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BASIC SCIENCE

Low-Intensity Pulsed Ultrasound Enhances Posterior Spinal Fusion Implanted with Mesenchymal Stem Cells-Calcium Phosphate Composite Without Bone Grafting

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Study Design. Experimental study on the effect of low-intensity pulsed ultrasound (LIPUS) on rabbit spinal fusion with mesenchymal stem cell (MSC)-derived osteogenic cells and bioceramic composite.

Objective. To investigate the efficacy of LIPUS in enhancing fusion rate and bone formation with porous tricalcium phosphate (TCP) bioceramic scaffold impregnated with MSCs without any bone grafts.

Summary of Background Data. The goal of spinal fusion in the corrective spinal surgery for spinal deformities is to achieve solid bony fusion between selected vertebral segments. Previous studies with bone morphogenetic proteins and genetically manipulated materials revealed significant difficulties in actual clinical application. Alternative such as LIPUS has been shown to be effective in enhancing healing of fracture and nonunion clinically. Its potential for enhancing spinal fusion warrants further in-depth study.

Methods. Posterolateral intertransverse processes spinal fusion at the L5 and L6 levels were evaluated in New Zealand white rabbit model. The animals were divided into three groups with (A) TCP alone, (B) TCP with differentiated MSCs, and (C) TCP with differentiated MSCs and LIPUS treatment. At week 7 postoperation, manual palpation, peripheral quantitative computed tomography, and histomorphometric assessments were performed.

Results. At week 7 postoperation, a statistically significant increase in clinical fusion by manual palpation was observed in group C animals treated with LIPUS (86%) in comparing with groups A (0%) and B (14%) without LIPUS. With peripheral quantitative computed tomographic analysis, the bone volume of group C fusion mass was significantly larger than the other two groups. Group C fusion also had better osteointegration length between host bone and implanted composite and more new bone formed in the TCP implants. Importantly, all the group C animals had osteochondral bridging—early stage of bony fusion histologically. Endochondral ossification was observed at the junction between the cartilaginous and osseous tissues at the intertransverse processes area. Quantitative analysis showed that the fusion mass in group C had significantly smaller gap and larger area of cartilaginous tissue between the transverse processes.

Conclusion. The present study showed that the combination of synthetic biomaterials, autologous differentiated MSCs, and LIPUS could promote clinical fusion in rabbit posterior spinal fusion model. The mechanism was likely to be mediated through better osteointegration between the host bone and implanted materials and enhanced endochondral ossification at the fusion site.

Key words: posterior spinal fusion, mesenchymal stem cell, low-intensity pulsed ultrasound, bioceramics, osteochondral bridging.

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Corrective surgery with spinal fusion is commonly used in treating spinal deformity of different ages.[1,2] To enhance good bony fusion between vertebral segments, autologous bone grafting with decortication is the "gold standard" as it possesses both osteoconductive, osteoinductive, and osteogenic characteristics.[3] The standard spinal fusion procedure with autologous bone graft, however, has been reported to be associated with 5% to 35% nonunion rate, intraoperative blood loss, and residual morbidity at the donor sites in as many as 30% of the patients.[4,5]

The search for alternative substitutes aiming at achieving equivalent or better fusion rate than the "gold standard" with less morbidity has stimulated multiple research approaches. With the discovery of its powerful osteoinductive potential, bone morphogenetic proteins have received a great deal of attention.[6,7] However, for effective enhancement of lumbar fusion, high doses of bone morphogenetic proteins need to be used. To maintain a continuous supply of the bone morphogenetic proteins at the fusion site, gene therapy and genetically manipulated cells have been suggested.[8,9] Possible serious adverse effects such as excessive bone formation and potential

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encroachment of the spinal canal are always of great concern.[10–13] The safety of application of viral vector-mediated gene therapy fusion has added on to the uncertainty and raised serious doubts clinically.[14,15]

