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ORIGINAL ARTICLE

Rabbit Mesenchymal Stem Cells Cultured in a Dynamic Culture System Displayed Superior Cell Proliferation and Osteogenetic Induction

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Objective: We evaluated the effect of three-dimensional static and dynamic culture on the proliferation, distribution, and differentiation of rabbit mesenchymal stem cells (MSCs) in a porous scaffold via autograft for bone regeneration.
Methods: Rabbit MSCs were seeded in a porous hydroxyapatite scaffold (MSCs/scaffold), and then cultured in petri dishes and a bidirectional flow reactor for 4 weeks for osteogenetic induction *in vitro*. Metabolic assay of the culture medium was carried out every 2 days; glucose, lactic acid, and calcium concentrations in the medium were also examined. Cell distribution in the scaffold was examined histologically. Cultured MSCs/scaffolds were implanted in rabbit condyles for the evaluation of bone regeneration *in vivo*.
Results: Histological sections showed that cells cultured in petri dishes grew only around the scaffolds and seldom in the inner part. However, the scaffolds cultured with MSCs in a bioreactor were almost fully occupied by cells. Metabolic results revealed that the average concentration of glucose in the medium was decreased as cells propagated. Glucose consumption was observed in both static and dynamic cultures, but higher lactic acid production was found in the static culture. Calcium ion concentration was reduced significantly in the dynamic culture after the addition of an osteogenetic induction medium, indicating progression of mineralization. The *in vivo* results showed that about 80% of the defect in the condyles with dynamically cultured MSCs/scaffold implants was filled with new bone tissue, a proportion much higher than that in the petri dish-cultured MSCs/scaffolds, in which only half of the bone regeneration occurred in the cavity.
Conclusion: This study provides evidence of the effectiveness of dynamic *in vitro* MSC culture for robust osteogenesis and indicates that it may impart superior potential for bone regeneration *in vivo*.

1. Introduction

Bone grafting is a common clinical orthopedic surgery. Autologous bone grafts supply osteoinductive growth factors, osteogenic cells, and structural supporting for bone defects and have thus become the gold standard for the surgical treatment of defects caused by trauma, congenital abnormalities, infection, or tumors. In aging populations, the number of bone reconstruction procedures is expected to rise notably, presumably increasing the demand for bone graft material. However, the amount of bone available for autografts is limited, and harvesting procedures for bone grafts are associated with a multitude of risks—pain, neurovascular injury, and infection—at the donor site.[1–3] The use of allograft bone as an alternative treatment carries the risks of disease transmission, host rejection, infection, and graft failure as a consequence of the reduced osteoinductivity of allograft bone. Accordingly, innovative approaches for bone regeneration in the field of orthopedic surgery are needed. Mesenchymal stem cells (MSCs), easily isolated and expanded from bone marrow aspirates, provide an excellent source of osteoprogenitor cells owing to their capacity for *ex vivo* proliferation and differentiation.[4–6]

MSCs are capable of differentiating into various types of cells such as osteoblasts, chondrocytes, myoblasts, and adipocytes, depending on various influences from bioactive factors.[5,7,8] However, these cells are present in low frequencies in bone marrow. Investigators thus have attempted to enlarge the MSC biomass by

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isolating, purifying, and expanding these cells *in vitro*. Currently, the engineering of tissue cultivation has been motivated by the need to create implantable tissue equivalents for clinical use. However, the structure and function of the resulting tissue constructs depend mostly on the flow and mixing conditions in the tissue culture environment.[9]

Tissue engineering protocols including *in vitro* expansion and differentiation of osteoprogenitor cells within scaffolds offer a promising strategy for *in vitro* formation of implantable autologous bone tissue.[1,3] However, three-dimensional (3D) cultures of MSCs on porous scaffolds present several challenges. Insufficient nutrient/oxygen transport and removal of waste from cells in the interior of the scaffold are major hurdles to 3D cultivation in static culture.[1,10] Consequently, decreased proliferation and differentiation occurs as a result of inhomogeneous distribution of cells in the middle of the scaffold, restricting the size of scaffolds that can be cultured under conventional static conditions.[2,11] Dynamic cell culture techniques have been adapted to solve this limitation.

