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Fixation compliance in a murine fracture model induces two different processes of fracture healing but does not lead to delayed union

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FIXATION COMPLIANCE IN A MURINE FRACTURE MODEL INDUCES TWO DIFFERENT PROCESSES OF FRACTURE HEALING BUT DOES NOT LEAD TO DELAYED UNION

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SUMMARY

Delayed unions are a problematic complication of fracture healing. Currently, they are diagnosed when already well developed and standard treatment is an operative procedure. In this study, a murine model for delayed unions was investigated to describe and further study the pathophysiological (delayed union) mechanisms of fracture healing. We hypothesized that interfragmentary instability combined with a cortical gap could induce delayed union. For this purpose, two internal fixators, one with a ¼ the bending stiffness of the other, as well as special surgical instruments, were developed for the mouse femur. μCT, radiographs, 4pt-bending tests and histological analysis demonstrated that the different fixator types led to two different healing pathways. The rigid plate induced only intramembranous ossification whereas the flexible plate induced a mixture of endochondral and intramembranous ossification. However, the different fixator types led to a delay in healing of only 3 to 5 days in the period between 14 and 21 post-operative days. Together, these results showed that fixation flexibility is necessary to induce secondary fracture healing in mice as that which occurs commonly in human cortical fractures, but that flexible fixation is not sufficient to induce a substantial delay in murine bone healing.

INTRODUCTION

Delayed unions remain a problematic complication of fracture healing with an incidence of 5 to 10% [15, 30]. Treatment usually involves a second operative procedure, often with significant economic impact and patient morbidity [27]. Some etiologies and their treatments are clear, but more often the reasons for why a fracture does not heal in a timely manner are not well understood [17].

Fracture healing is a process of bone regeneration recapitulating the embryonic processes of endochondral and intramembranous ossification [14]. It is a complex process involving differentiation, proliferation, migration and apoptosis of several mesenchymal cell types [21]. These cells and others communicate through growth factors and cytokines [9, 23] synthesizing matrix proteins as well as matrix remodelling enzymes [6] and angiogenic stimulators. This biological cascade is coordinated to restore mechanical stability and thus functionality of the fractured bone.

Functional tissue engineering through biological mechanisms, e.g., molecular agents, gene therapy, and cells is believed to be a potentially less invasive treatment option. Clinical trials have already shown some efficacy of local intra-operative applications of OP-1 [12] and BMP-2 [16] in certain type of fractures. However, these treatments are based on our general understanding and availability of these particular growth factors rather than on our understanding of the pathophysiological biochemical mechanisms during fracture healing [2, 22]. In an attempt to identify potential targets for biochemical diagnosis and intervention, it would be most helpful to first elucidate the physiological and pathophysiological mechanisms and then test appropriate agents for their efficacy as well as toxicity. However for this

purpose, well-standardized animal models of delayed fracture healing are required.

Overall, not many models to study delayed union can be found in the literature. One was established by Meyer et al utilizing an aging rat model [28]. However, they could not find a difference in protein expression, and hence this model did not seem to be useful for identifying therapeutic and diagnostic targets. Mice have been extensively used in basic research to investigate developmental biology issues such as bone and cartilage formation [18, 33]. In addition, availability of knockout mice and elucidation of the entire murine genome makes the mouse ideal for molecular biology based investigations [8]. But, established mice models are of non-unions rather than of delayed unions [4, 25], and their pathophysiological mechanisms may differ from each other. Recently, El-Zawawy et al reported a fracture healing delay in a smoking mouse model [10]. However, because smoking leads to special pathophysiology, which cannot be assumed for every delayed union, this particular model also may not be appropriate to identify general targets.

In humans, fixation instability and gap size are factors, which may lead to an increased incidence of delayed union. We hypothesize that this is also true in mice. Unfortunately until now, the use of mice as animal model in fracture healing has had a major limitation, specifically the size of mice bone. Due to this fact, the choices for fracture stabilization have been limited to intramedullary pins and external fixators [4, 7, 18]. Both of these methods have led to rather “unknown” as well as very flexible stabilizations. Rotational stability of non-interlocked pins is uncontrolled and often severe rotational malunions are observed. Unilateral external fixators often have a wide range

of stiffnesses resulting in variance of the callus size [3]. To make the fixators quite stiff, the fixator then became almost 25% of the weight of the mice and resulted in primary healing [32]. To achieve secondary healing, no fixation was attempted, but this resulted in malunions, which were difficult to quantify due to their wide variation in healing geometry [32].

To overcome this problem, a light plate with angle stable locking screws for mice was recently developed by Matthys-Mark and Perren [26]. This unique internal fixator can also be manufactured with different flexural rigidities. In this project, healing of a gap osteotomy in the mouse femur stabilized with two internal fixators of significantly different rigidity are characterized to test the hypothesis whether more flexible fixation will lead to delayed union. The development of this system will enable further study into the molecular events of delayed fracture healing.

MATERIAL AND METHODS

Mouse Model

In total, 120 female C57BL/6 mice, 20-25 weeks of age (RCC Ltd, Füllinsdorf, Switzerland), were used in this study. The mice were housed in group cages (except peri-operatively), with environmental enrichment such as a mouse house® (Tecniplast Deutschland GmbH, Hohenpeißenberg, Germany), under day/night light regime (12 hours light/ 12 hours dark), and with unlimited water and maintenance diet (Provimi, Provimi Kliba AG, Kaiseraugst, Switzerland). All procedures performed in this study were approved by the Animal Experimentation Commission of the Veterinary Office of the Canton of Grison, Switzerland and followed the guidelines of the Swiss Federal Veterinary Office for the use and care of laboratory animals.

The mice were randomly divided into two equal groups. The osteotomies in one group were fixed with a rigid plate and the other with a flexible plate (Figure 1a). The flexibility of the plates were measured in both the top/bottom and side/side directions, in a similar fashion to that of excised mouse femora (see below), while mounted on plexiglass cylinders of similar size to the mouse femur fragments, i.e. 2.1 x 2.1 mm cross-section with 0.45 mm gap (n = 7, each plate). In the side/side direction, the plates did not differ in stiffness, but in the top/bottom direction the flexible plate was only ¼ the stiffness of the rigid plate ($p < 0.0005$, independent t-test, Figure 1b).

