Histomorphochemical Comparison of Microfracture as a First-Line and a Salvage Procedure: Is Microfracture Still a Viable Option for Knee Cartilage Repair in a Salvage Situation?

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ABSTRACT: Microfracture is considered as the first-line procedure for knee cartilage repair, but the results of microfracture seem less predictable and rather controversial in a salvage situation. Thus, the purpose of the study was to histomorphochemically compare microfracture as a salvage procedure with microfracture as a first-line procedure in a rabbit model. We hypothesized that microfracture in a salvage situation would result in histomorphochemically inferior cartilage repair compared to microfracture as a first-line procedure, and the inferiority would be attributed to less migration of reparable marrow cells to the defect due to destruction of microarchitecture of the subchondral bone. Thirty-six New Zealand white rabbits were divided into three groups: (i) untreated full-thickness chondral defect, (ii) single microfracture treatment (first microfracture group), and (iii) repeated microfracture in 8 weeks after the first procedure (second microfracture group). In each group, rabbits were sacrificed at the end of 8 weeks, and osteochondral specimens at the repair sites were obtained for histomorphochemical analysis. Results showed that microfracture as a salvage procedure resulted in overall inferior cartilage repair histomorphochemically compared with microfracture as a first-line procedure, which correlated with deteriorative changes in the quality of underlying subchondral bone rather than intrinsic incapability to recruit the reparative cells in the defect area. In conclusion, although a comparable number of reparable cells and a mechanically weakened subchondral bone are anticipated, more study is necessary to clearly determine when a microfracture should be performed in a situation. © 2014 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 32:802–810, 2014.

Keywords: microfracture; bone marrow stimulation; mesenchymal stem cell; cartilage repair; subchondral bone; knee

One of the major reasons for insufficient and less durable cartilage repair is the lack of effectively reparable cells in the affected defect area due to the avascularity of the region. Effort to repair the cartilage defect has been focused on the recruitment of cells to the defect area either endogenously or exogenously. The bone marrow stimulation technique (BST) has long been adapted to allow migration of endogenous cells from bone marrow to the damaged area. Since the disclosure of cell origin of the repair tissue after BST,[1] mesenchymal progenitors or stem cells, and the cytokine-enriched microenvironments in the defect area have become an indispensable factors in repopulation of the defect. With its potential advantages such as relative technical ease, minimal invasiveness and cost-effectiveness, BST is considered as the first-line treatment option for the small- and medium-sized knee cartilage defects[2,3] and has already shown clinically satisfactory results overall in the short- and mid-term follow-up.[4,5] Yet, the repair tissues after BST are mostly unstructured and consist of mechanically vulnerable fibrocartilage,[6–8] which eventually causes deterioration of repair cartilage in the long-term follow-up.[1,9,10]

As the first-line procedure for cartilage repair, microfracture is deemed reasonable since it is not only simple, less invasive, and cost-effective but also it has been assumed surgically inert procedure that does not prevent the application of other cartilage repair procedures that may be needed in the future.[11–13] However, in salvage situations, the results of microfracture seem less predictable and rather controversial.[12–16] Mithoefer et al.[14] reported that microfracture in a salvage situation seems to be less promising compared to microfracture as an initial procedure, with significantly less numbers of athletes with prior surgery history could return to high-impact sport activities when microfracture was performed after failed prior procedure. A couple of recent studies reported that prior BST hinders the success of autologous chondrocyte implantation (ACI) as a salvage procedure.[17,18] Yet, Zaslav et al. have recommended the use of ACI after unsuccessful initial BST procedures since, although ACI after failed previous procedure resulted in rather greater failure rate compared to primary ACI,[17,18] ACI in salvage situation still grants significant enhancement induration of benefit compared to failed non-ACI prior procedure.[19] With regard to microfracture as an option for salvage procedure, some theoretical concerns remain; whether an adequate number of progenitor cells could egress out of the bone marrow and whether any changes in the underlying subchondral

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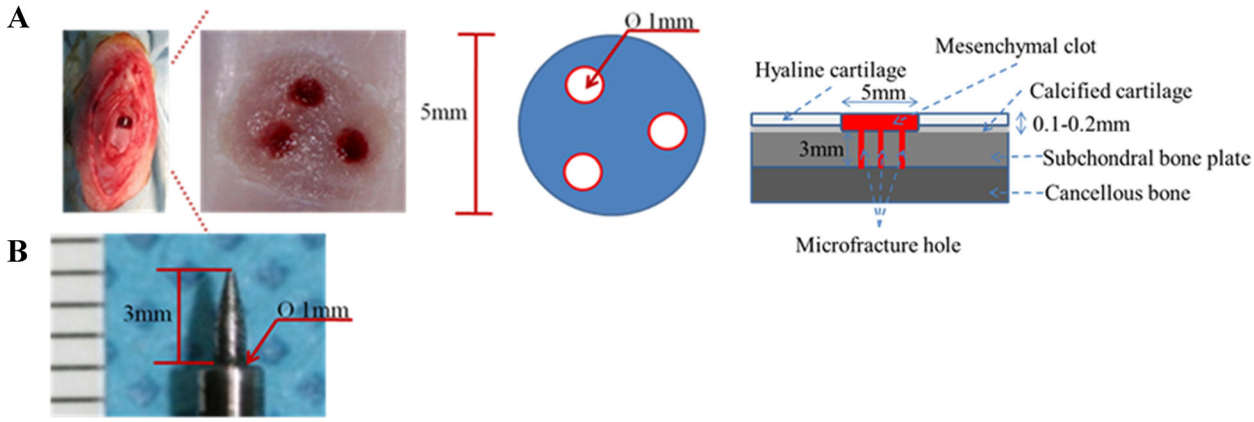