In the present study, we cultivated MSCs in a dynamic culture bioreactor that enables cell cultures in a bidirectional flow environment, in turn, eliminating channeling effects during medium flow. Oxygenation was achieved by alternately lowering the medium level and exposing the scaffolds to the air. We applied this dynamic culture system to enlarge the number of MSCs and induce them into osteogenic lineages *in vitro*, which can ultimately be applied to orthotropic bone regeneration.

2. Methods

2.1. MSC harvest and culture

Fresh bone marrow was obtained via iliac crest aspiration from New Zealand rabbits. Mononucleated cells (MNCs) from bone marrow were isolated via density gradient centrifugation (1.077 g/cm^3, Histopaque; Sigma–Aldrich, Saint Louis, MO, USA) and washed in phosphate-buffered saline. After centrifugation, the MNCs that remained in the buffy coat layer were collected. MNCs were seeded in an alpha-minimum essential medium (α-MEM; Sigma–Aldrich) supplemented with 10% fetal calf serum from Biological Industries (Kibbutz Beit Haemek, Israel), 2 mM L-glutamine, and 1% antibiotic–antimycotic solution (Gibco/BRL, Gaithersburg, MD, USA) named "basal medium". When cultures reached 80% confluence, cells were detached with a trypsin–EDTA solution (Gibco/BRL) to allow for continued passaging.

2.2. Induction of MSC differentiation

To assess the potential of MSCs for adipogenic, osteogenic, and chondrogenic differentiation, we detached the MSCs using a trypsin–EDTA solution and reseeded them in appropriate induction media. Three procedures were tested: (1) differentiation with an adipocyte induction medium (α-MEM supplemented with 1 μM dexamethasone, 60 μM indomethacin, and 5 μg/mL insulin–transferrin–selenium); (2) differentiation with an osteogenic induction medium (OS medium: α-MEM supplemented with 60 μM ascorbic acid, 10 mM β-glycerophosphate, and 0.1 μM dexamethasone); and (3) chondrogenic differentiation with Dulbecco's modified Eagle medium supplemented with 2 mM L-glutamine, 1% fetal calf serum, 10 ng/mL transforming growth factor β, 60 μM ascorbic acid, and 0.5 μg/mL insulin–transferrin–selenium (Sigma–Aldrich). All supplements were acquired from Sigma–Aldrich or Gibco/BRL. These induction media were replaced every 3–4 days. After 2–3 weeks, lipid vacuoles were observed and identified using Oil Red O (Sigma–Aldrich); alkaline phosphatase and calcium deposits were analyzed using an alkaline phosphatase kit and alizarin red-S stain, respectively; and chondrogenic extracellular matrix and glycosaminoglycan deposition were examined using Safranin-O stain.

2.3. Culture of MSC/scaffold constructs in bioreactors

The experiments described herein were performed after the third passage of MSCs. MSC cell suspension (200 μL) with a cell density of 5×10^5 cells/mL was loaded into porous hydroxyapatite (HAP) scaffolds. These scaffolds were 8–10 mm in diameter and 3–5 mm thick. After cell seeding, half of these scaffolds were cultured in petri dishes for 3D-static culture, and the other half was cultured in the bioreactor for 3D-dynamic culture (Figure 1). In the static cell culture, each cell/scaffold construct was maintained in one well of a six-well plate with 5 mL culture media. The bioreactor enables cell culture under a bidirectional flow environment, which eliminates channeling effect during medium flow. Oxygenation was provided by alternately lowering the medium level and exposing the scaffolds to air.