Recent development of application of osteogenic cell differentiated from bone marrow–derived mesenchymal stem cells (MSCs) for bone regeneration provided a new approach with great potential.[16] The source of MSCs can be derived from various tissues. It is possible to expand harvested autologous MSCs *in vitro* and hold them at the site of action with appropriate scaffold to enhance bone formation locally. The study by Matsushima *et al*[17] had shown that human MSCs cultured with porous calcium phosphate ceramics were prompted to proliferation and osteogenic differentiation. It has been found that large osteogenic cell population is required to achieve bony fusion.[18]

Low-intensity pulsed ultrasound (LIPUS) stimulation has been shown to enhance spinal fusion implanted with iliac crest autograft in animal models.[19–21] LIPUS has been used in clinical setting as an adjunctive therapy providing micromechanical stimulation to the target site, to accelerate bone healing of selected fresh fractures[22,23] and nonunions.[24,25] Recent studies have also shown that LIPUS could improve bone formation in distraction osteogenesis[26,27] and bone-tendon junction healing.[28] One of the advantages of LIPUS is that it can be delivered locally to the site of interest repeatedly without any known adverse side effect.

This study aimed to assess the efficacy of LIPUS on MSC-derived osteogenic cells impregnated on calcium phosphate bioceramic composite in enhancing spinal fusion in a standardized rabbit posterior spinal fusion model[6,29] without any bone grafts.

MATERIALS AND METHODS

Isolation of Bone Marrow–Derived MSCs

Bone marrow (15–20 mL) was aspirated from proximal femur, distal tibia, trochanter, and iliac crest of male New Zealand white rabbits (14 weeks) and collected in heparin tubes. After mixed with equal portion of Dulbecco's Modified Eagle Medium (Invitrogen, Carlsbad, NM), the bone marrow was centrifuged (1500 rpm, 10 min) for the collection of mononuclear cells at the bottom of the tube. The cells were seeded into 75 cm^2 plastic tissue culture flasks (Corning, Corning, New York) with Dulbecco's Modified Eagle Medium, 10% fetal bovine serum (Invitrogen), and 1% penicillin-streptomycin-neomycin solution (Invitrogen). Cells were then incubated at 37°C in a humidified atmosphere of 5% CO_2. The medium was changed twice a week. At 80% to 90% confluence, the isolated MSCs were trysinized with 0.25% Trypsin-EDTA solution (Gibco, Invitrogen, Vancouver, British Columbia, Canada) and subcultured into 150 cm^2 flasks (Corning) for further expansion. During the subculture, the cells were in Dulbecco's Modified Eagle Medium with basic fibroblast growth factor (2 ng/mL) and osteogenic supplements (5 mM ascorbic acid [Sigma, St Louis, Missouri], 10 mM β-glycophosphate [Sigma], and 10 nM dexamethasone [Sigma]) for 1 week to induce the MSCs to osteogenic linage.[30,31]

Preparation of MSC-Derived Osteogenic Cells-Tricalcium Phosphate Bioceramics Composite Implant

Five millions autologous MSCs were collected after expansion and loaded onto the β-tricalcium phosphate bioceramics (TCP; 6 × 8 × 30 mm; ChronOS, Synthes, Inc, West Chester, PA). The TCP was porous (60% porosity) with 200- to 400-μm functional pores.[32] Dulbecco's Modified Eagle Medium was then added carefully to 35-mm culture dish until the TCP block was covered with the medium. The cells-added TCP was incubated for another day before implantation.

Posterior Spinal Fusion Surgery

Grouping

The rabbits were randomly divided into three groups with different treatments. Group A used TCP alone, group B contained TCP and MSC-derived osteogenic cells, and group C consisted of TCP and MSC-derived osteogenic cells with LIPUS. The cells were added in an autologous manner.