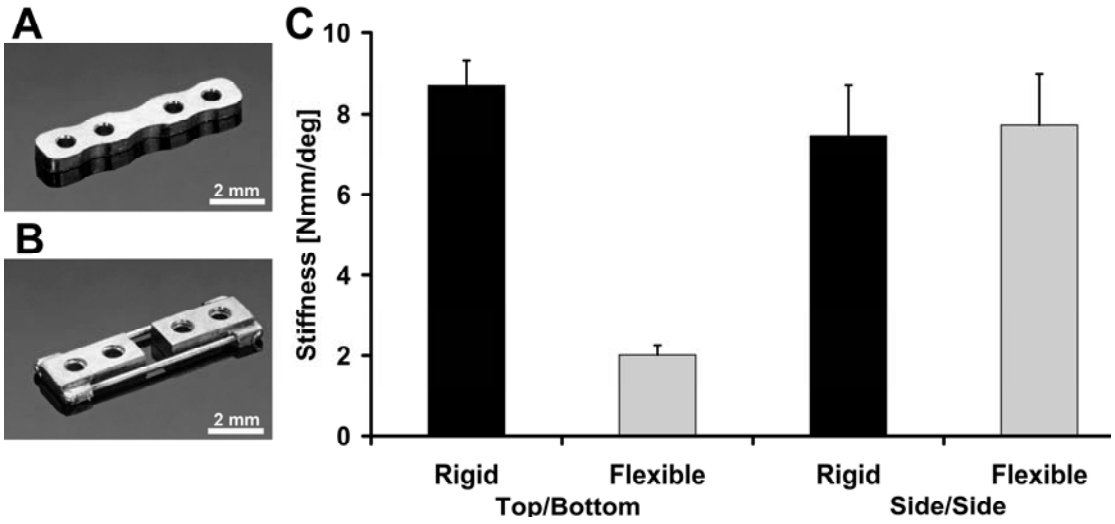


Figure 1: Oblique top view of MouseFix rigid (a) and flexible plates (b). In bending, the plates did not differ in side-to-side stiffness, but the flexible plates were ¼ as stiff as the rigid plate in top-to-bottom stiffness (c).

During surgery, general anesthesia was maintained with 2% isofluran (Isofluran Baxter ad us. vet.®, Baxter AG, Volketswil, Switzerland) at approx 200 ml/min O_2 via mask. A MouseFix™ plate (either rigid or flexible) was fixed to the femur. Briefly, the plate was attached to the anterior aspect of the mid-femur with four angle-stable screws through a lateral approach between the vastus lateralis and biceps femoris. Care was taken to avoid the sciatic nerve. Then a gigli saw with irrigation was used to make a 0.45 mm gap osteotomy at mid-shaft of the femur. After thorough irrigation, the wound was closed in layers (Vicryl® & Ethicon®, Johnson & Johnson Intl, St-Stevens-Woluwe, Belgium). Postoperatively, lateral radiographs were taken and skin staples were placed over the wound to discourage wound biting. For 48 hours post operatively, the mice received 0.1 mg/kg buprenorphine s.c. every 10-14 hr (Temgesic®, Essex Chemie AG, Lucerne, Switzerland). For 5 days post operatively, the mice also received paracetamol p.o. (Dafalgan Sirup für

Kinder®, 7 ml in 110 ml dinking water; UPSAMEDICA GmbH, Baar, Switzerland). Free weight bearing was allowed immediately after recovery from anesthesia.

Each fixator group of mice were equally subdivided into six groups, euthanized at 4, 9, 14, 21, 28, 42 days post-op (n = 10 mice/fixator/time-point).

Lateral view radiographs were taken post-mortem and both femora from each mouse were excised. After 9 days or more of healing, the osteotomy site was well encapsulated and the adjacent soft tissues could be easily removed. In the 4 day group, much of the adjacent soft tissue was left attached to the osteotomy site.

Mechanical Testing

In the mice euthanized after 21, 28 and 42 days of healing, the plate was gently removed and both femora were immediately tested in non-destructive 4pt-bending hydrated with Ringer's lactate. Femora with less duration of healing were not tested because they were difficult to bend without breaking. Femora were bent with the former plate position on the compression side at a deformation rate of 2.1 deg/min to a maximum of 4.5 Nmm/deg (°). Each femur was re-positioned and bent 3 times. The linear portion of the curve was used to calculate the bending stiffness. The two most similar stiffnesses were averaged and the healing femur stiffness was normalized by the contra lateral intact femur stiffness.

μCT

After excision or mechanical testing, all femora with osteotomy were fixed in 100% methanol for 10 days. In femora after 9, 14, 21, 28 and 42 days of healing, plates were gently removed, if not already, and the osteotomy site was evaluated using microtomographic imaging (μCT 40, Scanco Medical, Bassersdorf, Switzerland). The X-ray tube was operated at 70 kVp and 114 μA with a 200 ms integration time. The long axis of the femur was lined orthogonally to the axis of the X-ray beam by attaching two pins inserted in the most proximal and distal screw holes to a custom-made holder. Four hundred 2-dimensional microtomographic transverse cross-section images were scanned and reconstructed in 1024 x 1024 pixel matrices from 400 projections using a standard convolution-back-projection procedure. Images were stored in 3-dimensional arrays with an isotropic voxel size of 12 μm. A volume of interest within the two innermost plate screws on each side of the gap was selected. A 3D Gaussian filter with a sigma of 0.8 and a support of one voxel was used to partially suppress noise. A thresholding procedure, adapted from the protocol of Gabet et al to mice bones based on histogram of attenuation distribution [13], was used to segment tissue into three attenuation types, i.e. soft tissue (< 145); woven bone (low mineralization, 145 to 360); and lamellar bone (high mineralization, > 360, in per mille of maximal image gray value). Three-dimensional reconstructions were used to measure sub-volumes of the segmented bone types in a volume of interest between the most extreme screw holes as well as to qualitatively evaluate bone healing.

Histology

Femora of 8 mice per time point and fixator were embedded in Polymethylmethacrylat (PMMA), serially sectioned on a circular saw (Leitz 1600 Saw microtome®, Leica AG, Glattbrugg, Switzerland), and the mid-longitudinal section of each femur were ground and polished down to 80 – 100 μm thick sections (Exact Micro grinding System®, Exakt Apparatebau, Norderstedt, Germany). Their surface was etched with 1% formic acid (Fluka, Buchs, Switzerland) for 30 seconds, rinsed, stained with 1% Toluidin blue (Fluka) for 15 minutes and blot dried after washing in deionized water. Sections in the region between the outer screw holes were examined with a macrofluoroscope at x64 magnification (MacroFluo™, Leica Microsystems, Heerbrugg, Switzerland). Images were recorded in piecemeal with AxioVision software (Carl Zeiss AG, Feldbach, Switzerland) and stitched together with Adobe® Photoshop (Adobe Systems GmbH; Zurich, Switzerland). Eleven regions of interest, i.e. posterior-proximal-periosteal, posterior-gap-periosteal, posterior-distal-periosteal, posterior-gap-cortical, intra medullar-proximal, intra medullar-gap, intra medullar-distal, anterior-gap-cortical, anterior-proximal-periosteal, anterior-gap-periosteal, anterior-distal-periosteal, were delineated manually. In each region of interest (ROI), a custom macro (KS400, Zeiss) was used to measure the area of woven bone, lamellar bone, cartilage and total callus area. Each processed image was visually verified for proper segmentation and adjusted manually when necessary.