MSCs were cultured in basal medium in the first 2 weeks and medium were renewed once a week. After 2 weeks, MSCs were cultured in the OS medium for osteogenetic induction.[12] The culture medium was collected from the bioreactor and petri dishes once every 2 days. After 1 month of *in vitro* culture, these cultured scaffolds were fixed in 10% neutral buffered formalin for 3 days, decalcified in 10% EDTA solution (pH 7.4), and embedded in paraffin. For histology examination, the specimens were sectioned (5 μm thick) and stained with hematoxylin and eosin.

2.4. Metabolite assay

Concentrations of glucose (Glu), lactic acid (Lac), glutamine, ammonia, and calcium ions in the culture medium were measured using BioProfile 200 (Nova Biomedical Corporation, Waltham, MA, USA). Calcium concentration in the medium was estimated with a Sigma Kit 587, and the optical density was read at 590 nm with an enzyme-linked immunosorbent assay reader (SpectraMax M2, Molecular Device, Sunnyvale, CA, USA) to confirm the calcium deposition in the MSCs for mineralization.

2.5. MSC distribution in porous scaffolds

After 1 month of cultivation in petri dishes or the bioreactor, porous HAP scaffolds with MSCs were fixed in 4% paraformaldehyde. The specimens were decalcified with EDTA and embedded in paraffin. For each specimen, serial sections (5 μm thick, perpendicular to vessels) were cut at various levels (1 mm gap between levels). The specimens were stained with hematoxylin and eosin.

2.6. Surgical procedure and implantation in a rabbit model

Four New Zealand white male rabbits weighing 2.5–3.0 kg were used in this study. The in vivo experimental protocol was approved by the Institutional Animal Care and Use Committee of the Taipei Medical University (IACUC Approval No. LAC-99-0329). All surgical procedures were performed under general anesthesia administered via an intramuscular injection of a mixture of Zoletil 50 and 2% Rompun solution (1:2 ratio, 1 mL/kg). After shaving, disinfecting, and sterile draping of the operation site, the femoral condyles were exposed via a medical longitudinal incision. A bone defect 10 mm in diameter was created. The scaffolds of the static and dynamic culture procedures were implanted into the right and left legs, respectively, of the rabbits. After 3 months, the animals were sacrificed with an overdose of intravenous pentobarbital. Their legs were harvested and washed with phosphate-buffered saline. Figure 1A depicts the experimental process.

