

# Healing of Articular Cartilage Defects. An Experimental Study of Vascular and Minimal Vascular Microenvironment

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**ABSTRACT:** The repair potential in a chondral defect with (treatment A) or without access to bone marrow elements (treatment B) at the basis of the defect sealed by a rim-sutured periosteal flap was studied using adult New Zealand rabbits (22 weeks) as an experimental model. At sacrifice, macroscopical changes, synovial fluid contents, degree of filling, thickness of the cartilage rim, and the subchondral bone were evaluated. Histomorphometric measurements of extent of filling (mainly fibrous tissue) of the defect at 36 weeks postoperatively, showed 50% filling in treatment A compared with 33% in treatment B ( $p = 0.011$ ). A difference in height of the cartilage rim between the experimental groups and sham-control was measured ( $p = 0.005$ ). Cartilage degeneration was observed at the cartilage rim of the original defect, and included loss of chondrocytes and disruption of surface continuity in both experimental groups. In addition, treatment A resulted in a significantly increased thickness of the subchondral bone in the defect in comparison to treatment B at 2 weeks and at 36 weeks ( $p = 0.021$ ). The increased thickness of the subchondral bone may be of concern for the bone marrow stimulation techniques regarding the long-term outcome. © 2006 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. *J Orthop Res* 24:1069–1077, 2006

**Keywords:** subchondral bone plate thickness; cartilage repair; periosteal flap; bone marrow stimulation; chondral defect

## INTRODUCTION

Articular cartilage injury is relatively common. Of all knees subjected to arthroscopy 5–7% show a cartilage lesion larger than 1 cm<sup>2</sup> that is graded as ICRS grade 3 or 4.<sup>1,2</sup> One of the established treatment modalities to restore this cartilage defect is to use a periosteal coverage over multiple perforations of the subchondral bone plate in an attempt to create a biologically favorable microenvironment.<sup>3,4</sup> In this microfracture technique, multiple microperforations of the subchondral bone plate are used to create hemorrhage and access to the bone marrow.<sup>5</sup> Alternatively, when using the procedure of autologous chondrocyte

transplantation, it is considered important to preserve the subchondral bone plate because it is believed that the deposition of blood elements in the defect will interfere with the growth of the transplanted chondrocytes.<sup>5,6</sup> The importance of the vascular microenvironment in the defect in contrast to no or only minimal vascular environment has been discussed but not investigated.<sup>5</sup> Thus, the present study was conducted to evaluate the impact of vascular microenvironment on the healing process using periosteal flap coverage of a well-defined cartilage defect in an established animal model.

## MATERIALS AND METHODS

### Animal Care

Forty-one New Zealand rabbits of both sexes were used. They were kept in cages under standard laboratory conditions and feed standard diet with free access to water. At the initial surgery the experimental rabbits

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were 22 weeks of age weighing 3.6 kg (SD 0.42 kg). At sacrifice at 36 weeks the mean weight of animals was 5.2 kg (SD 0.6 kg). From the end of the first week after the final surgical procedure and until sacrifice the rabbits were allowed free activity in a 10 m<sup>2</sup> room. The experiment was performed according to the guidelines for animal research at the University of Oslo and approved by the Norwegian Government Committee for Experimental Animal Care.

### Experimental Groups

The rabbits were allocated into one experimental group (34 rabbits) with a chondral lesion made in both knees. Access to bone marrow was established at the base of the defect in one knee (treatment A, vascular microenvironment), whereas in the other knee the base was left untreated (treatment B, minimal vascular microenvironment) (Fig. 1A and B). After final surgery with repair of the defect 6 of these 34 rabbits were observed for 1 week, 12 rabbits for 2 weeks, and 16 rabbits for 36 weeks. In addition to these 34 rabbits in the experimental group 7 animals had an arthrotomy performed to one knee only with no cartilage intervention (sham-operated) and the contralateral knee was left untouched serving as normal control. These seven animals were observed for 36 weeks after surgery until sacrifice.