Two out of ten femora per time point and fixator were decalcified in 12.5% EDTA (Schweizerhall Chemie AG, Basel, Switzerland) with 1.25% Sodiumhydroxide (Fluka) for 7-14 days at room temperature until

decalcification, as confirmed with radiography, was completed. They were then stored in 100% methanol afterwards. Twenty-four hours prior to cutting, specimens were transferred to PBS with 5% sucrose. Mounted with freezing medium, mid-sagital 12 μm thick sections were cut with a cryostat (HM 560, Microm International GmbH, Walldorf, Germany). During the freezing process plates in the 4-day group were removed. Sections were immunolabeled with collagen II (CIICI developed by Holmdahl and Rubin, Developmental Studies Hybridoma Bank, NICHD, University of Iowa, Department of Biological Sciences, Iowa City, IA, U.S.A.) and with collagen X (polyclonal rabbit Ab kindly provided by Professor D. Chang, University of Hong Kong, Department of Biochemistry, Hong Kong SAR, China). After labeling, sections were stained with the Vectastain DAB™ Peroxidase Substrate Kit (Vector Laboratories Inc, Burlingame, CA, U.S.A.) and the Vecastain M.O.M.™-Kit (Vector Laboratories Inc) and DAB™ Kit, respectively. Both stains were counterstained with Meyer's hematoxylin (Sigma) and differentiated with 1 ml NaOH. Sections were qualitatively evaluated with the Axioplan® Imaging 2 microscope (Zeiss).

Statistics

All data subgroups were found not to differ significantly from a normal distribution (Shapiro-Wilkes). A univariate analysis of variance was performed with fixator type and healing time as factors in a full factorial GLM (significance threshold $p < 0.05$). Differences between fixator groups at specific time points were tested with an independent t-test (significance threshold $p < 0.01$, using a Bonferroni correction).

RESULTS

Radiographs, Mechanical Testing and µCT

From 14 days onwards, radiographs exhibited an abundant amount of callus around fractures that were treated with the flexible fixator reaching a peak size around day 21 (Figure 2b) and then decreasing by day 42. Hardly any callus was observed on femora treated with the rigid fixator (Figure 2a).

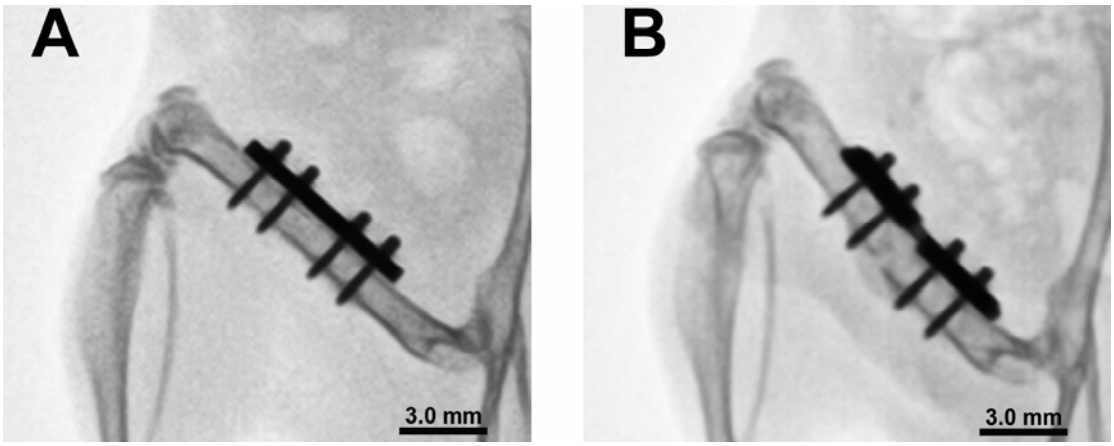


Figure 2: Radiographs (lateral view) exhibited hardly any callus on femora treated with the rigid fixator (day 21, a) but an abundant amount of callus was observed around fractures that were treated with the flexible fixator reaching a peak size around day 21 (b).

After 21 to 42 days of healing, the stiffnesses of all osteotomized femora were approximately 50-70 % of contralateral intact femora (Figure 3). The stiffnesses of the osteotomies were not significantly different between fixator types ($p = 0.41$). However, they tended to differ in their development over time with the osteotomies stabilized with the flexible fixator exhibiting a steeper

continued increase in stiffness compared to those stabilized with the rigid fixator (p = 0.08).

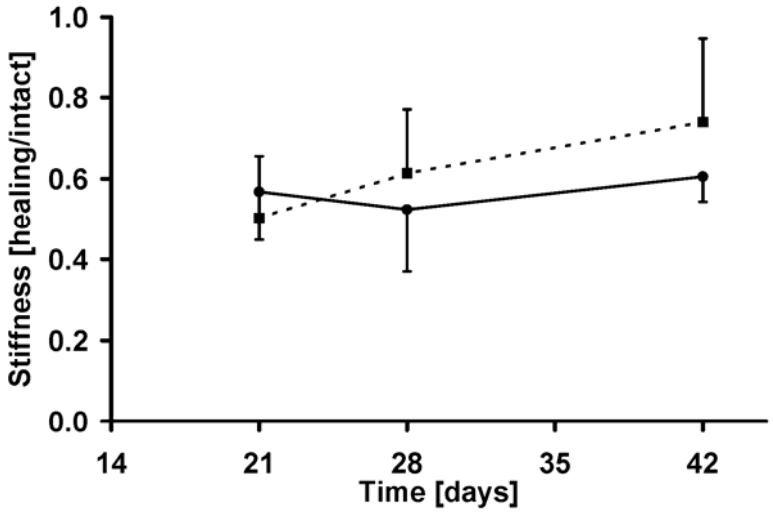


Figure 3: During 21 to 42 days of healing, the bending stiffnesses of all osteotomized femora were approximately 50-70 % of intact. The stiffnesses of the osteotomies were not significantly different between fixator types (p = 0.41). However, the osteotomies stabilized with the flexible fixator (–▪– –▪–) tended (p = 0.08) to continue to increase in stiffness, while those stabilized with the rigid fixator (•—•) did maintained a similar stiffness during the same period. The graph shows the bending stiffness (mean +/- SD) of the healed femur as percentage of contralateral intact femur.

Results observed in the radiographs were confirmed with quantitative µCT evaluation (Figure 4).

