new bone

3	51%-75% of the section is covered by new bone
4	>75% of the section is covered by new bone

Table 1: Osteoinductivity Scoring Scale and Criteria

RESULTS

Growth Factor Characterization

Results of IHC staining were visualized in Figure 1 using conventional microscopy imaging. In these images, growth factor presence in demineralized cortical fibers is represented by brown coloration, which can be seen throughout the fibers. A description of the different growth factors that were evaluated in this study as well as whether they were present in the fibers is seen in Table 2 below. Representative images are below in Figure 1.

Growth Factor	Role in Bone Healing Cascade	Present
BMP-2	Differentiation of MSCs into osteoprogenitor cells, chondrocytes and osteoblasts	✓
BMP-7	Differentiation of osteoprogenitor cells into osteoblasts	✓
PDGF-BB	Mitogenic for MSCs and osteoblasts and responsible for macrophage chemotaxis	✓
FGF-1	Mitogenic for MSC, chondrocytes, and osteoblasts. Promotes vascularization	✓
IGF-1	Promotes proliferation and differentiation of osteoprogenitor cells	✓
TGF-β	Pleiotropic growth factor responsible for simulation of undifferentiated MSCs	✓
VEGF	Promotes migration and proliferation of osteoblasts. Promotes angiogenesis	✓

Table 2: Presence of growth factors in Kore Fiber.

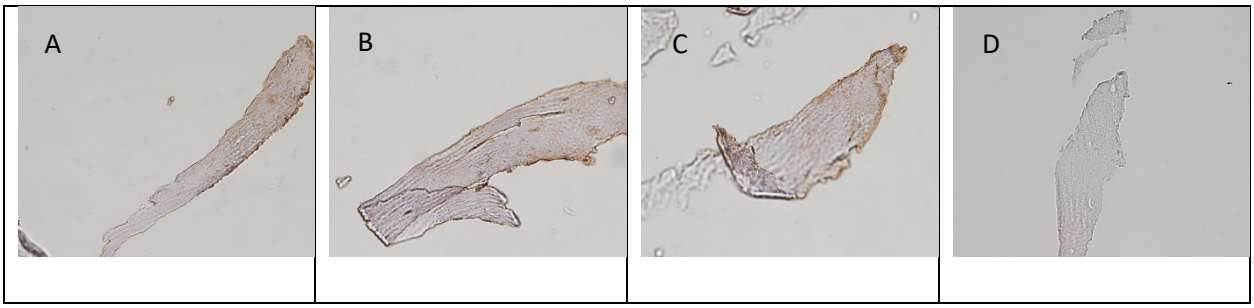

Figure 1: Representative immunohistochemical staining images of endogenous growth factors in Kore Fiber. Brown coloration indicates the presence of the targeted growth factor, BMP-2 (A), BMP-7 (B), PDGF-BB (C), and a Negative Control (D).

Cell attachment study

For both donors, there was a high degree of cell attachment and the cells that attached remained attached over time, as seen in the cell distribution images (Figure 2). For the cell distribution portion of the study, cells, dyed in green, are visible along the scaffold, dyed in red at each time point. The cell density increases from 24 hours to 7 days, and appears to maintain a high cell density at 28 days. A representative set of images from one of the donors can be seen in Figure 2.