Posterolateral Intertransverse Spinal Fusion Procedure

A widely used posterolateral intertransverse spinal fusion model in rabbits developed by Boden *et al*[29] for evaluating the effect of different types of bone grafts and substitutes on bony fusion was used. Briefly, rabbits were put under general anesthesia by intravenous low-dose pentobarbital (50 mg/kg/mL; Alfasan, Woerden, Holland). Through a dorsal incision, the transverse processes of the rabbit at L5 and L6 lumbar vertebra were exposed. The dorsal cortical layer of the transverse processes was decorticated with pneumatic bone burr down to the marrow with observable bleeding (Synthes, Mathys AG, Bettlach, Switzerland). The composite implants were put into direct contact with the decorticated transverse processes of L5 and L6. The muscles and skin were then closed in layers with resorbable suture.[6,33] No bone grafts were used in this study.

LIPUS TREATMENT

LIPUS Devices

LIPUS machine (Exogen 2000+, Smith & Nephew, Inc, Memphis, TN) was used to provide an ultrasound impulse at 1.5-MHz pulsed frequency, 200-μs pulse duration, and 1.0-kHz repetition rate. The power of the LIPUS devices was 30 ± 5.0 mW/cm^2 spatial average and temporal average incident intensity. The calibration of the LIPUS devices was done by the manufacturer.

Treatment Procedures

Transcutaneous LIPUS treatment was started on day 3 postoperatively (20 min/d; 5 d/wk for 7 weeks). Rabbits were put under general anesthesia with low-dose 10% ketamine (Alfasan) and 2% xylazine (Alfasan; 0.6 mL: 0.6 mL, v:v) during LIPUS treatment. The LIPUS probe with coupling gel was carefully placed on the skin of dorsal to the fusion site easily located through palpation (Figure 1). For other groups without LIPUS treatment, the same dosage of ketamine and xylazine was given during the same period of 7 weeks as the LIPUS-treated groups.

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Figure 1. Low-intensity pulsed ultrasound treatment at the fusion site of a rabbit with posterior spinal fusion surgery under general anesthesia. The hair was shaved before the application of the ultrasound.

ASSESSMENT OF FUSION MASS

The animals were euthanized at week 7 after surgery by overdosed pentobarbital. The L5 and L6 transverse processes, together with the TCP implant, were harvested *en block*.

Manual Palpation

After sample harvest, the fusion mass of transverse processes with implant was assessed with manual palpation by two assessors (C.H. & C.C.) in a blinded manner. Only those specimens with complete absence of motion between the transverse processes of L5 and L6 were rated as clinical fusion.[29,34] The interobserver correlation was 0.899 ($P < 0.05$).

Peripheral Quantitative Computational Tomography

The mineralized tissue at the fusion site was evaluated with a multilayer peripheral quantitative computed tomography (pQCT) (Densiscan 2000, Scanco Medical AG, Bassersdorf, Switzerland). Eight sections (8 mm) in each sample were scanned sagittally parallel to the longitudinal axis of TCP implant. The scanning resolution is 0.3 mm with 1-mm thickness per scanned section. The middle six computed tomographic sections were used for bone volume analysis to ensure that the analyzed area was within the TCP implant at the fusion site.

Histomorphometry

After pQCT measurement, the specimens were fixed overnight in 4% formaldehyde and dehydrated in graded alcohols for histomorphometry. The specimens were embedded in methyl methacrylate following an established protocol[6] for undecalcified histology. In brief, samples were dehydrated with graded ethanol and embedded in polymethylmethacrylate (Merck, Darmstadt, Germany).

Along the sagittal axis of the vertebra and TCP implant, the methyl methacrylate–embedded samples were then sectioned into 500-micron-thick slices using saw microtome (SP1600, Leica Microsystems GmbH, Wetzlar, Germany). The middle slice of the fusion mass was selected for histomorphometric analysis. The slice was then grounded and polished to approximately 100 μm (Phoenix 4000, Wirtz Buehler, Germany) for Stevenel's Blue and counterstained with van Gieson Stain.[35] Histological images were captured under light microscopy (Leica Application Suite and DFC295 digital microscope camera, Leica Cambridge Ltd, Cambridge, United Kingdom).

