

# Novel Application of HA-TCP Biomaterials in Distraction Osteogenesis Shortened the Lengthening Time and Promoted Bone Consolidation

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**ABSTRACT:** This study tested the hypothesis that use of biomaterials in distraction osteogenesis (DO) would reduce the treatment time and enhance bone formation quality. A 1.0-cm tibial shaft was removed in the left tibia of 36 rabbits. Rabbits were randomly divided into three groups: group A, the defect gap was reduced with the tibia shortened for 1.0-cm; group B, the defect gap was filled with 1.0-cm restorable porous hydroxyapatite and Tri-calcium phosphates cylindrical block (HA/TCP block, diameter is 0.5-cm); group C, The 1.0-cm defect gap was reduced 0.5 cm and the remaining 0.5-cm defect gap was filled with the 0.5-cm HA/TCP block. The tibia was then fixed with unilateral lengthener; for groups A and C; lengthening started 7 days after surgery at a rate of 1.0 mm/day, in two steps. Group A received lengthening for 10 days and group C for 5 days, there was no lengthening for group B. All animals were terminated at day 37 following surgery. The excised bone specimens were subject to microcomputed tomography (micro-CT), mechanical testing, and histological examinations. Bone mineral density and content and tissue mineral density and content, as well as the mechanical properties of the regenerates were significantly higher in group C compared to groups A and B. Micro-CT and histological examinations also confirmed that the regenerates in Group C had most advanced bone formation, consolidation, and remodeling compared to other groups. In conclusion, the combined use of biomaterials and DO technique can reduce the treatment time and enhance bone consolidation in bone defect management. © 2008 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. *J Orthop Res* 27:477–482, 2009

**Keywords:** distraction osteogenesis; hydroxyapatite; TCP; bone defect; rabbit

Distraction osteogenesis (DO) is induction of osteogenesis by means of an osteotomy, followed by fixation with an external fixator and subsequent controlled gradual lengthening.<sup>1</sup> During DO, new bone is formed rapidly and is accompanied with advanced angiogenesis and tissue remodeling.<sup>2–4</sup> The DO technique has been perfected by Ilizarov and others<sup>5–8</sup> over the last 2 decades, and it has been widely applied in the managements of bone defects, limb deformities, and fracture nonunions. However, for the management of larger bone defects caused by trauma, bone infection, and bone tumors, the long duration associated with DO treatment and the bone consolidation phase can cause considerable morbidity for the patients, such as refracture, nonunion, infection of pin hole, etc;<sup>7,9–11</sup> and in clinical practice, there is a need for shortening the treatment time of DO and augmentation of bone consolidation during DO. Various methods for promoting bone consolidation during DO treatment have been studied, such as using physical stimulations, ultrasound, surgical techniques, grow factors, and stem cells,<sup>11–24</sup> but these treatments are not always successful, and require higher cost and additional procedures/facilities/equipment, that may not readily available.<sup>25</sup>

In orthopedics and traumatology a variety of biomaterials has been developed and widely applied in spinal fusion, bone-defect management, and skeletal tissue engineering, with many satisfactory clinical outcomes. Hydroxyapatite (HA) and Tri-calcium phosphates (TCP)

are osteoconductive, porous HA–TCP biomaterials that have similar composition, structure, and characteristics as native bone with good biocompatibility.<sup>26</sup> HA–TCP-based biomaterials can guide bone tissue growth, facilitate bone formation or consolidation, and have been used clinically as bone substitutes.<sup>26,27</sup>

In view of the rapid bone formation and vascularization associated with DO and the readily available biomaterials, we hypothesized that combining the use of HA/TCP-based biomaterials and DO technique would greatly reduce the lengthening time and enhance bone formation quality in bone defect management.