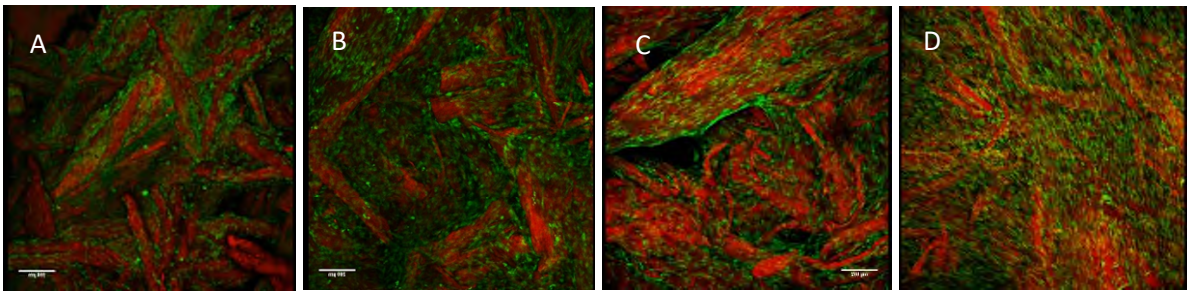

Figure 2: Time sequential confocal images of hMSCs seeded onto Kore Fiber that were obtained during the first day of culture. The cells were fluorescently stained green using CellTracker™ Green CMFDA while the scaffolds were stained red using Alexa Fluor 633. The time points were 24 hours (A), 7 days (B), 14 days (C), and 28 days (D).

For the cell differentiation portion of the study, adherent cells were observed on the surface of both donors at each timepoint for the H&E staining. At the day 28 and day 42 timepoints, new extracellular matrix was visible near the surface of the scaffold, where cells were present. For both test articles, positive Von Kossa and Alizarin Red staining were observed at days 28 and 42. Areas of positive staining

correlated between the two stains, confirming the presence of calcium in positively stained areas. Representative pictures of each of the staining types can be seen in Figure 3 and Figure 4.

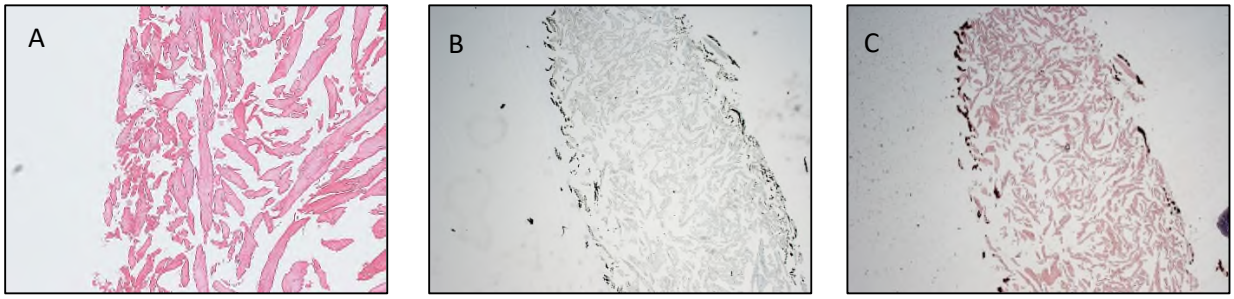

Figure 3: Histological image of hMSCs seeded onto Kore Fiber at 28 days after cell seeding. Sections were stained with hemotoxylin and eosin (A), Von Kossa (B), and Alizarin Red (C). Histological images of the von Kossa staining of sections (black color) and the Alizarin Red staining (dark red color) depict evidence of mineralization through the deposition of calcium phosphate by differentiated hMSCs on the fibers.

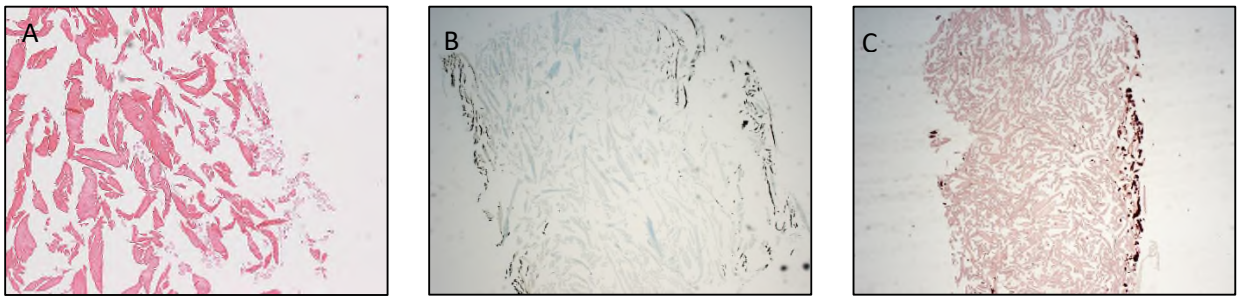

Figure 4: *Histological image of hMSCs seeded onto Kore Fiber at 42 days after cell seeding. Sections were stained with hemotoxylin and eosin (A), Von Kossa (B), and Alizarin Red (C). Histological images of the von Kossa staining of sections (black color) and the Alizarin Red staining (dark red color) depict evidence of mineralization through the deposition of calcium phosphate by differentiated hMSCs on the fibers.*