### Surgical Technique

A defect ( $\diamond = 4$  mm) was created in patella of both knees in the 34 experimental rabbits (Fig. 1A). The defect in the patella corresponds to 30% of the total articular cartilage area of the patella. Minimal vascular microenvironment was defined as a chondral lesion where all cartilage including the calcified layer was removed. The designation is used as no or only minimal communication between subchondral vessel and the base of the lesion may occur. Cover of the defect with a periosteal graft further reduces the influence of blood elements released into the joint immediately after surgery. In treatment A, four drillholes at the base of the defect supply bone marrow elements from the base of the defect into the chamber, thus representing the vascular microenvironment (Fig. 1A).

The surgery was performed under general anesthesia composed of a mixture of Hypnorm<sup>®</sup> and Dormicum<sup>®</sup> with dosages adjusted according to weight. Before each surgical intervention 2 mL of NaCl 0.9% were injected in the joint, and after the joint had been subjected to 50 repeated full range of motion one aliquot of this washout sample of minimum 0.75 mL was retrieved for analyses of proteoglycan concentration. In addition, local anesthesia (Marcain<sup>®</sup>) with epinephrine was injected locally at the wound edges at the start of surgery. An antibiotic (Vibramycin<sup>®</sup>) was given per orally as prophylaxis pre- and postoperatively for 5 days together with Temgesic<sup>®</sup> subcutaneously as pain relief. Medial parapatellar incisions were made in the skin, and the patella was dislocated laterally and inverted. A biopsy punch

( $\phi = 4$  mm) was used to induce the cartilage lesions. Three experienced surgeons specially trained in the procedure of harvesting periosteum and performing cartilage repair procedures in rabbits performed all the surgery. Dental instruments and a stereomicroscope (magnification  $\times 14$ ) were used to secure removal of all the cartilage in the defect. Care was taken to avoid any damage of the subchondral bone plate. Two weeks later the defects were repaired during rearthrotomy. Once again, the defects were debrided with the use of a stereomicroscope and dental instruments. Figure 1B shows the histology after this procedure demonstrating an intact subchondral bone plate in the defect with all or nearly all cartilage removed. In the experimental group of the defect in one of the knees underwent drilling of the subchondral bone plate. Four drill holes ( $\diamond = 0.6$  mm) were made by a hand-driven drill to a depth of 3 mm. In the contralateral knee the subchondral bone plate in the defect was left intact (treatment B). A periosteal flap was harvested from the medial part of proximal tibia in both knees and placed over the defect with the cambium layer facing the defect. Subsequently, the flaps were sutured with four or five 9.0 sutures to the rim of the defect, and the rim was covered with Tisseal<sup>®</sup> glue. The surgical procedure of treatment A and treatment B were consecutively changed between the right and left knee from one animal to the next without the surgeon changing position. Thus, difference between surgeons could not interfere systematically with the results. Furthermore, all three surgeons performed a similar number of treatment A and B.