Figure 1. Microfracture technique in a rabbit model. (A) An experimental method for microfracture in this study (B) a 3 mm-depth awl.

bone have a negative effect on the cartilage repair on the defect area with prior surgery. Progenitor cells in the bone marrow are restored to normal levels within a few days of egress and the overall concentration of mesenchymal stem cells (MSCs) is quite low and declines with age.[7] Also, subchondral bone changes have been demonstrated in up to one-third of patients treated with microfracture, and its structure can also influence the biomechanics of the entire osteochondral unit, the repair process, and ultimately the articular cartilage resurfacing outcome.[11,20] Although results of microfracture as a salvage procedure has been reported in clinical perspective, unfortunately, no previous report has histochemically or microscopically assessed the repaired tissue along with its underlying subchondral bone after microfracture procedure as a salvage treatment option. Our hypothesis was that microfracture in a salvage situation would result in histomorphochemically inferior cartilage repair compared to microfracture as a first-line procedure, and the inferiority would be attributed to less migration of reparable marrow cells to the defect due to destruction of microarchitecture of the subchondral bone. Therefore, the purpose of the study was to histomorphochemically compare microfracture as a salvage procedure with microfracture as a first-line procedure in a rabbit model.

MATERIALS AND METHODS

Experimental Design

The experimental protocol was reviewed and approved by the ethics committee for animal research of the Laboratory Animal Research Center of Ajou University Medical Center (Institutional Review Board, ACM 120). Twenty-week-old, male New Zealand white rabbits (3.5 ± 0.5 kg; KOATECH, Pyeongtaek, Korea) were used. Thirty-six rabbits were divided into three groups, each comprising 12 rabbits. The first group was the control group with untreated full-thickness chondral defect that did not open the subchondral bone marrow space. In the second group, a full-thickness chondral defect was made followed by microfracture (first microfracture group). In the third group, microfracture was repeated at the site of prior microfracture in 8 weeks after the first procedure (second microfracture group). In each group, rabbits were sacrificed at the end of 8 weeks, and osteochondral specimens at the repair sites were obtained for histomorphochemical analysis.

Surgical Procedures and Determination of the Depth of Microfracture

A 5 mm-diameter, full-thickness cartilage defect was created on the trochlea of each femur using a 5-mm biopsy punch (Miltex, York, PA) and a special curette until the cartilage and calcified layer was removed thoroughly in the defect area. For first MF group, microfracture with 3 mm depth was performed using a mini-microfracture awl to create three holes in each defect site, with each hole being 2.0–2.5 mm apart on the subchondral bone (Fig. 1). The reason for 3 mm-deep microfracture was that 3 mm depth in a rabbit model

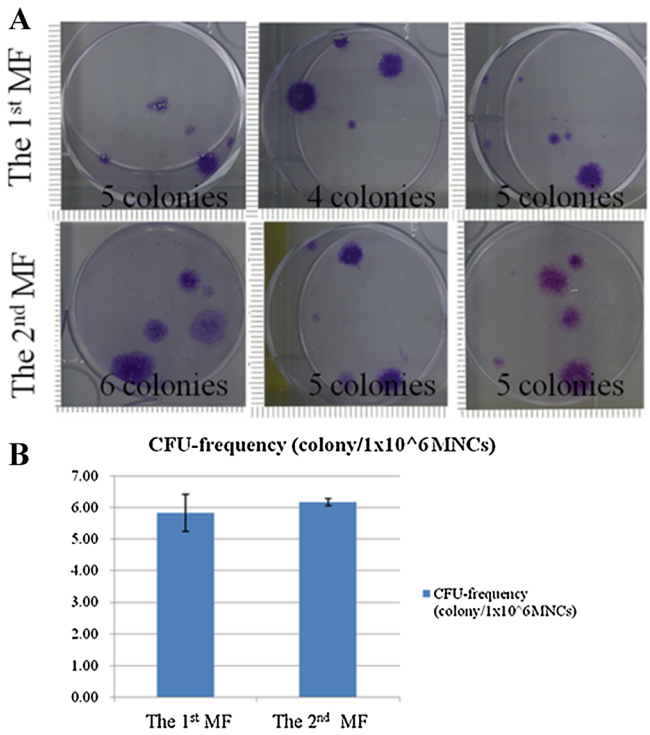

Figure 2. Fibroblast CFUs determined using crystal violet staining after the first microfracture and a second microfracture. (A) Representative photograph of colonies. (B) Comparison of CFU-frequency of MSCs between first and second microfracture. Note that there was no significant difference in CFU-frequency between first and second microfracture. MF, microfracture.