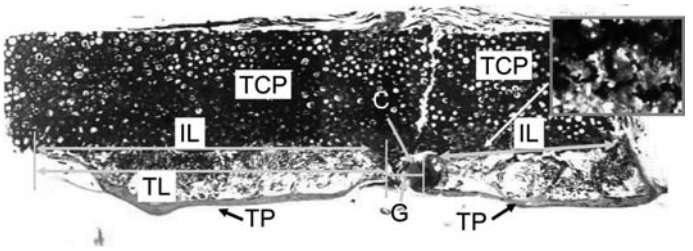

Figure 2. Parameters measured with the histomorphometry analysis using computer-aided image analysis mentioned in the "Material and Methods" section. G indicates gap distance between the transverse processes at the fusion site where the cartilaginous tissue (C; blue) was excluded in the measurement; IL, integrated length where bony tissue formed along the interface of the bioceramic implant (TCP); TL, the length of the transverse processes that is measured from tip to tip of individual transverse process; TP, transverse processes at the fusion site. The top-right insert showed that the bony tissue in the implant was stained with orange color, which was quantified with image analysis to assess the amount of bone formed within the bioceramic implant. The cartilaginous tissue (C) area was measured with the area of blue staining in the fusion gap. (Stevenel's blue and van Gieson staining: bone/mineralized tissues: red/orange. Cartilaginous/nonmineralized tissues: blue/green).

Using the Metamorph Image Analysis System (Universal Imaging Corporation, Downingtown, PA), the following measurements were taken (Figure 2): (1) bony gap distance between adjacent transverse processes (mm) and length of transverse processes (mm); (2) the length of osteointegrated interface between the transverse processes and TCP block; (3) total area of mineralized tissue in TCP (mm^2) was measured with a color threshold for orange in TCP to assess visible bony ingrowth into the TCP, and (4) area of osteochondral tissue (mm^2) was measured with purple color threshold to select the cartilaginous tissue at the intertransverse processes space.

Statistical Analysis

The results were expressed in mean ± 1 standard deviation. Differences in the various variables assessed by manual palpation were compared by Fisher exact test, while pQCT and histology were compared by the one-way analysis of variance among different groups and followed by a *post*

TABLE 1. Fusion Rate of Different Groups of Animals by Manual Palpation

Group	% of Fusion	*P*
A	0% (0/7)	0.005*
B	14% (1/7)	0.029*
C	86% (6/7)	

**Fisher exact test when compared with group C.*

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TABLE 2. Bone Volume Analysis of the Fusion Mass in Different Groups of Animal with Peripheral Quantitative Computed Tomography at Week 7 Postoperation

	Groups			
	A (mean ± SD)	B (mean ± SD)	C (mean ± SD)	*P*
Bone volume (mm^3)	476 ± 16	440 ± 65	582 ± 36	0.007* 0.001†

*Post hoc test, Bonferroni when group A compared with group C.
†Post hoc test, Bonferroni when group B compared with group C.

hoc Bonferroni test. Statistical significance was set at $P \leq 0.05$. All these statistical analyses were done with SPSS 13.0 software (SPSS, Chicago, IL).

RESULTS

Clinical Fusion Assessment with Manual Palpation

By manual palpation, groups B and C showed 14% and 86% clinical fusion at week 7 postoperation while 0% in group A. Group C showed the significantly highest clinical fusion rate when compared with other groups ($P < 0.05$) (Table 1).

Assessment of Bone Volume of Transverse Processes by pQCT Analysis

Comparing with group A, group B had similar bone volume of the transverse processes (Table 2). Group C showed significantly higher bone volume than group A (22%) ($P = 0.007$) and group B (32%) ($P = 0.001$).