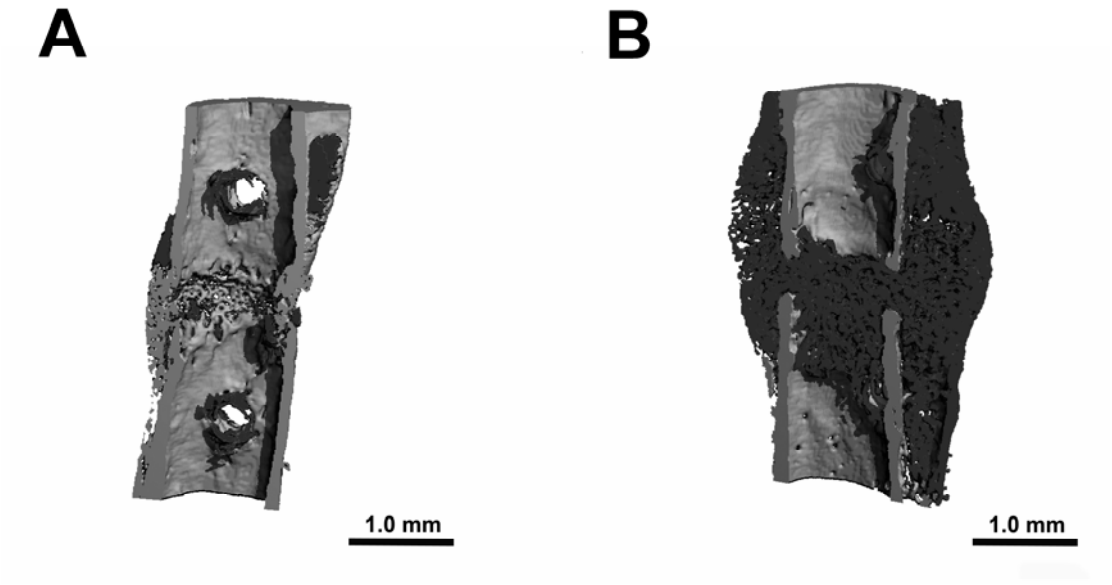


Figure 4: The segmented bones volumes (lamellar = light grey, woven = dark gray) resulting from the µCT-analysis showed that with a rigid fixator most of the woven bone was already remodeled into lamellar bone in the gap at day 21 post-op (a). On the contrary a larger callus with substantially more woven bone could be observed when the osteotomy was fixed with a flexible fixator (b, 21 days post-op).

With both fixators, the amount of woven bone started to increase at day 9 (Figure 5a). The amount of woven bone was significantly greater with the flexible fixator ($p < 0.001$), especially on days 14-28. The maximum woven bone volume occurred between day 14 and 21 with the rigid fixator and approx. at day 21 with the flexible fixator. With both fixators, as woven bone was resorbed, lamellar bone volumes increased similarly (Figure 5b). This remodeling started on approx. day 21. At day 42, there was hardly any woven bone observed in the rigid fixator group whereas there was still a small volume in the flexible fixator group. This difference was approx. equal to the

difference observed in lamellar bone volume between the two groups at day 42.

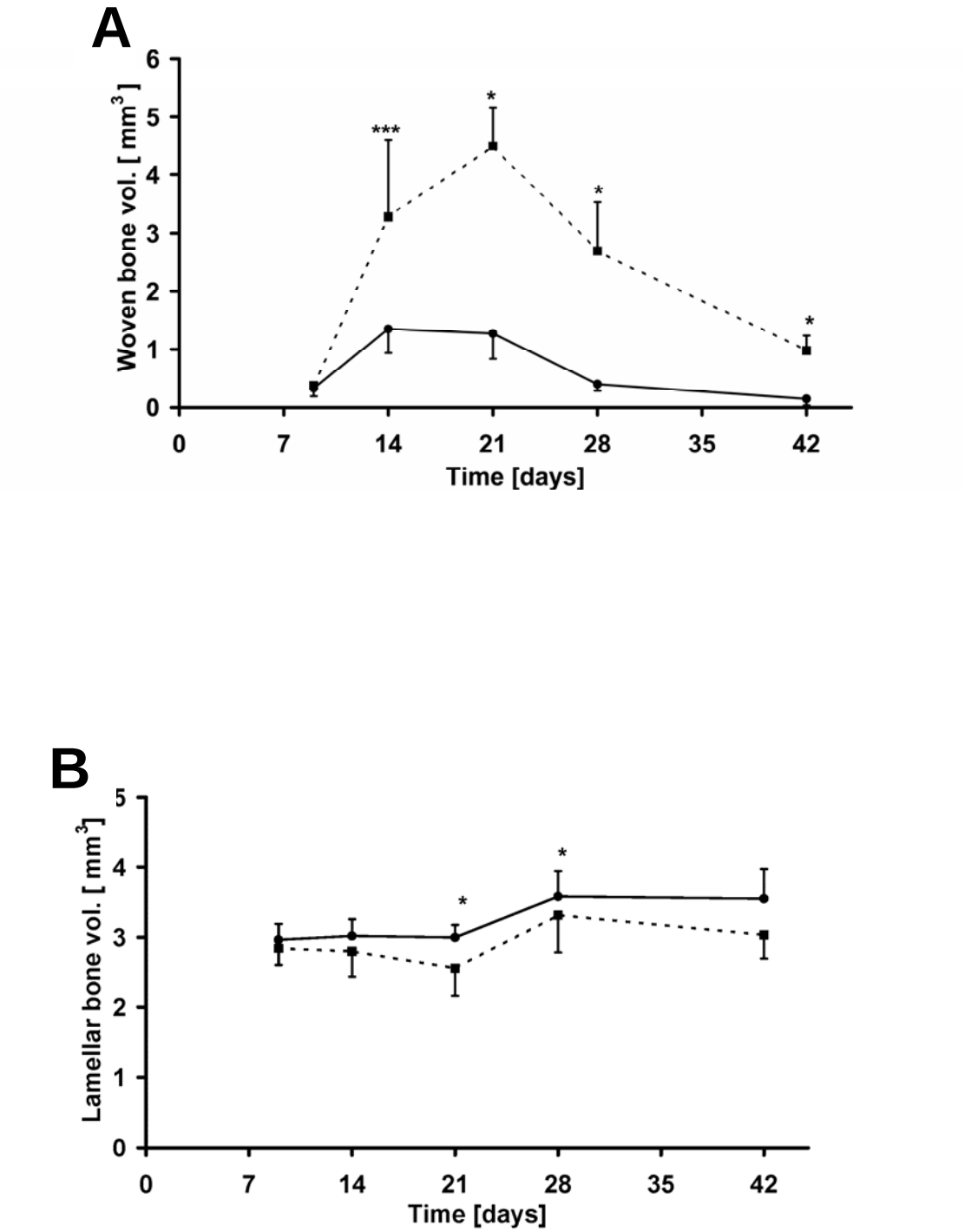


Figure 5: Quantification of µCT data indicated that the amount of woven bone started to increase at day 9 (a). Woven bone volume was significantly greater

with the flexible fixator ($p < 0.001$), especially on days 14-28, reaching a maximum between day 14 to 21 with the rigid fixator and at day 21 with the flexible fixator. As woven bone was resorbed, lamellar bone volumes increased similarly (b), starting on day 21 (rigid •—•; flexible –▪– –▪–; points represent mean +/- SD; *** is $p \leq 0.001$; ** is $p \leq 0.01$; * is $p \leq 0.05$).