significantly enhanced the volume of blood clot seeped out from the bone marrow but did not penetrate the epiphyseal scar, invasion of which caused massive bleeding and resulted in contamination of the defect area for the study (Figures S1 and S2). In the same manner as first microfracture group, second microfracture group underwent the identical microfracture procedure repeatedly: at 8 weeks after first microfracture, the repair tissue at the prior defect site was debrided meticulously again and 3 mm-deep microfracture was repeated at the previous hole position using the same awl with identical diameter.

Cell Culture and Assay of Fibroblast CFU

The number of MSCs derived from blood clot was measured by a CFU assay (Figure S3). The cells were cultured and stained with a 5% crystal violet solution (Sigma-Aldrich, St. Louis, MO) in 100% methanol for 10 min. The size of colonies was measured by an image analysis system.[21] Colonies with a diameter exceeding 2 mm were counted.

Histological Evaluation

Samples were fixed with 4% formalin and dehydrated through a graded series of alcohol before being cleaned in xylene. Samples were sectioned at a thickness of 4 μm. The sections were stained with Safranin-O for glycosaminoglycan (GAG), hematoxylin and eosin (H&E) for morphologic analysis. Sections were also immunohistochemically analyzed for type II collagen (Calbiochem, Sunnyvale, CA). Each sample was then graded histologically using modified O'Driscoll and Sellers scores accordingly.[22]

Biochemical Assay of Repaired Cartilage

At 8 weeks post-surgery, cartilage was extracted from microfracture area in each group and analyzed for biochemical contents. Water content was calculated by weighing before and after freeze-drying (IlShin Lab, Gyeonggi-do, Korea). Each sample was then digested with papain-degestion solution.[23] Total DNA content was determined using Hoechst 33258 dye.[24] Total GAG content was also measured spectrophotometrically using the 1, 9-dimethylmethylene blue colorimetric method[25] and shark chondroitin sulfate as a standard.

Radiological Evaluation

Each femur was scanned with a model 1076 X-ray micro-CT (SkyScan, Kontich, Belgium). Scanning was carried out with its resolution at pixel size of 9 μm. A three-dimensional (3D) polygonal region of interest (ROI) was developed to characterize the morphometric parameters of subchondral bone microarchitecture. The data from the first and second MF were compared at 8 weeks post-operation and were compared with control samples.