Histomorphometric Analysis

Histology of the transverse processes at lumbar vertebra L5 and L6 in contact with TCP bioceramic was stained with van Gieson Stain (Table 3). TCP implants in all groups showed a crack line at the middle of the implant after 7-week implantation. New bone ingrowth was found in the TCP implant in all groups. The gap distance between L5 and L6 transverse processes was shorter in group C (Figure 3). Fibrous tissue with small islet of cartilaginous tissue without true osteochondral bridging between the transverse processes was found in groups A and B (Figure 3). In contrast, all the group C fusion masses showed continuous osteochondral bridging (7 of 7; Figure 4). Figure 4B and C showed the details of the osteochondral bridge and significantly more bony ingrowth into the TCP implant (Figure 4D, E). In addition, more bony tissues from the transverse processes were found attached to the TCP implant in group C.

The gap distance of group C treated with LIPUS was significantly shorter (67%) than those of groups A and B without LIPUS ($P < 0.05$; Table 3).

The total length of the transverse processes was 30.9 ± 2.9 mm in group C at week 7, significantly longer than those of groups A (26.3 ± 2.5 mm) and B (25.0±1.6 mm) ($P = 0.023$ and 0.005, respectively; Table 3). The degree of osteointegration between host bone and the composite implants expressed as ratio of osteointegrated length to the transverse process length showed that group C (79.8 ± 11.8%) has higher ratio than group A (58.7 ± 4.3%) and group B (54.1 ± 9.2). This indicates 35% better bony integration in group C with LIPUS treatment (Table 3)

In parallel, the amount of bone mineralized tissue growing into the TCP composite implant was significantly larger in group C fusion mass (0.038 ± 0.014 mm^2) when compared with group A (0.012 ± 0.011 mm^2) and group B (0.012 ± 0.011 mm^2) ($P < 0.05$; Table 3). Furthermore, group C animal also had significantly larger area of cartilaginous tissue (1.73 ± 0.92 mm^2) when compared with groups A (0.15 ± 0.21 mm^2) and B (0.53 ± 0.64 mm^2) ($P < 0.05$; Table 3).

DISCUSSION

The efficacy of LIPUS in promoting clinical fusion in the rabbit posterior spinal fusion model with autologous MSC-derived

TABLE 3. Histomorphometric Analysis of the Fusion Mass in Different Groups of Animals at Week 7 Postoperation

	Groups			
	A (mean ± SD)	B (mean ± SD)	C (mean ± SD)	*P*
Gap distance (mm)*	2.97 ± 0.37	4.98 ± 1.98	0.97 ± 0.13	0.001†
Total length of transverse processes (mm)	26.3 ± 2.5	25.0 ± 1.6	30.9 ± 2.9	0.023‡ 0.005†
Integrated length/transverse processes length (%)	58.7 ± 4.3	54.1 ± 9.2	79.8 ± 11.8	0.035‡ 0.004†
Mineralized tissue in tricalcium phosphate bioceramic (mm^2)	0.012 ± 0.011	0.012 ± 0.011	0.038 ± 0.014	0.009‡ 0.007†
Cartilaginous tissue formation (mm^2)	0.15 ± 0.21	0.53 ± 0.64	1.73 ± 0.92	0.006‡ 0.026†

*In the gap distance measurement, the cartilaginous tissue is excluded.
†Post hoc test, Bonferroni when group B compared with group C.
‡Post hoc test, Bonferroni when group A compared with group C.