## MATERIALS AND METHODS

### Animal Model of Distraction Osteogenesis and Experimental Groups

All animal experimental procedures were performed under the guidelines for animal scientific procedures approved by the host institution's ethical committee and carried out in the General Hospital of Chinese People's Liberation Army, Beijing, P.R. China. Midtibial osteotomies were performed in 36 adult male NZW rabbits (age 20–24 weeks, body weight 2.3–2.8 kg), with the tibiae stabilized by external fixators. In brief, under general anesthesia osteotomy was made by a hand saw in the left tibia below the tibiofibular junction, and a 1.0-cm tibial shaft was removed through a second osteotomy below the first one, with the tibiae stabilized with external fixator (Orthofix M100, Orthofix, Italy). The Orthofix M-100 fixators were as previously described.<sup>23,24</sup> The animals were randomly divided into three groups (Table 1) as: group A: the 1.0-cm defect gap was immediately reduced, with the tibia shortened for 1.0-cm and fixed with a unilateral external fixator. Lengthening started 7 days after the osteotomy at a rate of 1.0 mm/day, in two steps, for 10 days. Once the lengthening (1.0 cm) was achieved, the regenerate was allowed to

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**Table 1.** Animal Experimental Group Details

Parameters	Group A	Group B	Group C
Defect created (cm)	1.0	1.0	1.0
Defected reduced (cm)	N/A	1.0	0.5
HA/TCP block used (cm)	N/A	1.0	0.5
Latency period (day)	7	N/A	7
Lengthening period (day)	10	N/A	5
Total lengthening (cm)	1.0	N/A	0.5
Consolidation period (day)	20	37	25
Total time at termination (day)	37	37	37

HA = hydroxyapatite; TCP = tri = calcium phosphates.

consolidate for further 20 days. Group B: the 1.0-cm defect gap was immediately filled with a 1.0-cm (length)  $\times$  0.5-cm (diameter) restorable porous hydroxyapatite/tri-calcium phosphates (HA/TCP) cylindrical block (OsteoStim<sup>TM</sup>, Millenium Biologix Inc, Kingston, Ontario, Canada) and the bone was stabilized by the unilateral fixator. OsteoStim<sup>TM</sup> materials contain multiphase composition, consisting of approximately 70% Silicon-TCP and 30% HA/TCP, with more than 70% porosity. There was no lengthening in this group and animals were left untreated for 37 days. Group C: the 1.0-cm defect gap was immediately reduced to 0.5-cm, with tibia shortened for 0.5-cm and the remaining 0.5-cm defect gap was filled with a 0.5-cm HA/TCP cylindrical block and held in position with the unilateral external fixator. Lengthening started 7 days after the osteotomy, at a rate of 1.0 mm/day, in two steps, for 5 days. Once the lengthening (0.5 cm) was achieved, the regenerate was allowed to consolidate for further 25 days. All animals were terminated at day 37 following initial surgery.

#### Radiographic Examination

Serial radiographs were taken at the day of surgery (day 0), day 12, day 17, day 27, and day 37 (termination point) after surgery, using a C-arm fluoroscopic machine. The exposure condition was 32 kV, 10 ms at 1 $\times$  magnification.

#### Microcomputed Tomography (Micro-CT) Imaging Examination

Specimens of the experimental tibiae including the bone defect gap and 0.5-cm adjacent intact bone at both ends were subject to micro-CT examinations using a micro-CT system (GE Healthcare Explore Locus; MicroView v2.1 software ABA, UK) before processing for mechanical testing. For scanning, samples were placed in a sample holder with specimen's flat anterior surface facing downward. This sample orientation allows for longitudinal scanning of the single specimen. A control file, or measurement protocol was created to define scanning parameters such as source energy, sample size, and image resolution. Parameters selected for this study included a source voltage of 80 kV and 450 mA. Sample measurements (scans) were performed on the samples using high resolution settings. The regenerate bone in the 1-cm distraction gap was chosen as the region of interest (Voxel size: 0.041  $\times$  0.041  $\times$  0.041, ROI type: box). The 3D reconstruction of the distraction gap was performed by 45  $\mu$ m-resolution using the software provided. For data analysis, the following parameters were used to generate reports: mean volumetric bone mineral density (BMD), bone mineral content (BMC), tissue mineral content (TMC), bone volume (BV), tissue volume (TV),

trabeculae thickness (Calib.Tb.Th.3D), and trabeculae space (Calib.Tb.Sp.3D).