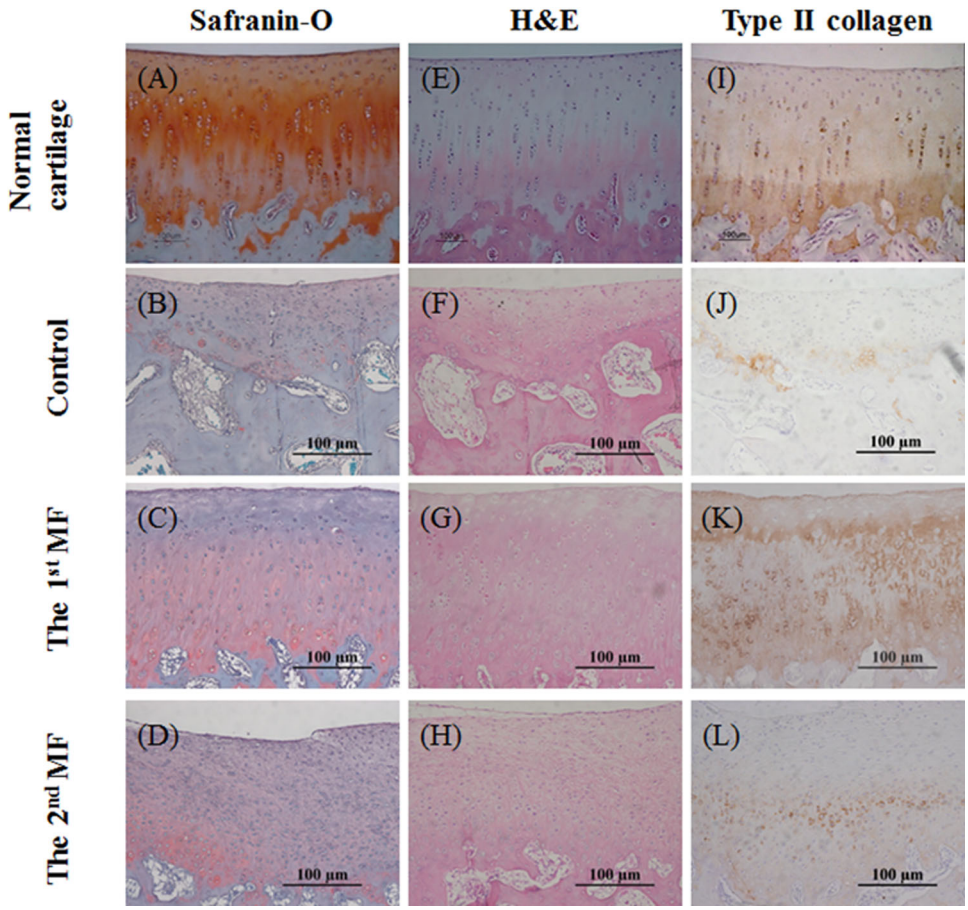

Figure 3. Histological assessment of repair cartilage after first and second microfracture in comparison with normal cartilage and the control. (A–D) Safranin-O, (E–H) H&E, and (I–L) type II collagen immunohistochemical staining. Bars = 100μm (original magnification ×200). MF, microfracture.

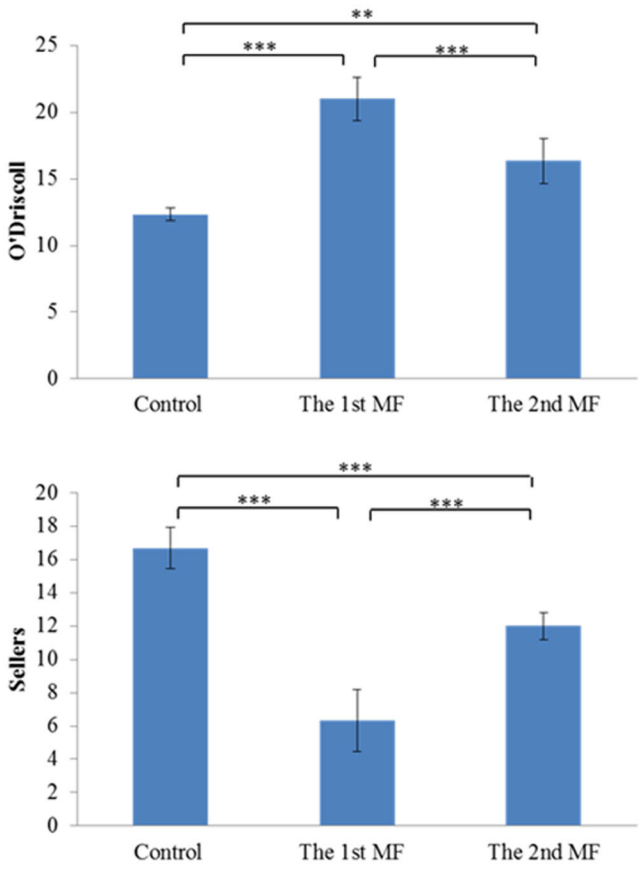

Figure 4. Quantiative assessment of repaired cartilage after 1st and 2nd microfracture using O'Driscoll and Sellers score. $^{**}p < 0.01$, $^{***}p < 0.001$. MF: microfracture.

Statistics

Statistical differences in the assays were determined using one-way analysis of variance (ANOVA) with Tukey–Kramer multiple comparisons test ($p < 0.05$, $p < 0.01$, and $p < 0.001$). The data are expressed as mean ± SD.

RESULTS

Fibroblast CFU Assay

On average, the CFU-frequency of MSCs was (5.83 ± 0.59): 1000000 for first microfracture and (6.17 ± 0.12): 1000000 for the second microfracture. There was no significant difference in colony formation capacity between the two (Fig. 2).

Histological Evaluation

Grossly, all defects in three groups (control, first microfracture, and second microfracture) were covered by a new repair tissue overall. Histologically, both first and second microfracture groups have shown overall intermediate degree of staining in safranin-O and immunostaining for type II collagen compared to normal cartilage and the control without microfracture. In comparison between first and second microfracture, first microfracture group has shown somewhat more significant safranin-O and type II collagen immunostaining overall (Fig. 3). Quantitative assessment has shown significantly superior repaired cartilage tissues after first microfracture compared with those after second microfracture, albeit, both first and second microfracture groups still yielded better histological scores than the control (Fig. 4).

Biochemical Assay of Repaired Cartilage

Total GAG contents in the repaired cartilage of first and second microfracture groups were 50.79 ± 1.37 ng/μg and 45.19 ± 5.97 ng/μg respectively, both of which being greater than that of control group (39.08 ± 2.08 ng/μg). Total DNA contents in the repaired cartilage were greater in the order of first microfracture (44.21 ± 2.63 ng/μg), second microfracture (31.81 ± 1.04 ng/μg), and the control group (29.71 ± 1.74 ng/μg). Similarly, water contents in the repaired cartilage were greater in the order of first microfracture (83.46 ± 2.10%), second microfracture (76.02 ± 4.01%), and the control group (53.31 ± 2.57%) (Fig. 5).