Histomorphometry

In general, the histological findings were consistent with the results observed with µCT analysis (Figure 6). Callus size was significantly smaller ($p < 0.001$) with rigid fixation whereas in the flexible group excessive callus formation was observed (Figure 6 and 7). In the flexible group a significant ($p < 0.05$) increase of the woven bone area could be observed between 9 and 21 days of healing. Afterwards the amount dropped as the bone remodeled till day 42. The amount of lamellar bone started to rise on day 14 and reached a plateau around day 21. Compared to this, in the rigid group the amount of woven bone was significantly lower ($p < 0.001$) and peaked already on day 14. The curve of the amount of lamellar bone followed the one of the flexible group but did not reach a lower plateau until day 28. Although the total amount of lamellar bone was lower, the difference was not significant. With a significant increase of cartilage in the flexible group between day 9 and 21 ($p < 0.05$), the greatest mean cartilage area was observed on day 21, which was significantly higher ($p < 0.001$) than with rigid fixation where hardly any cartilage formation could be observed. Finally, the overall percentage of mineralized callus over time was significantly lower ($p < 0.001$) with the flexible fixation compared to the rigid plating.

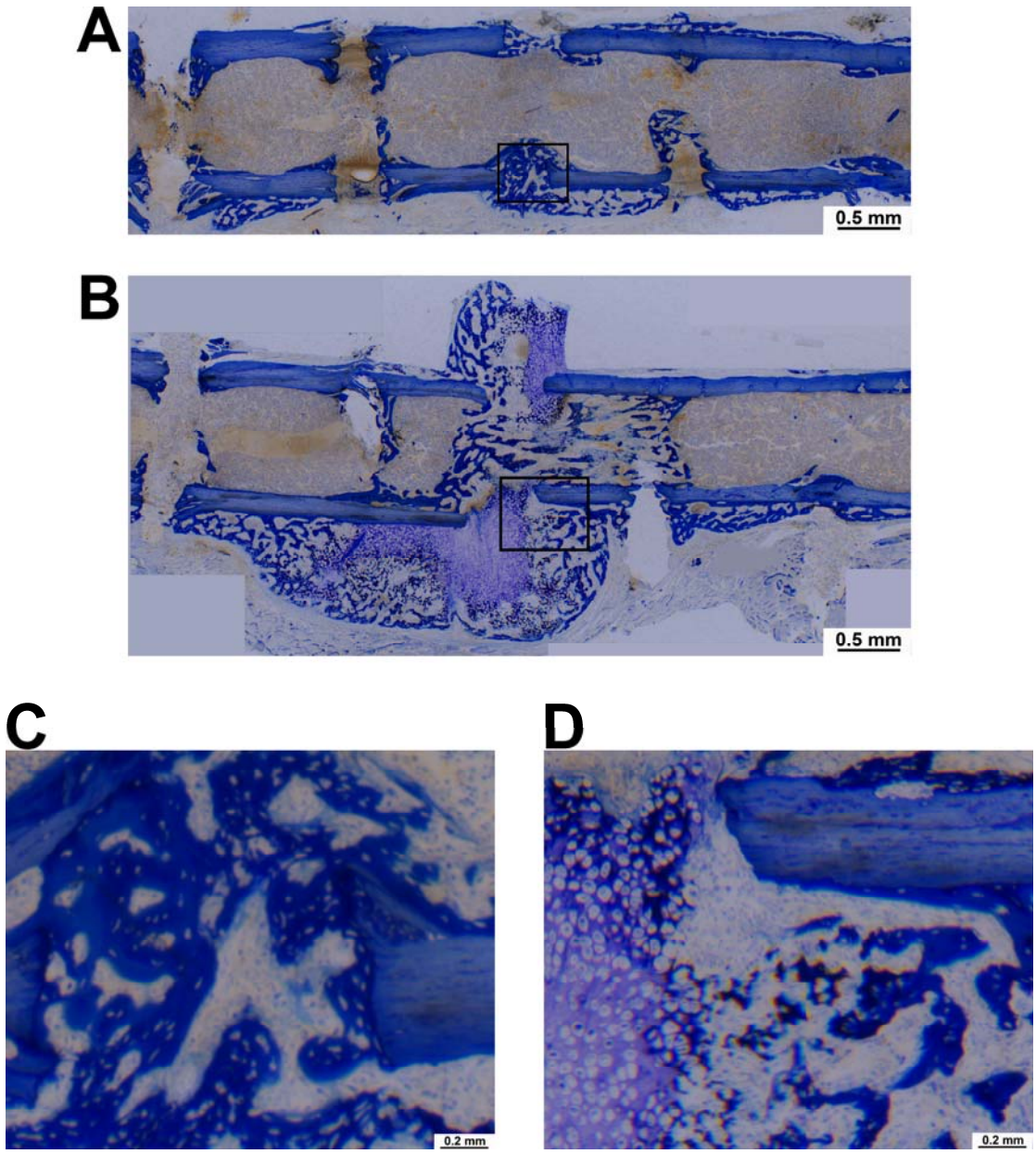
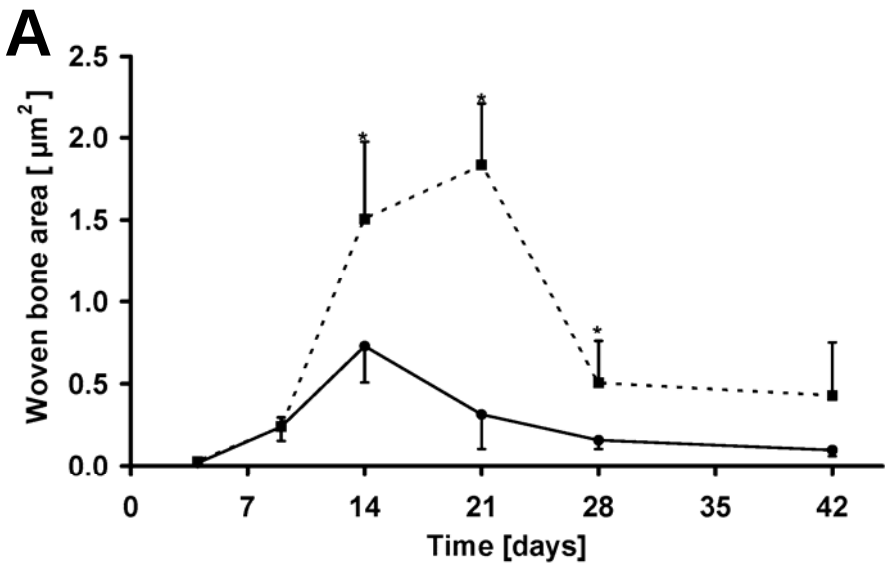
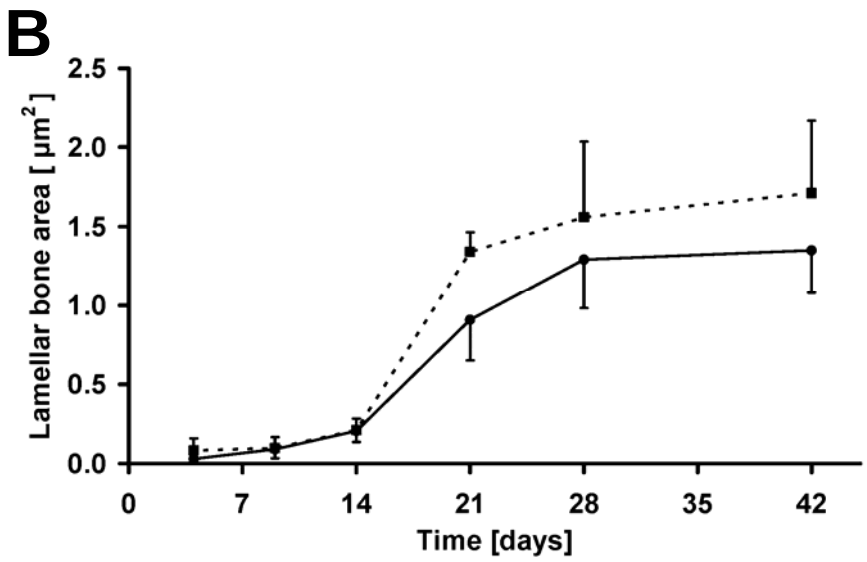