#### Mechanical Testing

Mechanical testing was performed on samples after the micro-CT examination. Torsional test was performed using a MTS 858 Mini BionixR11 test machine at room temperature (22°C). The rotation angle was applied at a rate of 4.5 deg/s and the torque and angular displacement was recorded by the builtin devices. The biomechanical properties of specimens were determined by the maximum torque, torsional stiffness, and the maximum angular displacement required for failure of the specimen.

#### Histological Examination

The samples were fixed in 10% buffered formalin for 48 h and decalcified at 4°C over a period of 4 weeks in 14% EDTA in 0.1 M Tris-HCl buffer, pH 7.2. All samples were then processed through graded alcohols, xylene, and embedded longitudinally (on their coronal plane) in paraffin wax. Sections (7  $\mu$ m) were cut at and stained with hematoxylin and eosin (HE) and Alcian blue/Sirius red. For Alcian blue/Sirius red staining, after deparaffin, rehydration, nuclear staining with Weigert's hematoxylin, sections were stained with Alcian blue 8GX (0.1% in 1% acetic acid) and Sirius red F3B (1% in saturated picric acid). Alcian blue stains the proteoglycan-rich cartilage matrix blue, while Sirius red stains the type I collagen fibril red.

#### Statistical Analysis

All quantitative data from micro-CT examination and mechanical testing were transferred to spreadsheets and analyzed using a commercially available statistical program SAS (Version 9.0, SAS Institute, Cary, NC). Data from each group were first tested by one-way analysis of variance (ANOVA) to identify significant difference between the groups. If a significant difference ( $p < 0.05$ ) is found, multiple comparisons were then performed using an SNK (Student-Newman-Keuls) test. Differences were considered significant at  $p < 0.05$  in all tests.

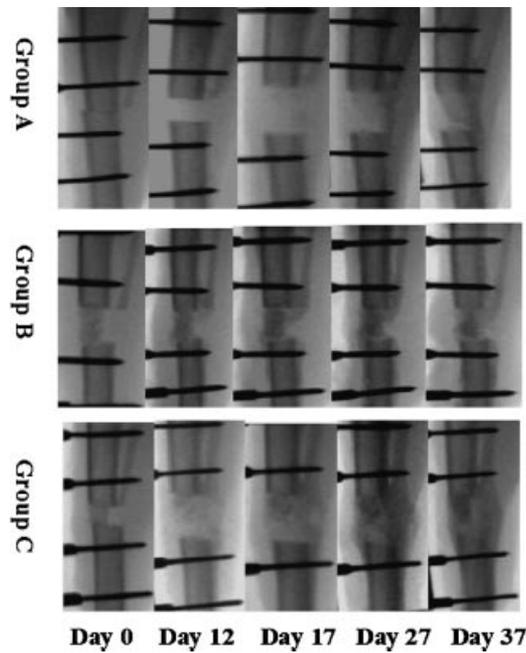
## RESULTS

### General Observations

At the first 2 weeks postoperation, all the skin incision wounds healed with no sign of pin site infection, swelling, erythema, or suppuration. During the last 3 weeks postoperation, no apparent difference was noticed in animals in all groups. The experimental details for the three groups are summarized in Table 1.