Radiological Evaluation

Microarchitecture of subchondral bones at 8 weeks after first and second microfracture was evaluated using micro-CT (Fig. 6). The overall percent bone volume was similar between the two groups, but overall bone density was significantly less in second microfracture group (Fig. 7 A and B). The two parameters were not significantly different between first microfracture group and normal control. Bony trabecula in subchondral bone after second microfracture was overall less in its number, more separated and less densely connected but thicker compared to that after first microfracture (Fig. 7C–F). Likewise, trabecular bone volume in microfracture hole area and the percent bone volume in the superficial subchondral bone surface were significantly less after second microfracture accordingly (Fig. 7G and H). Representative three-dimensional micro-CT images also revealed relatively less degree of bony tissue repair after second microfracture compared with first microfracture (Fig. 8).

DISCUSSION

The most important finding of the study is that microfracture as a salvage procedure resulted in overall inferior cartilage repair histomorphochemically compared with microfracture as a first-line procedure, which correlated with deteriorative changes in the quality of underlying subchondral bone rather than intrinsic incapability to recruit the reparative cells in the defect area. Our hypothesis was that cartilage repair after microfracture, when performed in a salvage situation, would be less favorable histomorphochemically because less amounts of reparable cells would be recruited after prior surgery. As expected, the overall repaired cartilage seemed histologically worse after second microfracture in the study, but comparable CFU-frequency for MSCs were obtained in

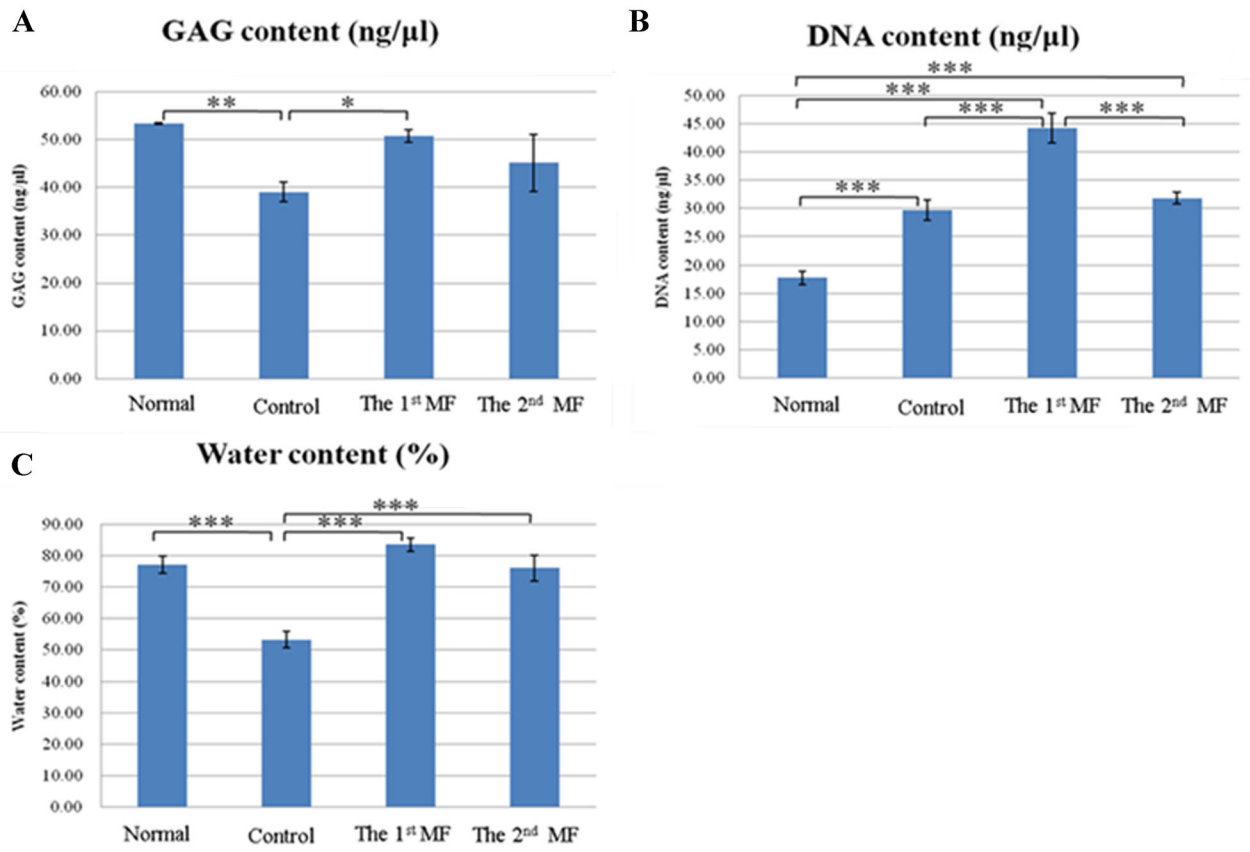

Figure 5. Biochemical comparison of repaired cartilage between first and second microfracture. (A) Total GAG content, (B) total DNA content and, (C) water content. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. MF, microfracture.

both first and second microfracture group. Thus our results indicate that some other factors besides the number of recruited cells is responsible for relatively inferior cartilage repair after repeated microfracture, which, we suppose, may be the significant changes in the microarchitecture of the underlying subchondral bone.