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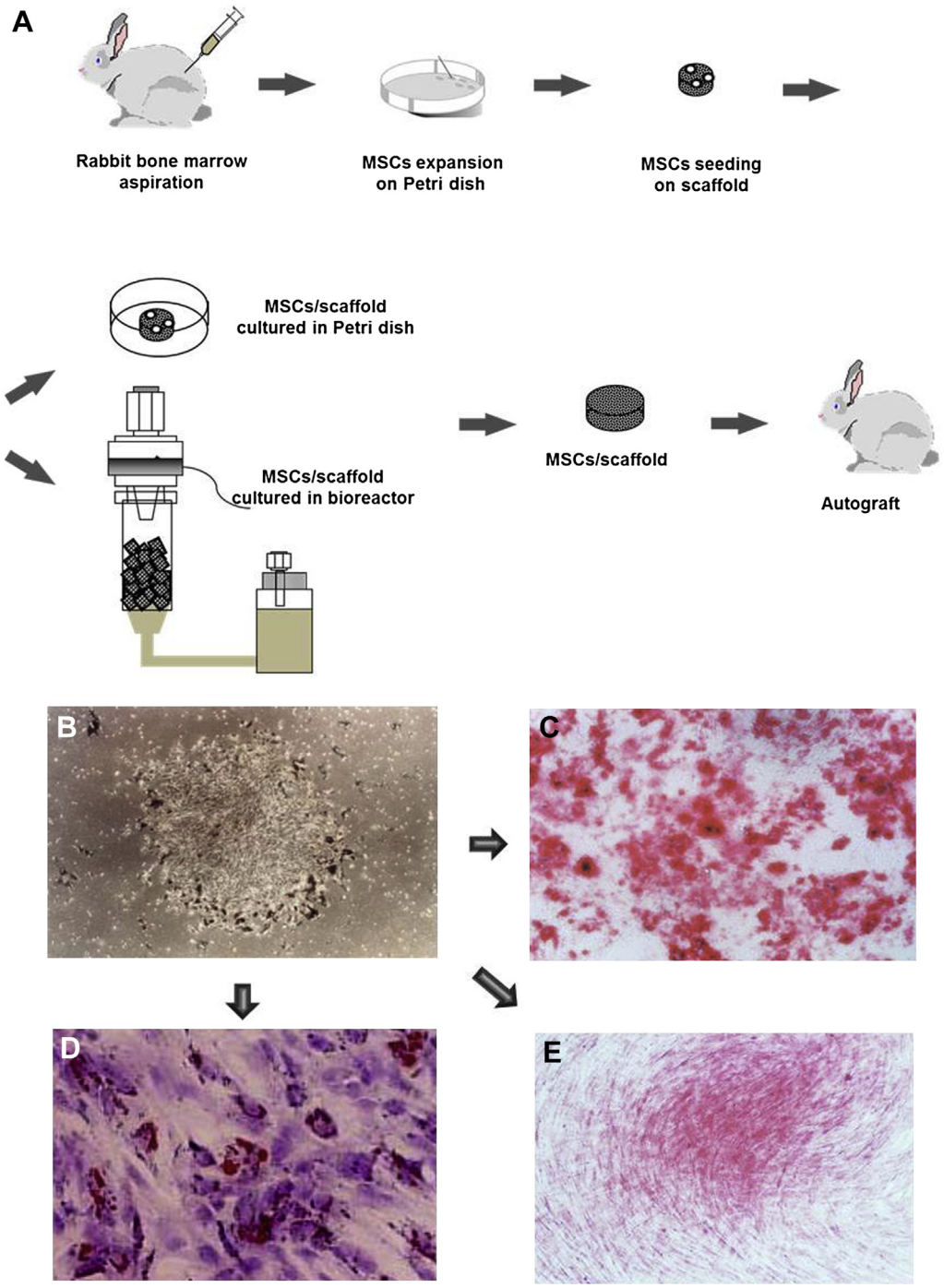

Figure 1 (A) Schematic depiction of MSCs from rabbit bone marrow culture in two environments. Multilineage differentiation of rabbit MSCs in specific induction medium: (B) spindle-shaped p1 MSCs, with colony formation (40×); (C) calcium deposition observed using Alizarin red-S stain (40×); (D) adipogenic induction, stained with Oil Red O (300×); and (E) chondrogenic induction; glycosaminoglycans stained with Safranin-O (40×). MSC = mesenchymal stem cell.

2.7. Histological analysis

Sampled bone tissues were fixed in 10% neutral buffered formalin for 1 week. For histology examination, the specimens were treated with a series of alcohol dehydrations. Samples were impregnated with methyl methacrylate for 4 days with one refreshment prior to being embedded in methyl methacrylate monomer solution at 4 °C for 4 days and then in methyl methacrylate monomer resin. After polymerization, the specimens were sectioned with a diamond saw into slices approximately 200 μm thick; the slices were polished with a grinding machine to a thickness of approximately 30 μm. Finally, the specimens were stained with a Von Kossa silver nitrate solution for the detection of mineralized tissue.

2.8. Statistical analysis

Data are presented as the means of three measurements and error bars represent standard deviation. Statistical comparisons were

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made using Student *t* test. A difference was considered significant at $p < 0.05$.