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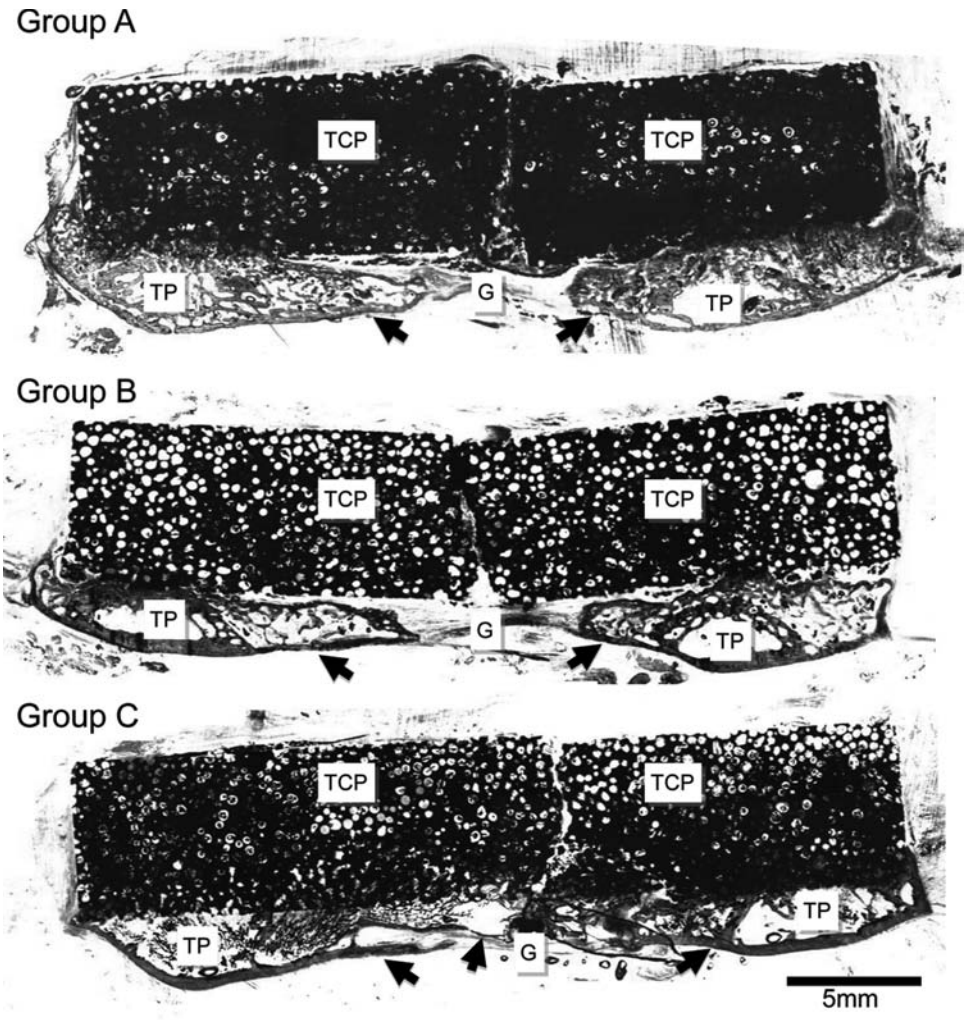

Figure 3. Histology of the fusion mass in different groups of animals at week 7 postoperation. **Group A:** Tricalcium phosphate bioceramic (TCP) only. **Group B:** TCP with osteogenic-induced bone marrow–derived stem cells. **Group C**: LIPUS-treated plus TCP with osteogenic induced bone marrow derived stem cells. The transverse process (TP) was no longer in oval shape cross-sectionally and changes to an irregular shape extending along the TCP implant. The new bone formed from the TP along the TCP implant interface (arrows). The fusion gaps (G) of group C were smallest when compared with groups A and B. In group C, the blue-stained cartilaginous tissue was observed at the fusion gap. The cartilaginous tissue in groups A and B was not obvious. (Stevenel's blue and van Gieson staining).

osteogenic cells and bioceramic composite was well demonstrated in the present study. Early osteochondral bridging between two adjacent transverse processes was observed in the LIPUS treatment group as early as week 7 postoperatively. Several studies have demonstrated that LIPUS could promote bone formation in spinal fusion with autograft.[19–21] Higher osteoblastic activity was reported in LIPUS-treated group.[19] With LIPUS, the degree of graft incorporation was found to be increased in another study.[20] In the current study, results of manual palpation, pQCT, and histomorphometry had demonstrated that LIPUS treatment with autologous MSC-derived osteogenic cell-biomaterial composite without bone grafting could promote both early clinical fusion and histological fusion as reflected by the increase in the size of fusion mass, osteointegration, and endochondral ossification at the fusion site.