### Radiographic Evaluations of the Regenerates

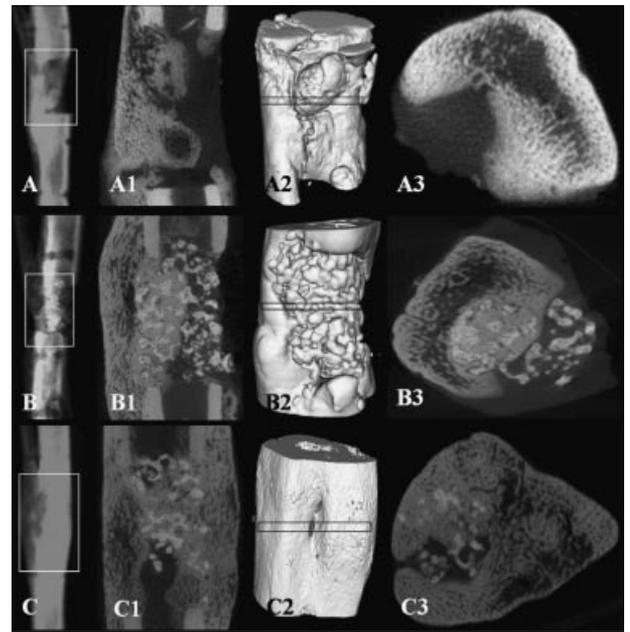
The average (mean  $\pm$  SD) lengthening of all the animals was 9.8  $\pm$  0.4 mm (Fig. 1). At day 27 and day 37 postsurgery, radiographs show that there was more bone formation in the group C (Fig. 2C) than that in groups A and B (Fig. 1 and Fig. 2A and B). Radiolucent regions representing focal defects in the distraction regenerates were frequently seen in group A (Fig. 1 and Fig. 2A and B).



**Figure 1.** Representative radiographs of all experimental groups at day 0, day 12, day 17, day 27, and day 37 postsurgery. Day 0: the 1.0-cm defect gap was reduced in the group A; The 1.0-cm defect gap was filled with 1.0-cm restorable porous HA/TCP cylindrical block in the group B; the 1.0-cm defect gap was reduced with the tibia shortened for 0.5 cm, and the remaining 0.5-cm defect gap was filled with 0.5-cm restorable porous HA/TCP cylindrical block in the group C. Day 12: groups A and C had finished 0.5-cm lengthening. Group C ended its lengthening and the HA/TCP block started disappearing. Group A still had 0.5-cm lengthening to go on. Group B had little increase in callus formation compared to day 0. Day 17: group A had finished lengthening, and there was little sign of callus formation in the gap. There was an increase in callus formation in groups B and C compared to day 12, and group C had more callus formation than group B. Day 27: there was a significant increase in callus formation in all three groups compared to day 17. Groups B and C had more callus formation than group A, and group C had more bone formation than group B. Day 37: all animals were terminated at this point. There was a significant increase in callus formation in all three groups compared to day 27. Bone defects were still visible in group A; the HA/TCP block was clearly evident in group B. The radiographic signs of cortical continuity was only seen in group C, which had the best bone quality compared to groups A and B.

#### Micro-CT Images and Data

Micro-CT images of the distraction gap tissues demonstrated enhanced bone formation in group C (where there was no focal bone defects; Fig. 2C1) compared to groups A and B (whereas focal bone defects were frequently seen; Fig. 2A1, 2B1). The newly formed bone was more evenly distributed across the distraction gap in group C (Fig. 2C1, 2C3) compared to groups A and B (Fig. 2A1, 2A3, and 2B1, 2B3). In group A, the newly formed bone in the distraction gap (Fig. 2A2) was less even and smooth comparing to the bone in group C (Fig. 2C2). In group B, HA/TCP biomaterials were still clearly visible (Fig. 2B2, 2B3). In group C, the callus was evenly formed across the gap with smooth shape of morphology, showing that bone remodeling was proceeding (Fig. 2C, 2C1–3); however, in group A, larger focal defects were frequently seen and the new bone formation was poor in the medial–anterior side of the regenerate (Fig. 2A, and 2A1–3).



**Figure 2.** Representative micro-CT images of the entire regenerates were shown. The far left panel A–B–C shows the representative radiographs of the regenerates of the three groups at day 37 postsurgery. The panel A1–B1–C1 shows the longitudinal CT scans of the corresponding specimens in the A–B–C panel. The panel A2–B2–C2 was 3D micro-CT reconstruction images of the same specimens as panel A1–B1–C1. The panel A3–B3–C3 was the corresponding cross-sectional images of the boxed areas in panel A2–B2–C2. The bone formation in group A was poor at this stage, with frequent focal defects and uneven bone formation (A, A1, A2, A3). The newly formed bone in group B was less well organized and uneven compared to group C, and the HA/TCP block was clearly evident in the gap (B, B1, B2, B3). The quality of newly formed bone was superior in group C as the callus was evenly distributed with signs of bone remodeling and new corticalization, the HA/TCP block has been almost resorbed (C, C1, C2, Cs).