3. Results

3.1. Multilineage differentiating potentials of rabbit MSCs

During expansion in monolayers, the individual cells composing the colonies exhibited a spindle-shaped and fibroblast-like morphology (Figure 1B). On approximately Day 21 of cultivation in an OS medium, the cells were positive for calcium deposition in MSC extracellular matrix mineralization with Alizarin red-S staining (Figure 1C). After treatment with an adipogenic medium for an average of 21 days, the cells displayed a reduced nuclear size, and accumulated lipid vacuoles within and around the cells were positive for the Oil red O staining (Figure 1D). After chondrogenic induction for 3 weeks, the cells stained with Safranin-O also demonstrated differentiation along with glycosaminoglycan production (Figure 1E).

3.2. Efficacy of dynamic culture for large tissue cultivation

MSCs cultured with scaffolds in petri dishes grew only around the scaffolds and were seldom found in the inner part, as shown in Figure 2A. The absence of cells in the central zone of the scaffolds was caused by necrosis. By contrast, scaffolds cultured with MSCs in the bioreactor (Figure 2B) were almost fully occupied by cells. Osteoid was formed in the scaffolds in the dynamic, but not in the static culture.

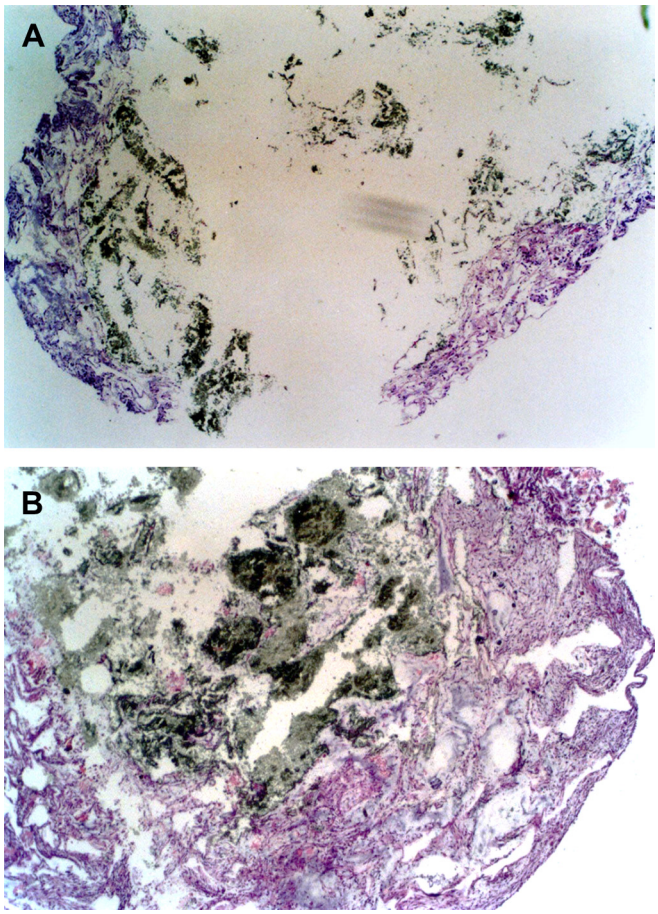

Figure 2 Histological sections obtained from decalcified specimens from MSCs/scaffolds cultured in (A) petri dishes, and (B) a bioreactor for 4 weeks in an osteogenesis medium (hematoxylin and eosin stain, 40×). MSC = mesenchymal stem cell.

3.3. Metabolites in medium

The profiles of Glu uptake rates and Lac production during the course of *in vitro* culture are shown in Figure 3A. Daily Glu