Figure 6: Histology of the mid-sagittal section through the osteotomy gap after 14 days post-op, stained with toluidine blue (plate position, above). With the rigid fixation woven bone formation was limited mostly to the gap (a and c). With flexible fixation, callus size always much bigger (b). The upper periosteal area is blocked by the plate. Additionally bridging in the external periosteal callus can be observed (b) and cartilage present in the gap is undergoing endochondral ossification (d).


A
Woven bone area [µm²]
2.5
2.0
1.5
1.0
0.5
0.0
0
7
14
21
28
35
42
Time [days]
*
*
*


B
Lamellar bone area [µm²]
2.5
2.0
1.5
1.0
0.5
0.0
0
7
14
21
28
35
42
Time [days]

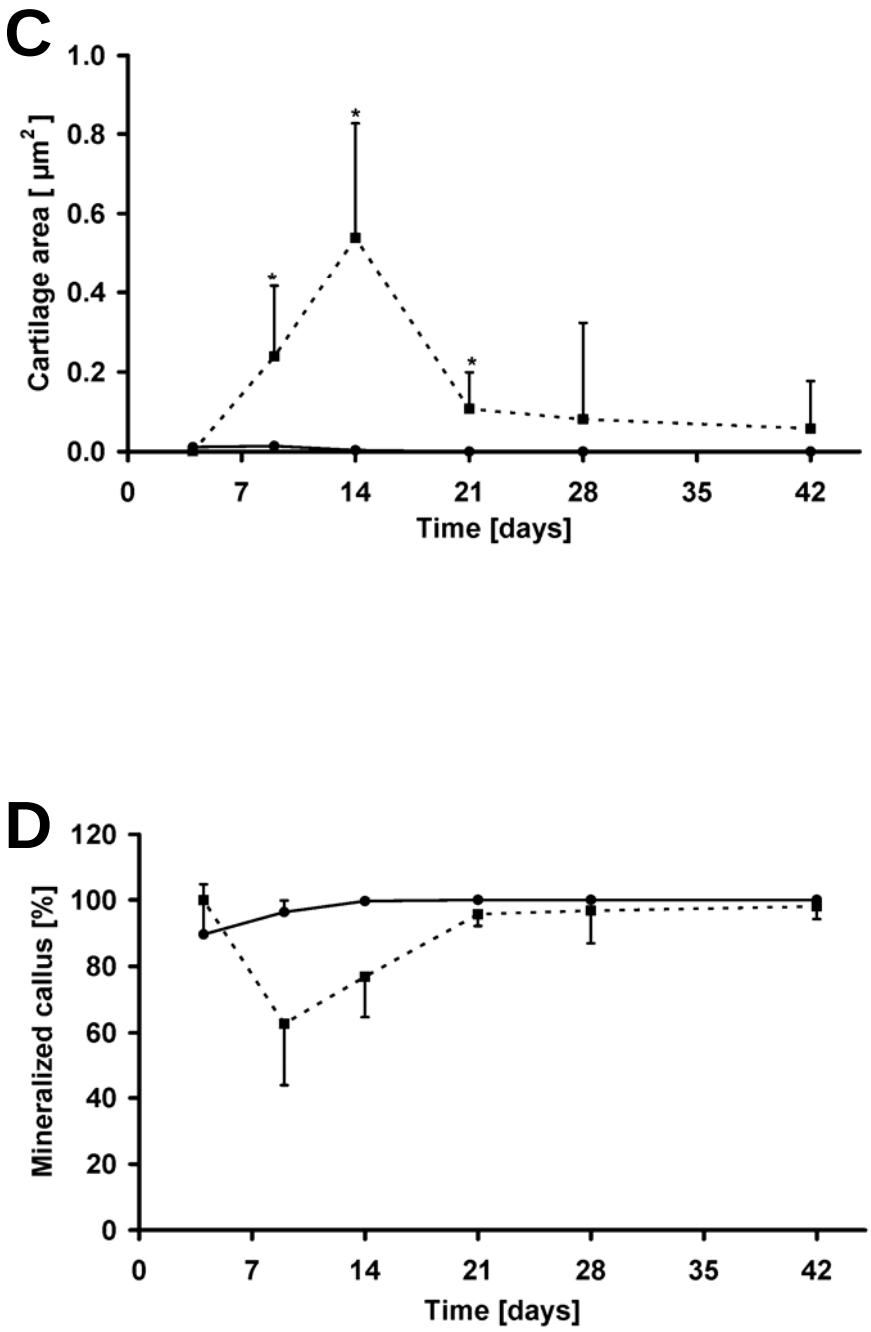


Figure 7: Histomorphometry indicated an increase in woven bone area between 9 and 21 days of healing for both fixator types, afterwards dropping until day 42 (a). Lamellar bone area started to rise on day 14 and reached a plateau around day 21 (flexible, b) and day 28 (rigid, b). In the rigid group,

woven bone area was significantly lower ($p < 0.001$, a) and peaked on day 14 compared to day 21 for the flexible group. Although the total amount of lamellar bone was lower for the rigid group, the difference was not significant. There was a significant increase of cartilage in the flexible group between day 9 and 21 peaking on day 21 (c). This was significantly greater than with rigid fixation where hardly any cartilage formation could be observed. Finally, the overall percentage of mineralized callus over time was significantly lower with the flexible fixation compared to the rigid plating (d) (rigid •—•; flexible –▪– –▪–; points represent mean +/- SD; *** is $p \leq 0.001$; ** is $p \leq 0.01$; * is $p \leq 0.05$).