Athymic Mouse Model

Following explantation after 28 days, samples from all 3 lots of samples that represent the Kore Fiber were found to show evidence of OI during histological examination. Histological findings included the observation of newly formed bone that bridged particles of original bone and the formation of marrow elements (Figure 5). Fiber samples were also noted to be consistently osteoinductive in this model with 100% of the samples exhibiting evidence of osteoinduction (Table 2). In addition, an average OI score of 1.79 ± 0.73 was determined.

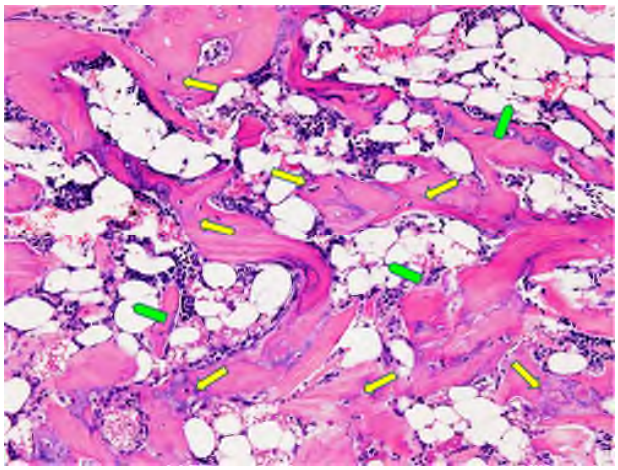

Figure 5: Explant of Kore Fiber demonstrating multiple regions of new bone formation (yellow arrows) and the presence of bone marrow (green arrows). H&E stain; 200X magnification.

Summary Statistics	Osteoinduction Score (0-4 Scale)		# Ranked Sample	Percentage of Osteoinductive Samples
	Mean	Std Dev		
Kore Fiber	1.79	0.73	24	100% (24/24)

Table 2: Osteoinductivity results including OI scores and the percentage of Kore Fiber samples found to be osteoinductive.

CONCLUSIONS

The efficacy of demineralized bone allografts has been previously shown to vary widely from samples that originate from one tissue processor to another[2]. While inherent donor variability may contribute to these differences, it has been recognized that tissue processing methods can significantly impact the

osteoinductive potential of demineralized allograft bone[3, 4]. Kore Fiber is a moldable cortical bone fiber allograft that is processed in a careful aseptic manner without extended exposure to harsh chemical reagents or being subjected to terminal sterilization by gamma irradiation in order to maintain its biologic activity.

Overall, the results generated by the characterization studies indicate that the inherent osteoconductive and osteoinductive properties of Kore Fiber are well-preserved following processing. Using immunohistochemical staining, an array of growth factors related to bone healing were found to be present in the fiber samples. When human MSCs were added to fibers, cells readily attached, and evidence of osteogenic differentiation was observed following culture in osteogenic medium. In addition, samples of Kore Fiber consistently elicited new bone formation in an ectopic site using the athymic mouse model. In particular, all 24 samples from 3 donor lots demonstrated osteoinduction when explants were assessed histologically. Based on the collective findings from the pre-clinical studies, Kore Fiber provide a cell-friendly environment along with preserved endogenous osteoinductive signals that can act as a suitable scaffold for bone repair.

REFERENCES

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2. DePaula CA, Truncale KG, Gertzman AA, Sunwoo MH, Dunn MG (2005). Effects of hydrogen peroxide cleaning procedures on bone graft Osteoinductivty and mechanical properties. *Cell and Tissue Banking, 6,* 287-298.

3. MTF, Data on file.

4. Takikawa S, Bauer TW, Kambic H, Togawa D (2003). Comparative evaluation of the osteoinductivity two formulations of human demineralized bone matrix. *J Biomed Mater Res A*, *65*(1), 37-42.