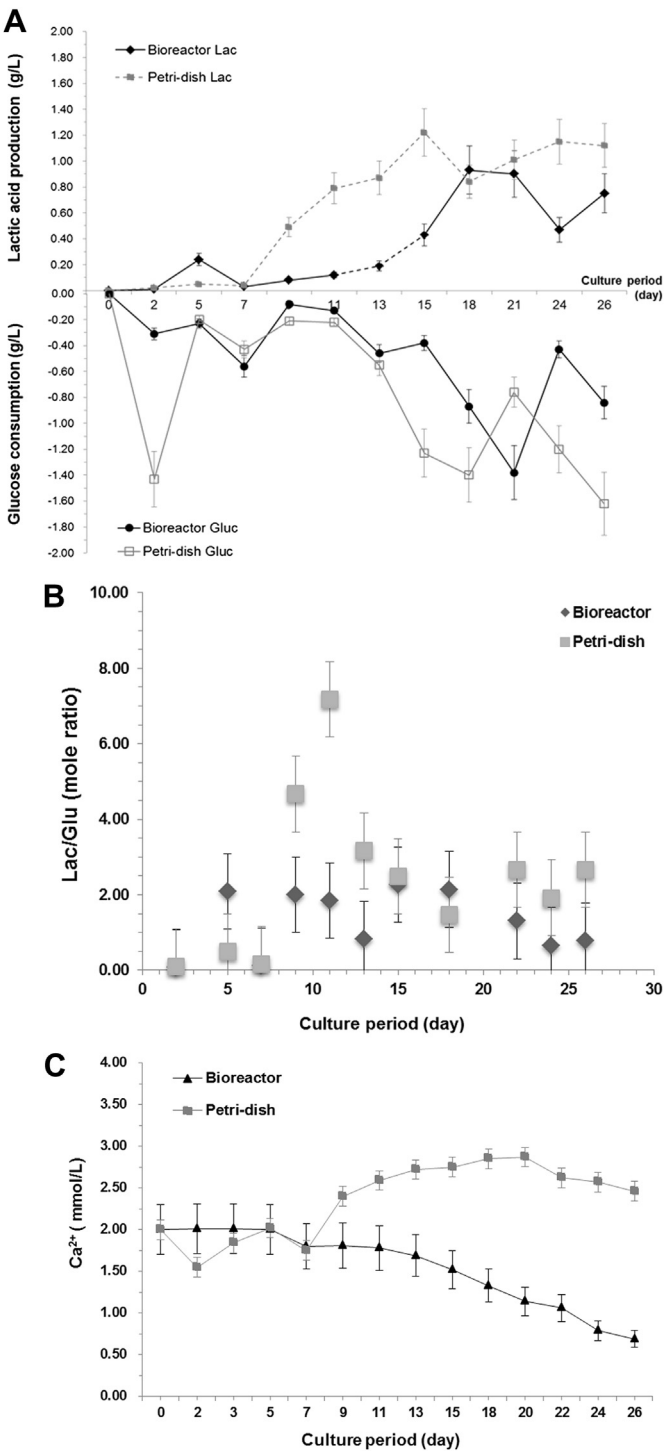

Figure 3 (A) Glu and Lac profiles in culture media from two culture systems. (B) Variations in the Lac/Glu mole ratio during the culture period. (C) Change in Ca^{2+} concentration in culture media: static culture in petri dishes and dynamic culture in a bioreactor. Glu = glucose; Lac = lactic acid.

consumption in media from the static/bioreactor group increased with cell proliferation. Figure 3B shows that the Lac/Glu ratio in the bioreactor group was 1.5–2.0. By contrast, the Lac/Glu ratio in the petri dish culture reached as high as 7 and averaged >2. The Ca^{2+} concentration in the petri dish culture group remained unchanged after the addition of the osteoinduction supplement (Figure 3C). By contrast, the Ca^{2+} concentration in the culture medium of the bioreactor culture group was reduced significantly after the addition of the OS medium.

3.4. *Ex vivo* MSCs/scaffolds cultured under different conditions causing diverse bone regeneration in vivo

The MSCs/scaffolds were implanted in rabbit condyles in a 10-mm-diameter defect created with a bone drill. On histological examination, after 3 months of implantation, the statically cultured scaffold showed little bone formation in the central part of the defect (Figure 4A). Nevertheless, new bone formed not only on the edge but also on the inner part of the defect area (Figure 4C). Direct bonding between the newly formed bone and the HAP scaffold was observed in both groups (Figure 4B and D). The *in vivo* results showed approximately 80% of bone regeneration in the condyle defects implanted with dynamically cultured MSCs/scaffolds, a level much higher than that of the petri dish-cultured MSCs/scaffolds (55% recovery).