The quantitative micro-CT measurement results are summarized in Table 2. The mean volumetric bone mineral density (BMD) of the regenerates was significantly higher in groups B and C compared to group A ( $p < 0.0001$ ). There was no significant difference in the mean BMD between groups B and C. The mean volumetric BMC was significantly higher in group C than that in groups A and B ( $p = 0.0002$ ), and there was no difference between groups A and B. There was significant difference in the mean volumetric TMD among the three groups ( $p < 0.0001$ ); group B was the highest and group C was higher than that of group A. The mean volumetric TMC was significantly higher in group C than groups A and B ( $p = 0.0002$ ); and there was no difference between groups A and B. The BV/TV ratio was higher in group C compared to group A ( $p < 0.05$ ), and there was no difference between groups A and B. For Calib.Tb.Th.3D and Calib.Tb.Sp.3D data, there was no significant difference among the three groups.

#### Mechanical Testing Results

All mechanical testing results are also summarized in Table 2. There was no significant difference in the maximum rotation angle for failure among the three

**Table 2.** The Micro-CT and Torsional Test Data

Parameters	Group A	Group B	Group C
BMD (mg/cc)	199.93 ± 46.62	346.57 ± 39.46 <sup>a</sup>	336.63 ± 23.30 <sup>a</sup>
BMC (mg)	279.47 ± 37.83	309.15 ± 35.06	454.44 ± 89.98 <sup>b</sup>
TMD (mg/cc)	566.60 ± 54.90	875.29 ± 96.98 <sup>c</sup>	760.96 ± 79.76 <sup>a</sup>
TMC (mg)	279.42 ± 37.84	309.11 ± 35.06	454.40 ± 89.97 <sup>b</sup>
BV/TV	0.354 ± 0.084	0.407 ± 0.069	0.451 ± 0.036 <sup>a</sup>
Calib.Tb.Th.3D (mm)	0.91 ± 0.51	0.64 ± 0.21	1.03 ± 0.38
Calib.Tb.Sp.3D (mm)	4.37 ± 1.17	4.36 ± 0.94	4.78 ± 0.56
Max rotation angle (deg)	-9.04 ± 6.18	-5.79 ± 3.42	-7.51 ± 2.02
Maximum torque (N-m)	-0.70 ± 0.25	-1.05 ± 0.27	-2.39 ± 0.44 <sup>b</sup>
Torsion stiffness (N-m/deg)	0.17 ± 0.13	0.37 ± 0.23 <sup>a</sup>	0.46 ± 0.12 <sup>a</sup>

BMC = bone mineral density; BMC = bone mineral content; TMD = tissue mineral density; TMC = tissue mineral content; BV = bone volume; TV = tissue volume.

<sup>a</sup>The value was significantly higher when compared to group A ( $p < 0.0001$ ).

<sup>b</sup>The value was significantly higher when compared to groups A and B ( $p = 0.0002$ ).

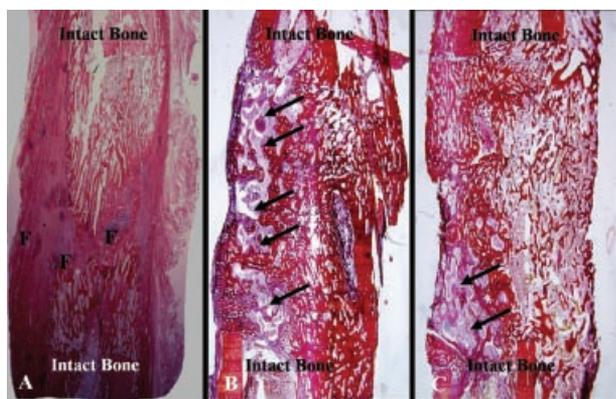
<sup>c</sup>The value was significantly higher when compared to groups A and C ( $p < 0.0001$ ).

groups. The maximum torque was significantly higher in group C compared to groups A and B ( $p < 0.05$ ), but there was no significant difference between groups A and B. For the torsional stiffness, group C was significantly higher than that of group A ( $p < 0.05$ ), but no difference was found between groups B and C.