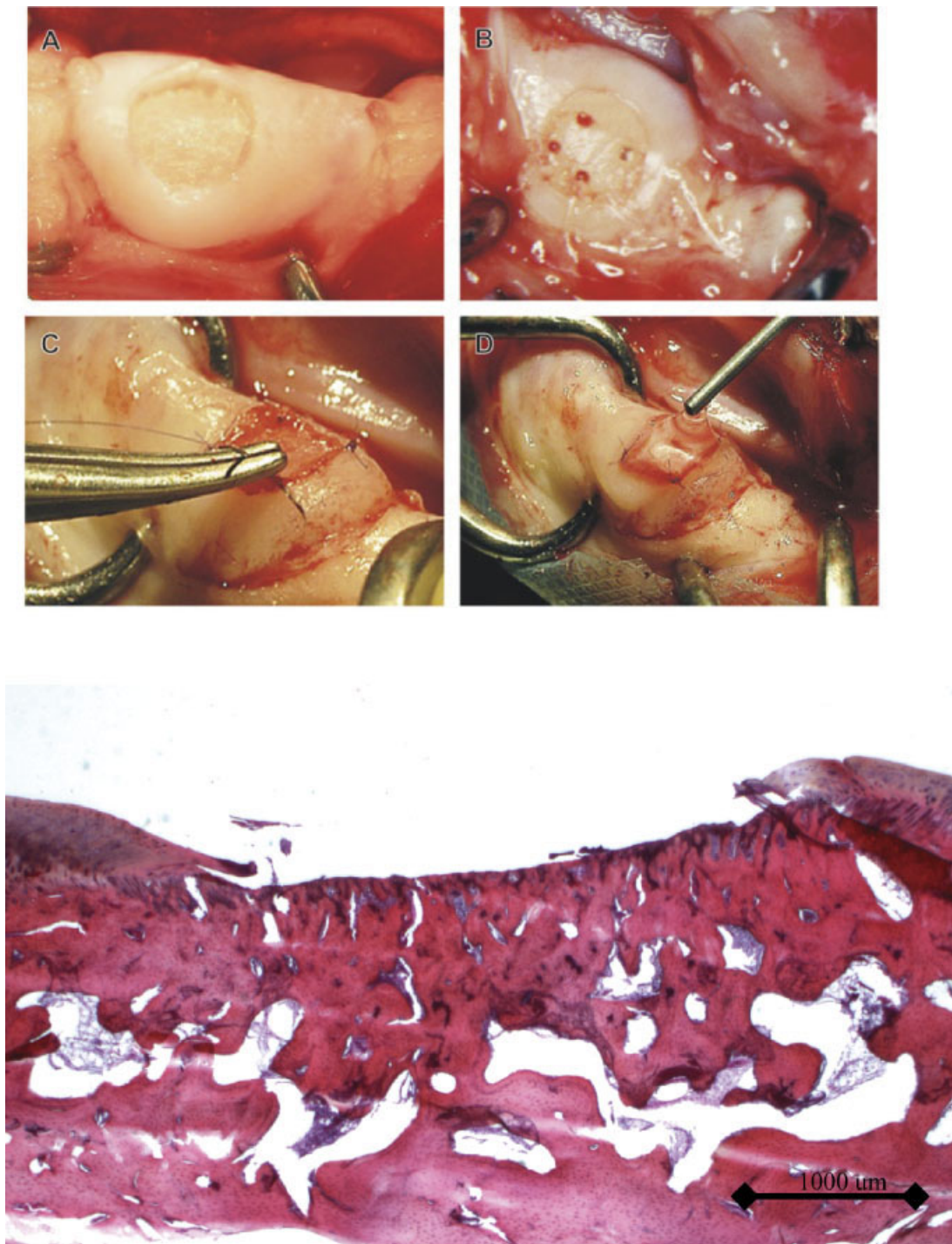
In the control group consisting of seven rabbits only a unilateral procedure was performed consisting of an arthrotomy and inversion of the patella to air 20 min before closure of joint incision as described. The unilateral procedure was repeated 2 weeks after the initial surgery, ensuring a similar overall setup as the experimental group. The other knee was left untouched until sacrifice 36 weeks after surgery. Postoperatively, no harmful effects were observed on gait among the animals subjected to bilateral surgery compared with rabbits undergoing a unilateral procedure only. By visual observations no difference in level of activity or locomotion pattern between the two groups was detected. Furthermore, the experimental group and the control group showed a similar degree of weight gain through the experiment until sacrifice.

### Macroscopical Evaluation

The knees were evaluated macroscopically at sacrifice. Signs of degeneration on the opposing articular surface in the patellar groove were examined to evaluate the long-term effects of containing such an articular cartilage defect in the joint.

### Histology

After sacrifice the patellas were dissected free and fixed in phosphate-buffered 4% paraformaldehyde for 48 h, and

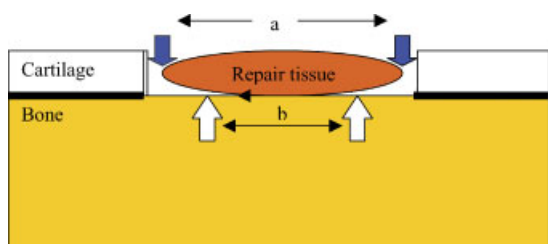


**Figure 1.** (A) Illustration of the surgical procedure to create the biological chamber in treatment group A. Photo A illustrates the creation step of the defect, following by step B showing penetration of the subchondral bone plate; step C is suturing the periosteal flap; and finally, step D is covering this repair with Tisseel<sup>®</sup> glue sealing off the biological chamber. Treatment group B is similar except that step B is omitted to keep the subchondral bone plate intact. (B) Empty defect with removed calcified layer. The micrograph illustrates also the bone marrow elements below the subchondral bone plate in patella. This is 1 week after repair, and demonstrates some wear of the edges and loss of the periosteal flap (HE, original magnification  $\times 1.25$ ).