The importance of subchondral bone in cartilage repair after microfracture has gained more attention recently. Unlike normal hyaline cartilage, cartilage tissues formed by microfracture is known to be rather mechanically vulnerable fibrocartilage, which is less durable and may eventually cause functional deterioration in long-term follow-up.[15] Recent studies have demonstrated that microfracture technique itself can cause changes in microarchitecture of subchondral bone such as thickening of the subchondral bone, formation of subchondral cyst, and intralesional osteophytes,[3,11,26] and such remodeling in subchondral bone can be regarded as a potential explanation for the deterioration and failure of microfracture.[11,27] As for the changes in subchondral bone microarchitecture by micro-CT, repeated microfracture in our study resulted in overall comparable bone volume, but decrease in overall bone density, trabecular number and separation, indicating rather weakened mechanical support compared to first-line microfracture. Previous studies have shown that mechanical instability by subchondral bone changes can cause cartilage matrix degradation,[28] and adequate mechanical support by subchondral bone remodeling plays an important role in the results of cartilage resurfacing.[11,27–29] Our results also can imply that weakened mechanical support by subchondral bone has a certain influence on overall histologically inferior cartilage repair after repeated microfracture, which is accordant with previous studies.[28,29]

Our study infers that the number of cells recruited after repeated microfracture can be maintained as many as that after first-line microfracture as long as the bony channel remains healthy and fully penetrated. Thus, it seems plausible to conclude that microfracture as a salvage procedure may not be prioritized as a viable option because of rather indecent and unhealthy subchondral bone condition and not because of inadequate number of recruited cells for cartilage repair. Also, our study implies that an elaborate microfracture technique should be addressed for adequate cartilage repair. In terms of its technique, several previous studies have compared the effect of microfracture versus drilling and deep versus shallow drilling on cartilage repair and pointed out that