### Histology Results

At day 37 following surgery, the regenerates in group A was still not fully consolidated with fibrous and cartilaginous tissues in the middle of the regenerate (Fig. 3A); whereas groups B and C mainly consisted of woven bone (Fig. 3B and C). In group B, the new bone was formed on the biomaterials similar to fracture healing (Fig. 3B); but in group C, the new bone was formed in parallel to the distraction forces, with signs of callus remodeling (Fig. 3C). In group A, there were

mixtures of fibrous, cartilaginous, and bony tissues in all of the regenerates; even in the regenerates that were considered consolidated on X-ray, focal defects with fibrous and cartilaginous tissues were frequently seen (Fig. 3A). In group B, the gaps had various degrees bone formation with the HA/TCP remains clearly seen, the callus formation was less even (Fig. 3B). In group C, all the regenerates had shown advanced consolidation, and the callus was formed evenly in the distraction gap. There were small areas of HA/TCP residues in the regenerates, but no cartilaginous or fibrous tissues were seen (Fig. 3C). In comparison, bone consolidation and remodeling was most advanced in group C as the regenerates were completely united with well-organized woven bone, whereas new callus formation was still going on with little sign of bone remodeling in group B and in group A fibrous and cartilaginous tissues were still visible.



**Figure 3.** Representatives of histological sections of the regenerates from the three experimental groups are shown. (A) Group A, showing that fibrous (F) and cartilaginous tissues were still present in the centre of the regenerate. (B) Group B, the regenerate mainly consisted of newly formed woven bone, the bone formation was induced by the HA/TCP block, which was not evenly distributed among the gap, and a larger amount of HA/TCP blocks were still visible (arrows). (C) Group C, the newly formed callus was in parallel to the distraction force and evenly distributed throughout the gap; there were signs of callus remodeling and small areas of HA/TCP residues (arrow). Sirius red/Alcian blue staining;  $\times 4$  original magnification.

### DISCUSSION

In clinical practice, for small bone defect (<30% of the total limb length), internal fixation, or external fixation combined with autologous bone or artificial bone grafts (similar to the method we studied in group B) is the first choice treatment.<sup>7</sup> Larger bone defect (>30% of the total limb length), distraction osteogenesis technique like group A of our study will be employed.<sup>11</sup> During DO, bone formation occurs in parallel with the tension force, leading to rapid bone formation and remodeling.<sup>2-4</sup> The DO technique has been widely used clinically, such as limb reconstruction following bone loss caused by trauma, infection, and tumor resection.<sup>1,2,7,8,11</sup> However, for larger extent lengthening it requires a longer time, as the lengthening rate is around 0.5–1 mm/day and a lengthy period of external fixation is usually needed to allow the newly formed bone to consolidate, during which period complications such as refracture or nonunion of the regenerate at the distraction gap are often seen.<sup>8-10</sup> To reduce the risk of complication, we need to reduce the lengthening gap, and therefore,

we hypothesized that we could use synthetic biomaterials like hydroxyapatite to fill some of the bone defect and reduce the distance needed for lengthening, followed by distraction osteogenesis to promote rapid bone formation, angiogenesis, and tissue remodeling. The key point of this study was to prove the concept that the combined use of HA/TCP biomaterials and distraction osteogenesis technique would reduce the treatment time and enhance bone consolidation compared to the established treatment methods, such as using DO technique or biomaterials alone. We used a rabbit model of 1-cm tibial bone defect to test the hypothesis; although this model is not a critical bone defect model, we want to use this model to prove our concept first before moving to use larger bone defect model (>30% of the total limb length) for further research.