decalcified in 7% EDTA with phosphate-buffered 0.5% paraformaldehyde. Subsequently, the patellas were divided in two through the center of the defect and embedded in paraffin wax according to a routine protocol. From each of these two parts five sections of 4–5  $\mu\text{m}$  thickness were made and the four sections that gave the best overview over the defect from each sample were subjected to morphometric measurements. For measuring filling of the defect micrographs of each of the four best sections of the defect from each sample were subjected to point counting according to the guidelines described by Romppanen and Collan in 1983.<sup>7</sup> The median value of these four measurements represented the value for each animal and served as basis for group mean values used for further statistical analysis. As was obvious, histologically which samples that had been subjected to perforation of the subchondral bone plate a blinded histological analysis was not possible to perform. Criteria according to previous described methods were used to quantitate the changes observed by histology.<sup>7,8</sup>

The proportion of the repair tissue in contact with the basis of the lesion was measured in four sections of each sample. Detachment was noted if there was a cleft between the repair tissue and the subchondral bone plate. Binding was defined as the distance of repair tissue in contact with basis of the lesion divided on the distance of repair tissue in the defect. The method for quantifying this binding is illustrated in Figure 2.

The thickness of subchondral bone plate was estimated using a semiautomatic interactive image analysis system (Analyses Soft Imaging System, Münster, Germany). Thus, the distance between the most advanced bone tissue islands (facing the defect/repair tissue in the experimental group and the calcified cartilage in the control group) and the most proximal parts of the bone marrow cavity<sup>8</sup> was measured. The identification of the borders was facilitated by the use of polarized light. The thickness of the subchondral bone plate was the median value of five measurements in each defect. The measurements were performed with the center of the defect as a starting point. In the control sham group this would be



**Figure 2.** Drawing of the method to evaluate subchondral binding of the repair tissue. Binding is defined as the distance between the white arrows (b) divided on the distance between the blue arrows (a). Tidemark is the upper border of the black broad line representing the calcified cartilage separating bone and uncalcified articular cartilage.

corresponding to the area with the thickest cartilage. From this center point two other measurements were performed 200 and 400  $\mu\text{m}$  lateral to the center point, respectively. A similar approach was also used to measure the articular cartilage thickness from tidemark to the surface in the center of the defect and 2.0 mm lateral from the center point at one side of the cartilage defect in the experimental group. In the control and sham-operated knees corresponding measured points from the center of the patella were made to enable comparison of articular cartilage thickness between the experimental group and the control-sham group.

### Analysis of Synovial Fluid

In the long-term observation group of 36 weeks and in the sham-operated knees of the control-sham group, a wash out sample of the synovial fluid was aspirated before each surgical procedure as previously described. Synovial fluid was analyzed for proteoglycan concentration using standard ELISA technique.<sup>9</sup>

### Statistics

According to previous experimental studies a filling percent difference of more than 25% was considered as a proper level to disregard the  $H_{01}$ -hypothesis of no difference between treatment A and B. Because all animals in the experimental group underwent both treatment A and B, a paired Student *t*-test could be applied for statistical analyses. Preexperimental analysis using power of 0.80 and a significance level of 0.05 to detect sample size indicated a need of nine animals in the experimental group. The long observation period and the risk of losing experimental animals were the reasons for the decision to use 16 experimental rabbits for the long-term observation. To test the  $H_{02}$ -hypothesis with no difference between the experimental group and the sham-control group the need of animals would be more than 12 due to the unpaired experimental situation. However, our results did show a filling percent difference of almost 50%, which means that the number of experimental animals in the control-sham group was sufficient. Standard Student *t*-test was used according to pairwise or unpaired comparison using. To check for reproducibility repeated morphometric measurements were performed on the same sections for the different evaluated parameters and the range difference between the measurements were 5–8%.