Immunohistochemistry

Staining with collagen II antibodies confirmed that the tissue identified as cartilage with conventional staining and classified as such in histomorphometric analysis was indeed cartilage. In general the rigid group showed very little collagen II positive tissue (Figure 8).

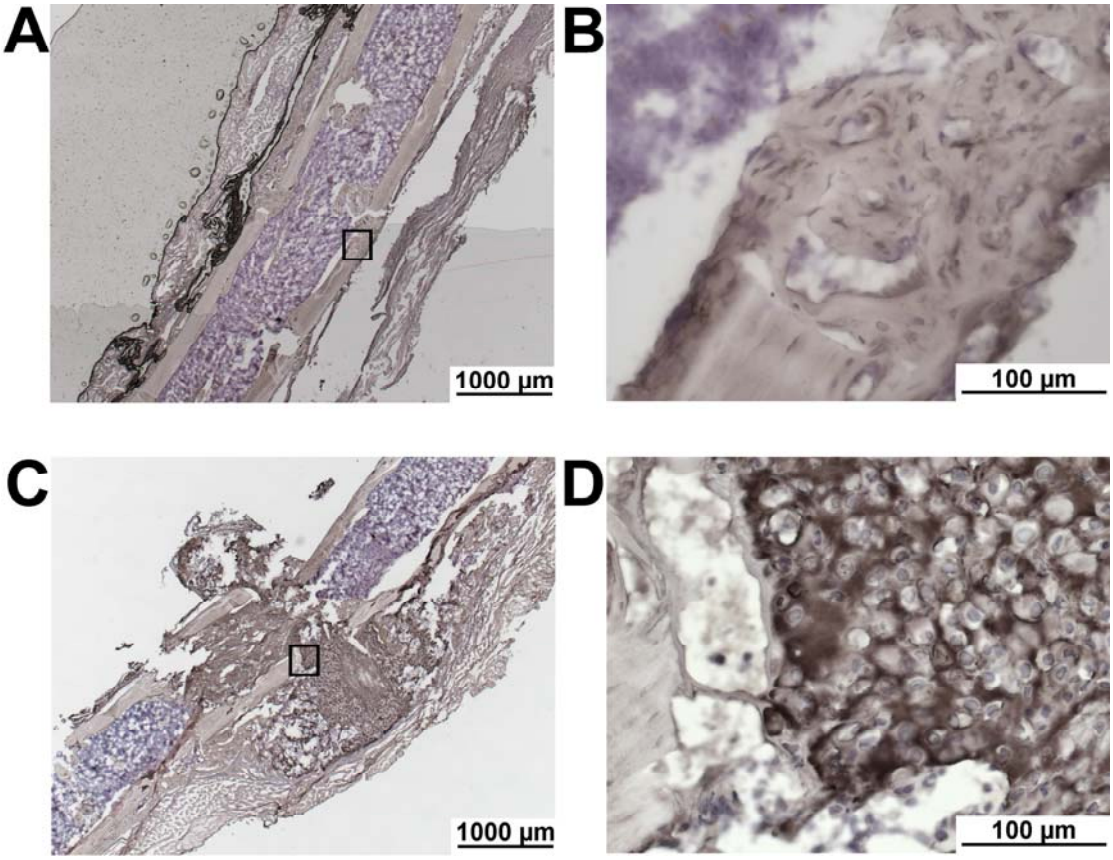


Figure 8: Immunohistochemistry with collagen II antibody and DAB confirmed the absence in the rigid (a and b) after 14 days of healing but the existence of cartilage in the flexible (c and d) fixated group. The larger field images are 2.5x and the small field at 40x magnification.

In contrast, with flexible fixation, collagen II positive tissue was first observed as early as day 9, peaked at day 14 and dropped again thereafter. The amount of the collagen X positive tissue followed a similar pattern to that of collagen II but appeared approx. 5 to 7 days later (Figure 9).