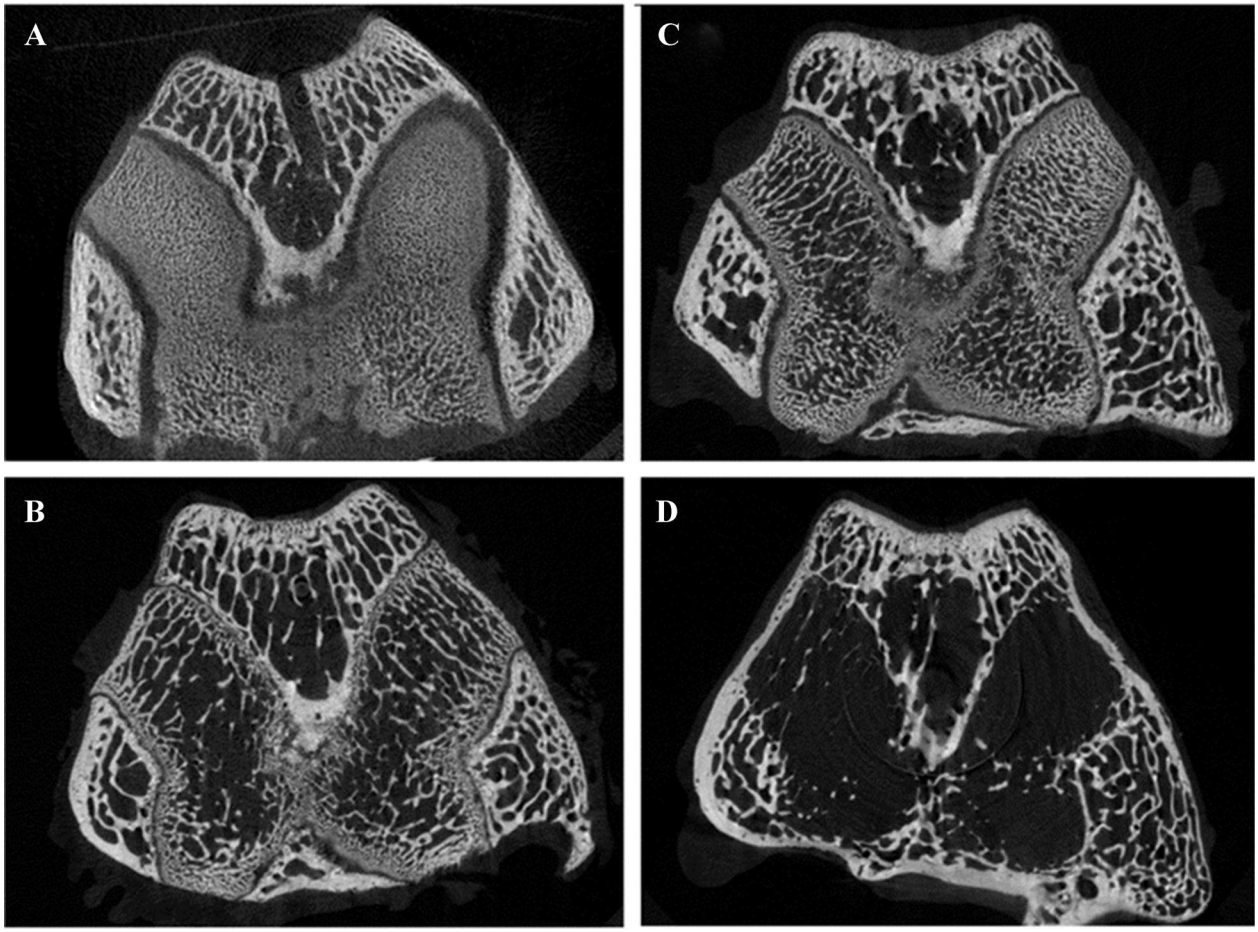

Figure 6. Evaluation of subchondral bone microarchitecture by micro-CT. (A) At immediate post-operation, (B) control (C) at 8 weeks after first microfracture, and (D) at 8 weeks after second microfracture. MF, microfracture.

fractured and compacted bone around holes blocks bony channel, hindering migration of viable bone marrow and potentially impeding cartilage repair.[20,30,31] Regarding the depth of drilling, deeper versus shallower drilling induced a larger region of repairing and remodeling subchondral bone that positively correlated with improved cartilage repair.[20,30] However, drilling deeper to 6 mm penetrated the epiphyseal scar in a mature rabbit model and led to greater subchondral hematoma.[31] Our preliminary study on adequate depth for microfracture also showed greater amount of marrow blood volume on deeper microfracture hole, but 5 mm depth invaded epiphyseal scar and caused massive bleeding that we determined 3 mm depth being appropriate for the study because it allowed significant amount of marrow blood volume but did not invade epiphyseal scar.

Several limitations should be addressed in this study. First, 8 weeks of period may not be long enough to allow complete healing of subchondral bone and to represent a chronic failed condition after prior microfracture. Previous studies also indicated incomplete reconstitution of normal bone structure and continued remodeling at 3 months after bone marrow stimulation in mature rabbits.[20] Another study also reported continuing fragility of subchondral bone even at 6 months after subchondral drilling in a sheep model.[29] Another study has reported subchondral bone remodeling at 1 year after microfracture in a sheep model,[27] but unfortunately, there seems scarcity in the evidence on the time period enough for complete bone remodeling in preclinical animal studies. Needless to say, longer period of study time could allow more chronic condition, but we mainly tried to compare the cartilage status at the same point after initial and secondary microfracture, and such 8 weeks of time period seems long enough to assess repaired cartilage in a rabbit model as in other studies.[32–37] Also, 8 weeks of study period in our study already presents some changes in trabecular bone volume and subchondral bone plate which represent worse environment than initial status. Most of all, we were afraid that chronic condition after cartilage defect may provide another detrimental factors, such as inflammation which is irrelevant to our concern in this study. Secondly, during the second microfracture procedure, the holes