4. Discussion

This study provides evidence that rabbit MSCs cultured in a bioreactor have superior capability for osteogenesis *in vitro*. Furthermore, MSCs/scaffolds cultured in a dynamic environment facilitated bone regeneration after autologous implantation. Adult human MSCs have been expanded as undifferentiated cells in culture for more than 27 passages, indicating their proliferative capacity.[11] Rabbit MSCs (passages 3–6) retain the capability for multidifferentiation, as confirmed via culture in different media (Figure 1). MSCs treated with OS, adipogenic, or chondrogenic media differentiated into osteogenic, chondrogenic, and adipogenic cells, respectively.

The supply of oxygen and soluble nutrients becomes critically limiting during the *in vitro* culture of 3D tissues, especially for large scaffolds.[1,6] The consequences of such a limitation are exemplified by early studies showing that cellular spheroids larger than 1 mm in diameter generally contain a hypoxic, necrotic center surrounded by a rim of viable cells.[13] External mass-transfer limitations can be reduced by culturing constructs under a flow condition. A laminar flow environment has been demonstrated to be an efficient way to reduce the diffusive limitations of nutrients and wastes.[10] Therefore, the bidirectional flow condition created by the novel bioreactor used in this study induced mixing of oxygen and nutrients throughout the flow medium and reduced the concentration boundary layer at the construct surface (Figure 2). Moreover, internal mass-transfer limitations can be minimized if the culture media are perfused through the porous network of the scaffolds.[14] Therefore, mass exchange of a medium occurs via internal pore interaction, and cells survive inside the scaffolds.

Glucose consumption and lactate production ratio can be used to estimate the oxygen content in the culture medium during cultivation. In aerobic metabolism, 1 mole of Glu produces 36 moles of adenosine triphosphate via the tricarboxylic acid cycle with effective energy transfer.[15] However, in anaerobic metabolism, only 2 moles of adenosine triphosphate are produced by 1 mole of Glu via glycolysis, with the coproduction of 2 moles of Lac.[15] Therefore, the static conditions of the petri dish culture lead to insufficient oxygen exchange, causing an aerobic environment (Figure 3B). Subsequently, the Lac content fluctuated greatly with time owing to the partial change of culture media and the medium circulation. The Lac/Glu ratio in the bioreactor was below 2, demonstrating that