## RESULTS

At sacrifice at 36 weeks after repair the mean weight of the experimental rabbits was 5.1 kg (range 4.6–6.0 kg) and of the control sham group 5.0 kg (range 4.7–6 kg). Two animals were lost during surgery due to problems with anesthesia and two in the follow-up period. The postmortem

investigation at the Norwegian School of Veterinary Science did not detect the reason for death of these two last rabbits. Supplementary animals replaced the four lost during the experiment to avoid skewed groups.

### Macroscopical Evaluation

All knees showed full range of motion. The main finding was incomplete filling of the defect and the poor quality of the repair tissue in the defect compared to normal cartilage. No free bodies were observed. In the 2-week observation group the periosteal flap was delaminated in several cases and found incorporated in the synovial tissue. At 36 weeks no sign of the periosteal flap was seen either in the defect or in the synovium. Macroscopic signs of cartilage degeneration were not observed.

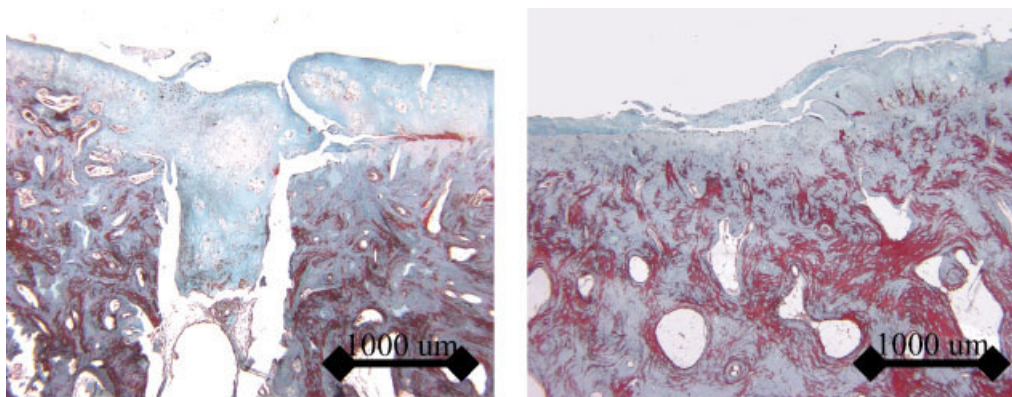
### Histomorphology

Histologic analysis of the defects verified that the defects were only partly filled. The repair tissue was fibrous tissue except close to the drill holes where some areas of unorganized cartilage was observed (Fig. 3). Clusters of chondrocytes embedded in the fibrous tissue with an incomplete binding to the subchondral bone indicative of a failed repair were evident even in the best cases. None of the experimental cases showed normal articular cartilage although some of the defects were almost filled. At 36 weeks wear of cartilage rim and loss of chondrocytes at the edges were evident in the experimental groups (Fig. 4).

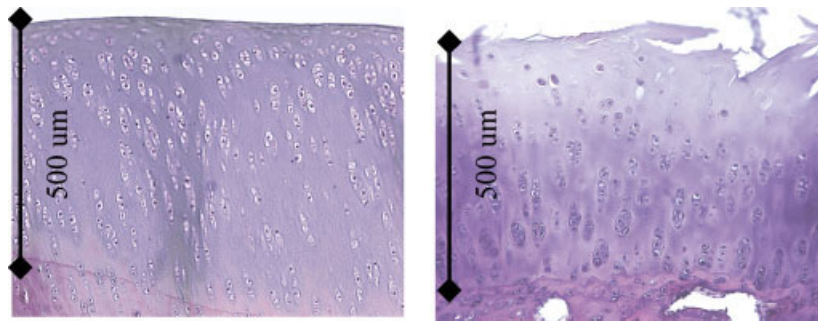
### Histomorphometry

One week after repair a mean filling as high as 40% was measured in treatment A (vascular microenvironment) and 30% in treatment B (minimal vascular microenvironment) (Table 1). This apparently high degree of filling was due to remnants or attached periosteal flaps. At 2 weeks after repair the filling had decreased to 14% in treatment A and 12% with treatment B. At 36 weeks after repair there was a statistical significant difference in filling between treatment A and treatment B ( $p = 0.011$ ). There was a considerable variation within the groups: the filling was up to 92% in the best cases of the vascular group and 89% in the minimal vascular group.