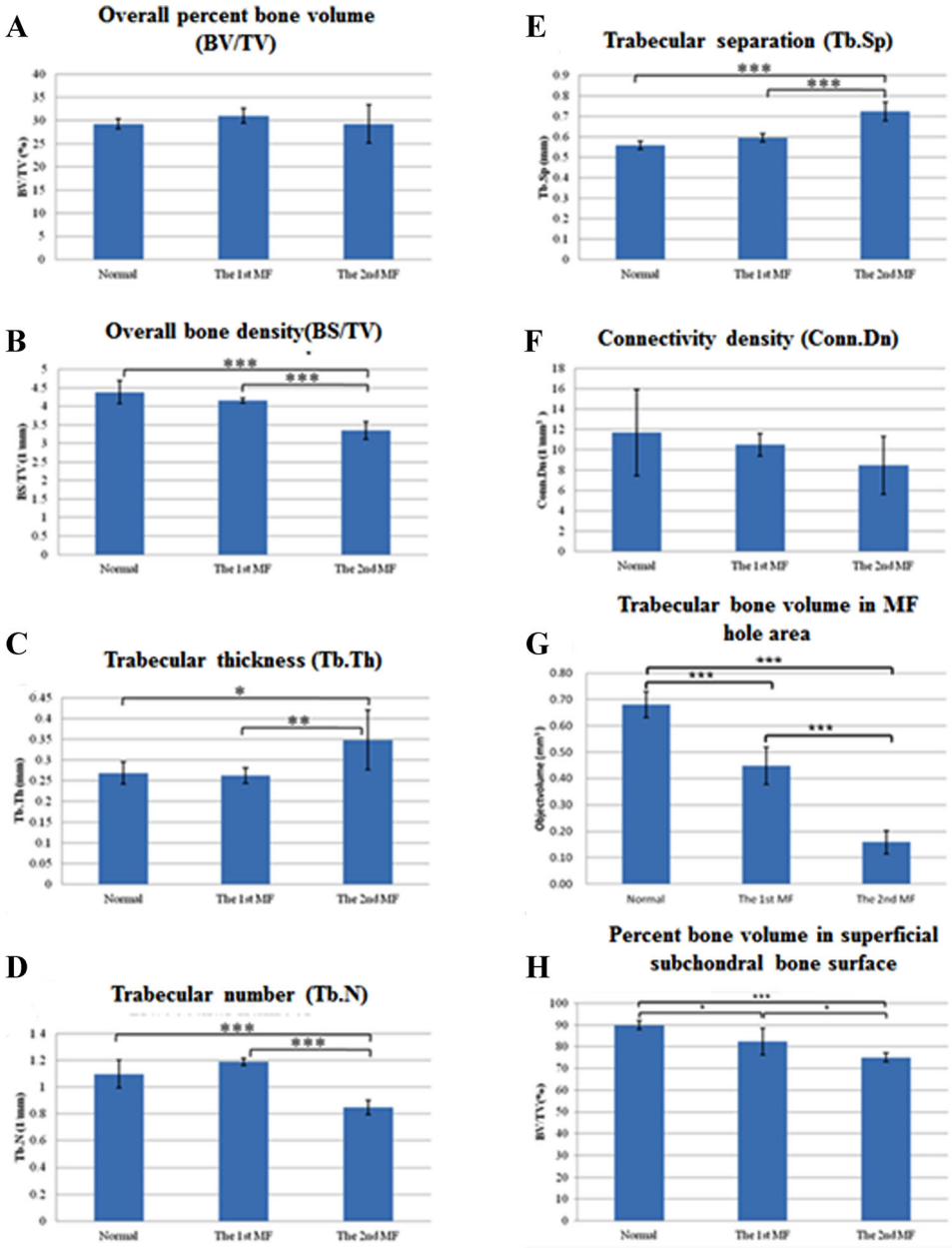

Figure 7. Comparison of subchondral bone architecture at 8 weeks after first and second microfracture. (A) Overall percent bone volume (BV/TV); (B) overall bone density (BS/TV); (C) trabecular thickness (Tb.Th); (D) trabecular number (Tb.N); (E) trabecular separation (Tb.Sp); (F) connectivity density (Conn.Dn); (G) trabecular bone volume in microfracture hole area, and (H) percent bone volume in superficial subchondral bone surface. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$. MF: microfracture.

had to be made at the identical corresponding point with the initial procedure due to limited spaces available in the defect with 5 mm diameter. Also, in 8 weeks after the initial procedure, we had to dig out and curet the repaired cartilage again in order to proceed onto the second microfracture. These may be totally different from the real clinical situation and may not fully represent the chronic failed status with scanty repaired cartilage. We still believe that such experimental conditions cannot be reproduced perfectly and technically limited in preclinical studies. Yet, our study is worthy of notice in that it is the very first to histomorphochemically compare microfracture as a first-line procedure with microfracture as a salvage procedure in a preclinical setting.

In conclusion, our preclinical study showed that microfracture as a salvage procedure resulted in overall inferior cartilage repair histomorphochemically compared with microfracture as a first-line procedure, which correlated with deteriorative changes in the quality of underlying subchondral bone rather than intrinsic incapability to recruit the reparative cells in the defect area.

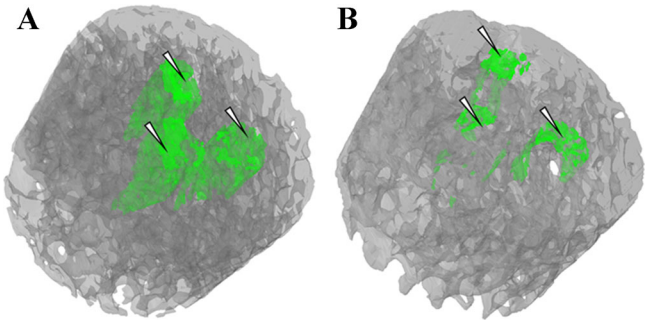

Figure 8. Comparison in the degree of bone healing at the microfracture hole by three-dimensional micro-CT. (A) At 8 weeks after first microfracture. (B) At 8 weeks after second microfracture. The microfracture hole area was repaired to lesser degree after second microfracture. Green areas represent repaired bone in the microfracture hole, and the arrowheads denoted the site of microfracture.

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SUPPORTING INFORMATION

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