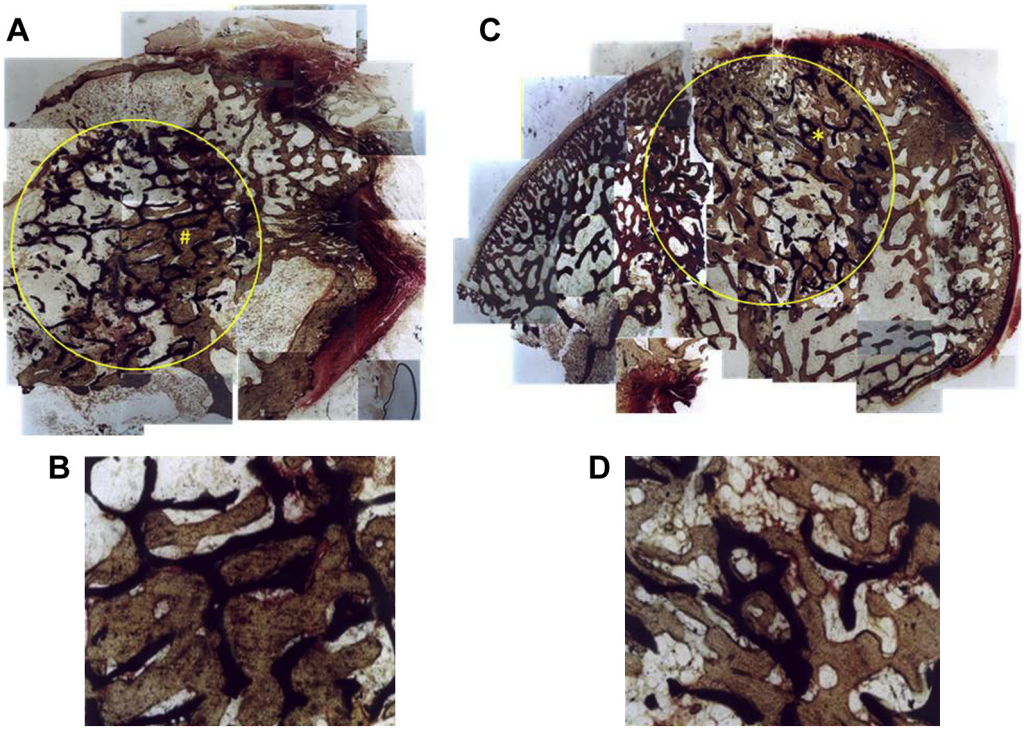

Figure 4 Histomorphological analysis with Von Kossa stain of condyle defects implanted with (A,B) statically cultured MSCs/scaffolds and (C,D) dynamically cultured MSCs/scaffolds after a 3-month implantation biopsy. Photos showed in A and B originally were captured under a magnification of 40×, then piece up to observe the whole defect zone. The images in (B) and (D), and the portions marked by # and * in (A) and (B) were acquired at a higher magnification (100×). MSC = mesenchymal stem cell.

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the bioreactor culture provides an excellent expanding capacity for cells *in vitro* because nutrients/wastes are effectively circulated and sufficient oxygen is provided.

The Ca^{2+} concentration in the petri dish culture medium remained unchanged after the addition of the OS medium, whereas that in the bioreactor medium was reduced significantly after OS medium addition. These results indicated mineralization progression of MSCs, with calcium deposition in extracellular matrix proceeding via Ca^{2+} acquisition from the medium (Figure 3C). This finding is in accordance with the results reported by Stiehler et al,[1] who also observed a significantly increased calcium content in dynamically cultured cell/scaffold constructs compared with statically cultured constructs. Dynamic culture resulted in a general mechanical stimulus to the cells that may assist in osteogeneration with MSCs.[16,17]

More extensive new bone formation was observed in the bone defects implanted with dynamically cultured MSCs/scaffolds (Figure 4C). MSCs propagated well, and osteogenesis as well as mineralization was induced properly in the bioreactor culture system prior to implantation. New bone lining on the implant surface was obviously observed. A greater proportion of cells survived and proliferated in the inner scaffold after bioreactor culture with a dynamic fluid, which provided oxygen and nutrients; therefore, a large bone-like tissue was obtained prior to implantation. Filling with more bone-like tissue (OS-induced MSCs/scaffolds) recovered the bone defect after a short term.

In conclusion, the average concentration of Glu decreased intensely in both dynamic and static cultures, with MSCs proliferating in the scaffolds. The Ca^{2+} concentration in the medium was reduced significantly in the dynamic culture after the OS medium was added, indicating progression of mineralization, whereas that in the petri dish cultures did not change. Histological sectioning showed that cells cultured in petri dishes grew only around the scaffolds and were seldom found in the inner part. However, the MSCs/scaffolds in the bioreactor culture were almost fully occupied by cells throughout the scaffold. The *in vivo* results revealed bone defects with a greater bone volume in the condyles implanted with dynamically cultured MSCs/scaffold implants compared to that in implants with statically cultured MSCs/scaffolds. These results demonstrated that MSCs from bone marrow proliferate and differentiate toward osteogenesis, and proceed to mineralization after cultivation under dynamic culture conditions.

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