At 36 weeks the repair tissue detachment from to the subchondral bone plate in the defect was apparently more pronounced in treatment group B [43% binding, CI 95% (25–60%)] in comparison with treatment A [58% binding, CI 95% (44–72%)], but the difference did not reach statistical significance ( $p = 0.20$ ) (Table 1). Subchondral bone plate thickness was significantly increased in treatment A both at 2 weeks and after 36 weeks ( $p = 0.021$ ) (Table 1). Articular cartilage thickness was significantly thinner in all experimental groups compared with control-sham at both measured time points. Compared with the corresponding area in control and sham-operated knees, the cartilage rim showed significantly lower values in both treatment groups ( $p < 0.05$ ). Micrographs of the cartilage defect during the experiment are illustrated in Figure 5.



**Figure 3.** Micrographs of specimens after 36 weeks showing vascular microenvironment to the left including a drill hole with repair plug. Minimal vascular microenvironment is illustrated to the right from the contralateral knee in the same animal (trichrome staining,  $\times 2.5$ ). Evident in the micrograph to the right is the degeneration of the defect edge as a result of the improper filling of the defect.



**Figure 4.** Illustration of the wear and degeneration at the cartilage rim in the experimental group to the right in comparison to the control-sham group to the left (HE,  $\times 10$ ).

### Synovial Fluid Analysis

The proteoglycan content was not significantly different in the experimental group between treatment A and B ( $p = 0.22$ ) (Table 2). Neither could any significant changes in proteoglycan levels be recorded in any of the two treatment groups from the start of the experiment until sacrifice at 36 weeks later ( $p_A = 0.11$  and  $p_B = 0.52$ ). Comparison between the control and sham knees at 36 weeks also turned out without significant difference ( $p = 0.74$ ). At sacrifice none of the treatment groups were different from the untouched control knees regarding proteoglycan level ( $p_A = 0.75$  and  $p_B = 0.56$ ).

### DISCUSSION

The vascular environment (treatment A) resulted in higher filling percentage than the minimal vascular group (treatment B). Thus, it appears that vascular microenvironment is beneficial to the repair process. However, the repair tissue in