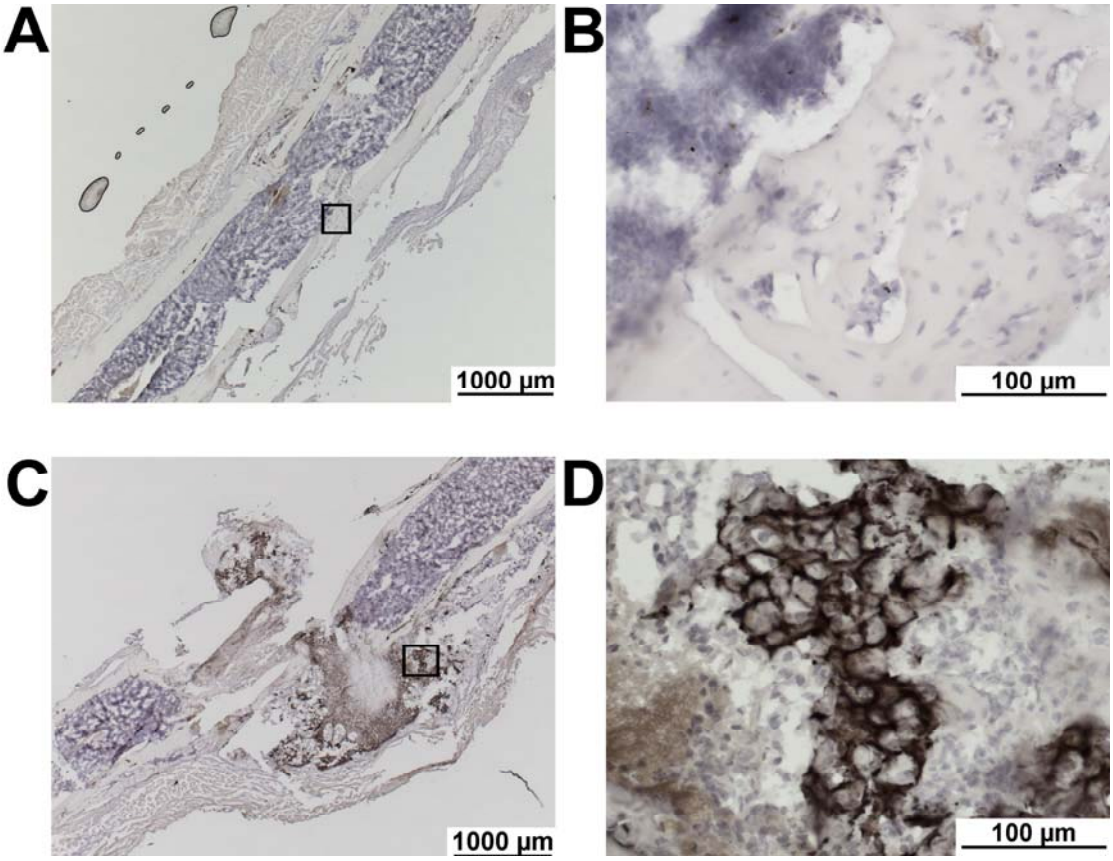


Figure 9: Staining with collagen X antibody, M.O.M™-Kit and DAB could not detect collagen X in bone fixated with the rigid plate (a and b, at 14 days post-op). In contrast the same staining demonstrated hypertrophic cartilage observable during enchondral ossification near the bone/cartilage interface in bone fixated with the flexible plate (c and d, at 14 days post-op). The larger field images are 2.5x and the small field at 40x magnification.

DISCUSSION

The objective of this study was to develop and characterize a delayed union model of cortical bone healing in mice. In humans [27] and large animals [5] increased interfragmentary motion and a cortical gap are often associated with delayed unions. It was hypothesized that this would also be true in mice. However, healing with a flexible plate that was only a quarter of the stiffness of a more rigid plate resulted in a delay of only a few days to a week as indicated by bone formation, measured by μCT and histomorphometry, and by continuation of increasing stiffness, measured by mechanical testing. This may be a consequence of faster bone healing rates in mice. However, when normalized by averaged healing time in mice (4 weeks) [1, 18] and humans (16 to 24 weeks [20]), a delay of a few days in mice would at best be 3 weeks in humans. Although a delay, this would not be substantial enough to make this a reliable murine model of delayed healing comparable to that in humans commonly observed in the clinic. A more likely reason for the lack of a substantial delay is that the difference in flexibility induced two different mechanisms of bone healing making the temporal rates of healing not comparable. This difficulty to achieve a murine delayed union model has also been previously experienced by others. Recent murine models had difficulty to achieve standardization in their course of healing [24] or lead to more non-unions rather than reproducible delayed unions [4].

In humans, shaft fractures heal either primarily through osteonal remodeling or secondarily through a combination of intramembranous and endochondral ossification [31]. In the present study two different paths of secondary fracture healing were observed. With rigid fixation, the gap was directly filled with

woven bone till day 14 and then remodeled into lamellar bone by day 21. Hardly any periosteal callus and cartilage tissue were detected. Thus, the rigid plate induced only intramembranous ossification similar to that observed in human cancellous bone healing but not commonly observed in cortical shaft fractures [35]. In contrast, fixation with the flexible plate led to a mixture of endochondral and intramembranous ossification in the classical pattern. First, a callus formed through intramembranous ossification from the periosteum. Then, cartilage developed in the gap leading to abundant callus growth radially. The cartilage then underwent endochondral ossification with the formation of woven bone, which was finally remodeled into lamellar bone. In this case the remodeling started also around day 21 but was not completed till then end of the observation period. This is the classic healing pattern observed in human cortical secondary bone healing [20].

These interspecies differences in bone healing between mice and humans may be simply a difference in scale. The cells and matrix molecules do not differ significantly in size between species, but the fracture gap and interfragmentary motion differ by several orders of magnitude. Hence physical processes, such as diffusion, fluid flow and cell migration distances, which would affect the behavior of cells, would not be similar between species. Alternatively, there may be biological process which are indeed different between species, e.g. osteonal remodeling [19]. Although the reasons for this difference in healing are unknown, this same bone healing reaction in mice has also been observed by others. Hiltunen et al showed that rod fixation in the mouse tibia allowed certain amount of bending and other fracture site movement, which was necessary for the formation of a fracture callus [18].

Cheung et al found that altering the stiffness of their external fixator led to differences in callus size resulting in a smaller callus size when using a stiffer fixation method [3]. Consistent with these findings, Thompson et al used a stiff external ring fixator to create a model for intramembranous ossification during fracture healing and compared this to non-stabilized fractures [34], which healed through endochondral ossification with through a large cartilage [11, 23].

The methods used in our study to assess bone healing are generally well established methods. The μCT provides valuable 3-dimensional information quickly and at high resolutions. However, segmentation is based solely on absorption of radiation energy and is only an approximate measure of less (woven) and more (lamellar) dense bone material [13]. Hence, histomorphometry analysis was used in this study and confirmed μCT measurement. This is also the reason why only the mid-sagittal sections were analyzed and only a few samples were analyzed with immunohistochemistry, i.e. to confirm other methods of measurement. Finally, mechanical testing was the least satisfactory of all analyses done in this study. Although it did indicate difference in the temporal development of stiffness between fixation types, 4pt-bending required a few repetitions to achieve reliable measurements. In our study, bending was selected instead of torsion, because it is the dominant physiological load applied to the femur in mice whose hips and knees are always flexed, and because the plate induced a non-axisymmetrical geometry of bone healing.

Regardless of the differences in healing, the other outcome of this study was the successful use of the MouseFix™ plate with angle stable screws [26].

Internal fixation with pins have mostly been implemented in the straight mouse tibia which is a rather small bone compared to the femur making assessment of fracture healing more difficult [18]. In contrast, the femur is bigger, easy to stabilize and easy to access. The relatively greater muscle mass over the femur necessitates an open approach, but the fracture can be well reduced with a controlled gap size. Additionally, other factors such as osteogenic material or additive injuries to the fracture site are possible. Our approach utilizing an internal fixator and adequate instruments eliminated difficulties concerning the small dimensions in the mouse and overcame the problem of mechanically uncontrolled healing. Moreover, light weight internal fixation was similar in proportion to body weight as in humans and was more comfortable for the animal than external fixation [3]. Consequently, our method seems to be applicable for great number of research questions in fracture healing in mice.

In conclusion our hypothesis was proven to be false: this flexible plate and gap size does not lead to a substantial delay in fracture healing compared to a rigid plate. Nevertheless the study clearly demonstrated that flexible fixation is needed to obtain secondary bone healing in the mouse femoral diaphysis, similar to that observed in humans.

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Fixation compliance in a murine fracture model induces two different processes of fracture healing but does not lead to delayed union; Manuscript submitted to JOR, July 2007

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