Significantly higher clinical fusion rate (86%) in LIPUS-treated group (Group C) was observed as early as week 7 postoperation when compared with other groups without LIPUS treatment (groups A and B: 0% and 14%, respectively). In a similar rabbit posterior spinal fusion study using manual palpation as clinical fusion indicator,[21] LIPUS enhanced autograft-mediated fusion rate of 93% when compared with 64% fusion rate in the control group with autograft alone.[21] Another study of similar rabbit model using muscle-pedicled bone graft [19] showed a 85% clinical fusion by manual palpation. The present treatment regimen with synthetic bioceramic impregnated with autologous MSCs without the addition of bone graft compared favorably with LIPUS-treated autologous bone grafting in other studies.[19,21]

The better clinical fusion in the LIPUS-treated animals can be explained by the increased bone formation at the fusion site observed in pQCT and histomorphometry. From the pQCT analysis, it was observed that bone volume at the fusion site was significantly larger in LIPUS-treated group (group C) when compared with other groups. This is also observed in smaller gap distance (0.97 ± 0.13 mm) and longer transverse process length (30.9 ± 2.87 mm) of LIPUS-treated animals from the histomorphometric analysis. The early clinical fusion in LIPUS-treated animal also reflected better osteointegration between the host bone and the implanted composite. In group C, the percentage of osteointegrated length is 79.8 ± 11.8% significantly larger than those of groups A (58.7 ± 4.3%) and B (54.1 ± 9.2%). In a previous study,[36] LIPUS stimulation was found to significantly increase the osteoblast number and bone area in the central part of the porous calcium phosphate bioceramic implanted in the femoral condyle as compared with the control bioceramic without LIPUS. LIPUS treatment is one of the noninvasive stimulators that could enhance bone formation activity of the implanted cells[19] and accelerated

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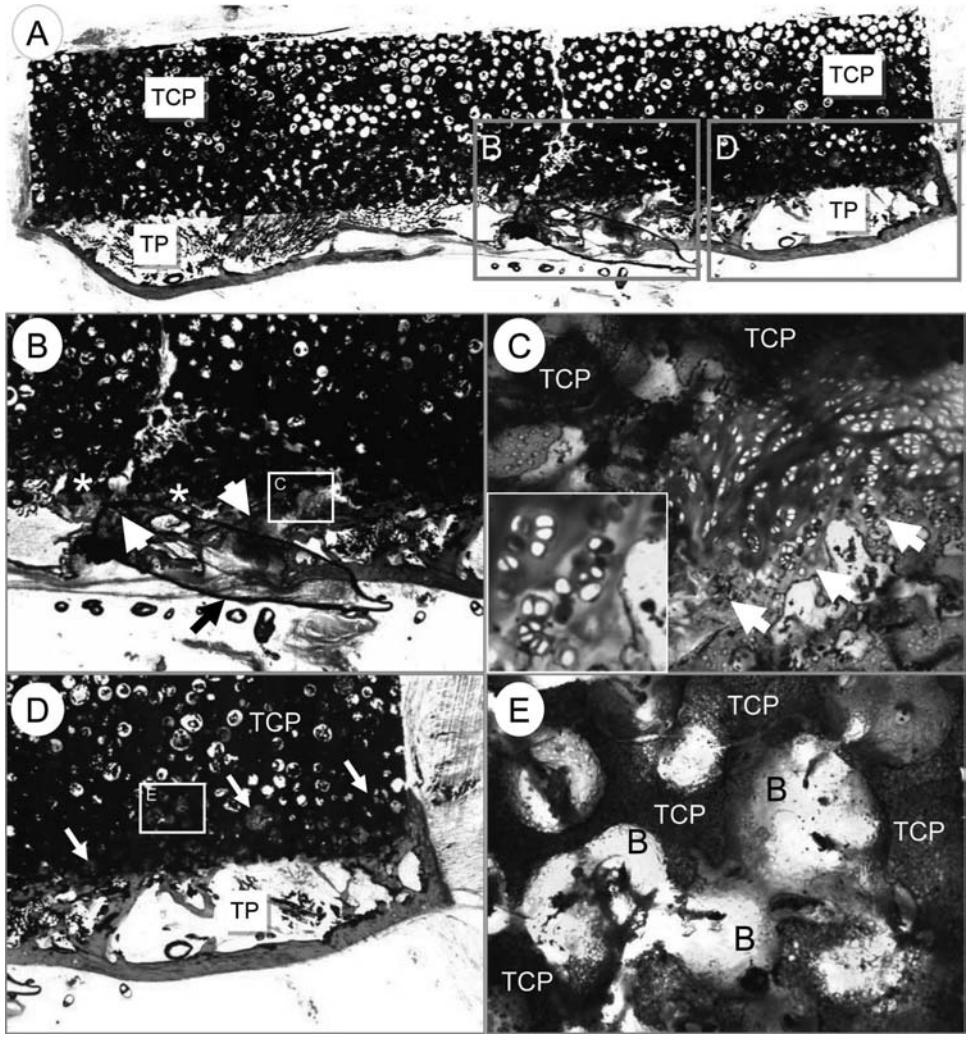