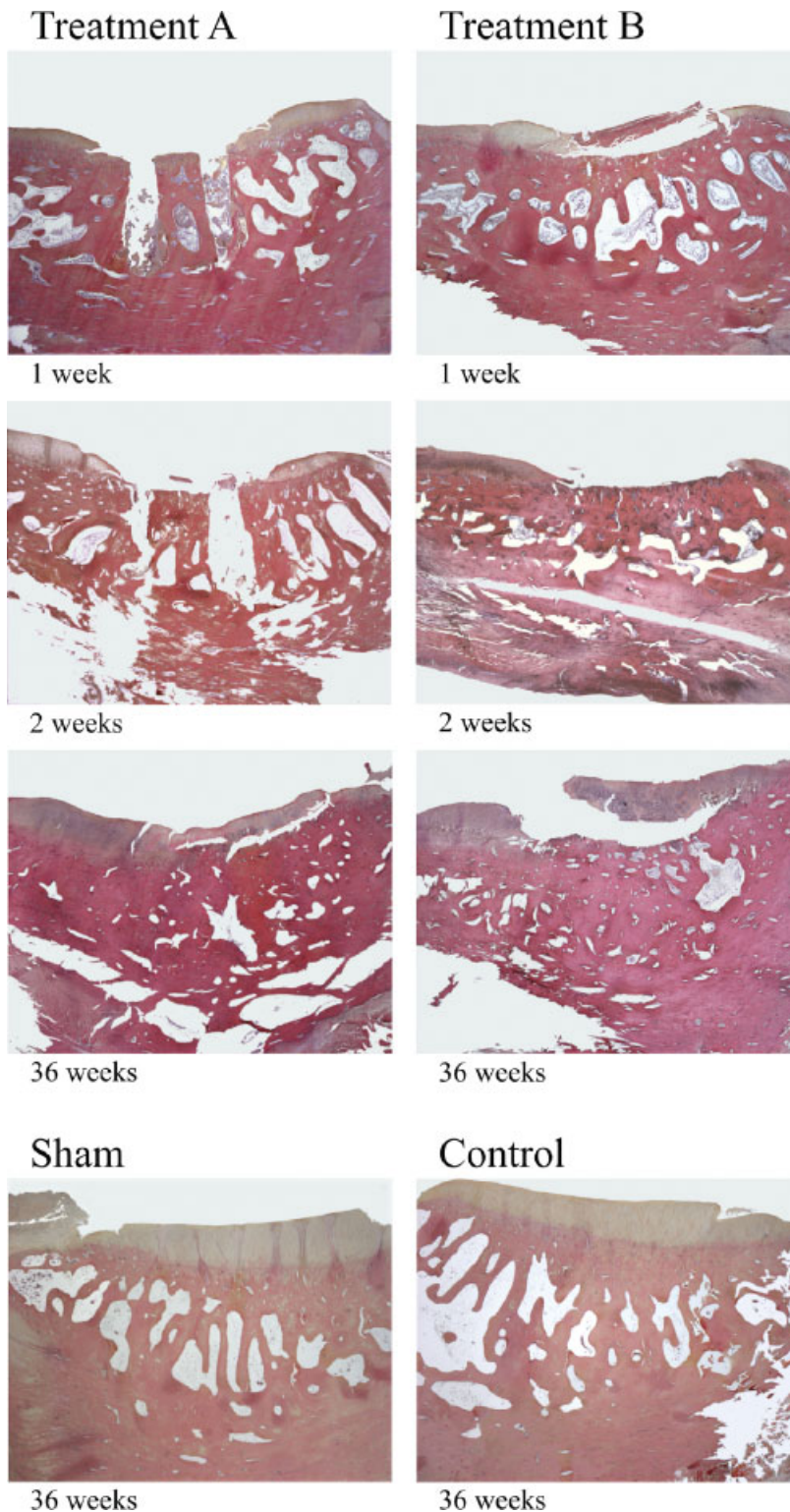
both groups was fibrous tissue, although small islands of cartilage tissue could be observed close to the drillholes in treatment A. Furthermore, inferior binding to the subchondral bone of the repair tissue was evident with both treatment A and B, although most profound in treatment B where only 44% of the repair tissue demonstrated binding to the subchondral bone. Similar experience has also been reported in clinical cases where delamination of the repair tissue has been observed after autologous chondrocyte transplantation.<sup>10</sup> The chondrocyte transplantation procedure is a minimal vascular method as the subchondral bone plate is not perforated.<sup>6,11</sup> In terms of filling our result in the minimal vascular group (treatment B) is comparable to those of other authors using the same experimental model.<sup>6</sup> Brittberg et al. (1996) found in his experimental study using only periosteal flap coverage of the defect with 52-week observation a filling of the defect of 30.9%. Our results show a filling of 33% 36 weeks after repair. Our study adds further information in showing that creating access to the

**Table 1.** Basic Evaluation Parameters of the Groups

Group	No of Knees	Mean Filling % of Defect	Subchondral Bone		Cartilage Thickness	
			Binding %	Thickness $\mu\text{m}$	Center $\mu\text{m}$	Rim $\mu\text{m}$
Control 36 w	7			622 $\pm$ 23	652 $\pm$ 27	495 $\pm$ 37
Sham 36 w	7			652 $\pm$ 59	805 $\pm$ 35	555 $\pm$ 48
T.A 1 w	6	40 $\pm$ 9	24 $\pm$ 10	580 $\pm$ 93	288 $\pm$ 87	356 $\pm$ 17
T.B 1 w	6	30 $\pm$ 8	12 $\pm$ 9	534 $\pm$ 86	156 $\pm$ 43	398 $\pm$ 43
T.A 2 w	12	16 $\pm$ 2	46 $\pm$ 11	706 $\pm$ 44	49 $\pm$ 16	366 $\pm$ 26
T.B 2 w	12	14 $\pm$ 3	34 $\pm$ 12	540 $\pm$ 22	26 $\pm$ 8	336 $\pm$ 24
T.A 36 w	16	50 $\pm$ 6	58 $\pm$ 6	687 $\pm$ 67	225 $\pm$ 41	351 $\pm$ 34
T.B 36 w	16	33 $\pm$ 6	43 $\pm$ 8	544 $\pm$ 34	175 $\pm$ 57	333 $\pm$ 48

The results are presented as mean  $\pm$  SEM of median values in each group.