As the radiographs, micro-CT and histology data showed the quality of the regenerate in group C (combined treatment) was much better quantitatively and qualitatively comparing to group A and B (single treatment). Focal defects (fibrous and cartilaginous tissues) in the regenerates were frequently seen in group A; this group had 20 days for consolidation following distraction, and it was clearly not enough time for the bone to fully consolidate. In group B, the bone formation was evident as the radiodensity of the HA/TCP block gradually increased over time; the quality of bone formation was acceptable, as this group had a total 37 days for bone formation. It is expected that this group should heal as the current management is sufficient to allow the 1-cm defect to heal in the rabbits. In group C, where animals had used HA/TCP blocks and DO together, and the bone formation quality was more superior than that of groups B and A. In group C, callus formation and partial bone union was seen as early as at day 17; and at day 27 the density of the regenerates further increased with radiographic signs of cortical continuity; and at day 37, the regenerates began remodeling. It is worth mentioning that group C had 25 days for bone consolidation, which was only 5 days longer than group A, but 12 days shorter than group B.

It is not surprising to find that the mean volumetric BMD and TMD of the regenerates were significantly higher in groups B and C when compared to group A, as both groups B and C had an HA/TCP block implanted, which itself has relatively higher mineral density. However, the volumetric BMC value in group C was significantly higher than those of groups A and B; indicating that there were more bone formation in group C than the other groups. Group C had only a half size HA/TCP block as group B implanted, yet had significant higher BMD than group B, indicating that the speed and amount of new bone formation in group C was the fastest and biggest among the three groups. It also indicated that the combined treatment had promoted bone consolidation of the regenerates, because there was no difference in trabecular bone thickness and space among the three groups, suggesting that the bone microstruc-

ture of the regenerates was similar among the three groups.

The torsional test is the golden standard for determining the functional quality of the regenerates. Group C had the higher maximum torque than that of groups A and B; the torsional stiffness of groups B and C was significantly higher than that of group A; suggesting that in the current study the regenerate quality was in the following order: Group C > group B > group A. The histology results demonstrated that the quality of the regenerates with the HA/TCP block as bridging materials in groups B and C was more superior than that in group A. In comparison, bone consolidation and remodeling was most advanced in group C, followed by group B and group A. In agreement with our finding, Watanabe et al. have recently reported that bone transport using HA loaded with rhBMP-2 was capable of regenerating living bone tissue, and they concluded that bone transport with devitalised bone and bone substitute with growth factors may be used for bone defect management.<sup>28</sup>

There were some variables, such as the bone lengthening length difference and time difference in lengthening and bone consolidation period among the experimental groups, especially in groups A and C. When designing the experiments, we have considered the time difference factors, which is closely linked to the total amount of bone lengthening required, for example, small lengthening requires less time and larger lengthening requires a longer time. However, the current study compared three treatment methods for managing bone defect: (1) bone lengthening, (2) biomaterial grafting, and (3) combined use of biomaterial and bone-lengthening techniques. We decided to mimic the clinical situation, that is, to keep the overall treatment time for all the experimental groups the same (37 days) for the same amount of bone defect (1 cm), regardless of the other experimental variables such as latency period or consolidation period in each group, as these variables are interlinked, and in clinical management the total time needed for the treatment is the most important end point. Hence, we have used the same total treatment time for all the groups (37 days) and compared the quality of bone formation with the three different managements in the same time frame. The data clearly demonstrated that the combined use of biomaterial with a bone-lengthening technique has reduced the bone-lengthening time required and increased the bone consolidation time, and significantly improved the overall bone formation quality (group C) for the same amount of bone defect within the same time period.

There are limitations in this study: the model we have used is not a critical bone defect model, and it has a narrow window to study the difference between the groups. However, this is a proof-of-concept study, and the data supported our hypothesis. Further investigations using a cortical bone defect model is undergoing and to test this novel bone defect management in larger animals before a clinical trial.

In summary, we have demonstrated that the combination of biomaterials with a distraction osteogenesis technique could be a new and cost-effective means to reduce the treatment time and enhance bone consolidation in the management of larger bone defect(s). The newly described technique in this study, which is essentially the same as the DO technique combined with an acute reduction of the bone defect gap by appropriate biomaterials, may be applied clinically for the management of larger bone defects caused by trauma, bone infection, and bone tumors.

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