Figure 4. A, Overview of the fusion mass with cell-bioceramic composite treated with LIPUS. **B**, The intertransverse processes were bridged with osteochondral tissue. Asterisks (*): osseous tissue formed at the fusion gap. Arrow heads: Cartilaginous tissue (blue). Black arrow: bubble artifact created during the plastic embedding procedures. **C**, Endochondral ossification was observed at the fusion gap. Arrows: bone formation front during endochondral ossification. TCP: bioceramic implant. The insert shows the embedded chondrocytes (blue) within the mineralized tissue and the extracellular matrix consisted of both mineralized tissue (red) and cartilaginous tissue (blue). **D**, Under low magnification (8.8×), ingrowth of osseous tissue (arrows, stained red) was observed within the bioceramic implant (TCP). **E**, With higher magnification (70×), the osseous tissue (B) could be clearly identified in the pores of the bioceramic implants (TCP). TCP indicates tricalcium phosphate bioceramic; TP, transverse process.

bone ingrowth into the pores of calcium phosphate bioceramic.[36] Findings of these studies matched well with the findings of the current study, showing threefold more mineralized tissue in the TCP block of the LIPUS-treated group C animals (0.038 ± 0.014 mm^2).

The early osteochondral bridging in group C implied that LIPUS was effective in enhancing clinical fusion rate as early as week 7 postoperation through endochondral ossification (Figure 4).[6,33] Studies[37,38] showed that LIPUS could upregulate aggrecan gene expression, a marker for chondroctye differentiation in endochondral ossification. The previous study[21] has observed cartilaginous tissue at the fusion site without detailed description. The present study showed the presence of different types of chondrocytes in the process of endochondral ossification. Therefore, the effect of the LIPUS in the present spinal fusion model with autologous MSC-derived osteogenic cells and bioceramic not only is on the direct enhancement of osteoblastic activity of the osteogenic cells but also acted through the enhancement of endochondral ossification at the fusion site.

In summary, results of this study have demonstrated that the use of LIPUS enhanced the fusion mass volume, osteointegration, and endochondral ossification of the fusion mass with associated increased clinical fusion rate in posterior spinal fusion implanted with autologous MSC-derived osteogenic cells and bioceramics composite. This could have significant clinical implication and potential for clinical application on posterior spinal fusion by reducing the use of bone grafting, bone morphogenetic proteins, or genetically manipulated materials.

➢ Key Points

- ❑ Low-intensity pulsed ultrasound enhanced clinical fusion and histological osteochondral bridging in rabbit posterior spinal fusion with MSC-derived osteogenic cells and bioceramic.
- ❑ Enhanced clinical fusion rate was likely due to better host-implant integration and enhanced endochondral ossification at the fusion site.
- ❑ The present study using synthetic bioceramic and cells, together with LIPUS, to replace the use of bone grafts showed that a proper combination of materials, cells, and biophysical intervention could reduce the use of bone grafts clinically.

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