T. A = treatment A with drillholes through the subchondral bone plate (vascular microenvironment). T. B = treatment B without drill holes (minimal vascular microenvironment).



**Figure 5.** Micrographs of the cartilage defect at different time points illustrating the measured increased subchondral thickness as a result of the cartilage repair procedure. The increased thickness is most pronounced in treatment A, where a significant difference was noted at the 36 weeks interval (Table 1). At the time point of 1 week the periosteum sutured to the defect is still visible. The tendency towards loosening of the repair cartilage is illustrated at the time point of 36 weeks after repair (HE, original magnification  $\times 1.25$ ).

bone marrow under a periosteal flap also results a somewhat higher degree of filling. Even though the increase is significant the quality of the repair tissue remains low. The procedure with perforating the subchondral bone under a periosteal flap coverage is in clinical use,<sup>4</sup> but based on our experimental results the prospects of restoring cartilage integrity must be low. Our results following microfracture are in accordance with another animal study using the same size and type of cartilage lesion.<sup>6</sup>

A new finding relevant to the treatment of focal cartilage defects is the observation that the subchondral bone plate over time increased in thickness following the penetration of the subchondral bone plate to access the bone marrow elements. Increased subchondral bone thickness may represent a risk factor for later development of cartilage degeneration. However, the fact that the macroscopic evaluation did not show degenerative changes in the joint supported by unchanged proteoglycan concentration in the synovial fluid suggest that the lesions did not induce major osteoarthritis during the observation period of the current study.

Another new aspect observed in the current study is that the incomplete filling appears to initiate a degenerative process at the microscopic level. The observation of reduced height of the neighboring cartilage of the patella in the experimental group may indicate that the adjacent cartilage was subjected to an extra wear. Alternatively, the procedure of suturing the periosteal flap to the rim of the cartilage might be responsible for the initiation of the degenerative process especially when the reparative procedure failed.

In clinical studies a cartilage defect with size of more than 2 cm<sup>2</sup> (14% of the cartilage area of human femoral condyle<sup>12</sup>), is used as inclusion criteria for surgical repair of the lesion. In comparison, in our model 30% of the articular joint surface of patella is removed and as it is not likely to obtain natural healing of a lesion of this size, such an injury would be eligible for cartilage repair treatment in the human knee joint.

As control we used the knees that were not subjected to surgery. This was done because a successful cartilage repair procedure means restoration of the anatomical structure of the cartilage and therefore normal cartilage is the "gold standard" control of all cartilage repair procedures. From this point of view the results in our study with incomplete filling of the chondral lesion in both treatment modalities represent failed procedures.

**Table 2.** Proteoglycan Concentrations Measured at the Different Time Points in the Experiment

Group-Timepoint	Mean (µg/ml)	CI-95 % (µg/ml)	No of Missing Values
T.A 0 w	6,4	4,7–8,0	0
T.A 2 w	41,2	3,4–78,9	0
T.A 36 w	10,4	7,7–13,1	1
T.B 0 w	8,5	5,8–11,3	1
T.B 2 w	23,3	5,1–41,4	0
T.B 36 w	13,2	7,1–19,2	3
Control-36 w	11,0	8,4–13,6	0
Sham-0 w	4,0	1,1–6,8	0
Sham-2 w	5,6	2,5–8,7	0
Sham 36 w	10,3	6,7–13,7	0

## CONCLUSION

Creating a vascular microenvironment in healing of a focal cartilage defect with a periosteal flap increases the filling of the defect significantly in comparison to a minimal vascular microenvironment. However, both approaches end up with incomplete filling of the defect and mostly in fibrous tissue instead of hyaline cartilage. Furthermore, a reduced thickness of adjacent cartilage in the experimental group compared to the control sham group was noted. Additionally, the increased thickness of the subchondral bone in treatment A may be of concern for the bone marrow stimulation techniques for the long-term fate of the repair tissue obtained as indicated previously.<sup>13</sup>

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