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Establishment of a Non-union Model Using Muscle Interposition without Osteotomy in Rats

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INDEXING WORDS

fracture; non-union; muscle interposition; rat

SYNOPSIS

Many attempts have described a standard experimental model for fracture non-union in laboratory conditions, but the majority of them produced after an experimental osteotomy, so it is different from clinical disturbed fracture healing. The purpose of this study is to establish a standard method for producing fracture non-union with only muscle interposed into the fracture site in rats. Bilateral tibial fractures were made in forty-eight male Wister rats by three point bending and we surgically interposed the distal end of the tibialis anterior muscle into the fracture site. They were sacrificed at 1, 2, 3, 6, 12, 24, 48, and 96 weeks after fracture. The rentgenograms were obtained, and the fractured tibias were harvested in each time period and investigated histologically and immunohistochemically.

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The rentgenogram at six weeks, in the non-union rats, showed abundant callus at each end of the fracture fragments, but no bridging callus. The histological finding with hematoxylin and eosin, at this point, shows no bridging soft callus, and small isolated regions of cartilage were observed only where the bone was not covered by the muscle. The proliferating cell nuclear antibody immunostaining which is associated with cell proliferation was abruptly lost in chondrocytes at two weeks. This early disappearance of chondrocytes without endochondral ossification may be a significant etiological factor in the development of a non-union. This non-union model is technically simple and reproducible, and dose not require periosteal stripping or surgical osteotomy to produce an artificial bone gap.

INTRODUCTION

In the last decade, the processes of normal and impaired fracture repair and the various factors affecting them have been extensively studied.[6,9,10] With the aid of advanced techniques, we have come close to understanding of the cascade-like process and complex regulation mechanisms of normal fracture repair. Although numerous studies of normal bone repair have been published, little basic research has been done on the factors that disturb normal fracture repair and the development of a fracture non-union.

Numerous factors that negatively influence normal fracture healing have been described.[1,7,8,11,13,17,19,20,22] These factors may include interposition of tissue into the fracture gap, instability of the fracture fragments, insufficient blood supply to the fracture and low oxygen tension at the fracture site, widened fracture gap, and infection.

Many of these negative factors have been used in previous attempts to describe a standard experimental model for fracture non-union in laboratory conditions.[1,2,3,15,16,21,23] However the majority of the published studies produced a non-union after an experimental osteotomy. It is possible to speculate that the surgical trauma required to produce the experimental osteotomy may have also

influenced the development of the subsequent non-union. This would alter the relevance of the experimental data when compared disturbed traumatic fracture healing in humans. Moreover, some of the described non-union models are very complex and differ from clinical disturbed fracture healing.

Several studies have demonstrated that muscle interposition into the fracture site is a primary factor contributing to fracture non-union.[14,18] König et al.[12] reported 120 cases of open reduction in which 30% of the patients had muscle interposition in the fracture site. In the majority of these patients the interposed muscle was necrotic. It is well established that a delayed union or non-union may develop when a large amount of soft tissue is interposed into the fracture site.

In this paper the authors present a standard method for producing and investigating fracture non-union in rats. This model is produced by creating a closed tibial fracture with only muscle interposed into the fracture site. Osteotomy of the rat tibia was not performed in the present study. This animal model is very simple and reproducible.

MATERIALS AND METHODS

Fracture model

Bilateral tibial fractures were made in 48 male Wister rats of 6 months age by three point bending. On one side, an experimental non-union was made by muscle interposition into the fracture site. The fractured tibia on the contralateral side was served as a control. The tibia fractures were then stabilized using intermedullary pins as described by Bonnarens et al.[4] The distal end of the tibialis anterior muscle were interposed into the fracture site in 48 fractured tibial shafts. The proximal fracture fragment was covered with the tibialis anterior muscle that was held in place with a nylon suture(Fig. 1). In both the experimental non-union and control tibia, meticulous attentions were directed toward preserving the periosteum. In the control leg the distal insertion of the tibialis anterior was divided but left in situ. Rentgenograms were obtained at weekly intervals to monitor fracture healing. Six rats were sacrificed at 1, 2, 3, 6,

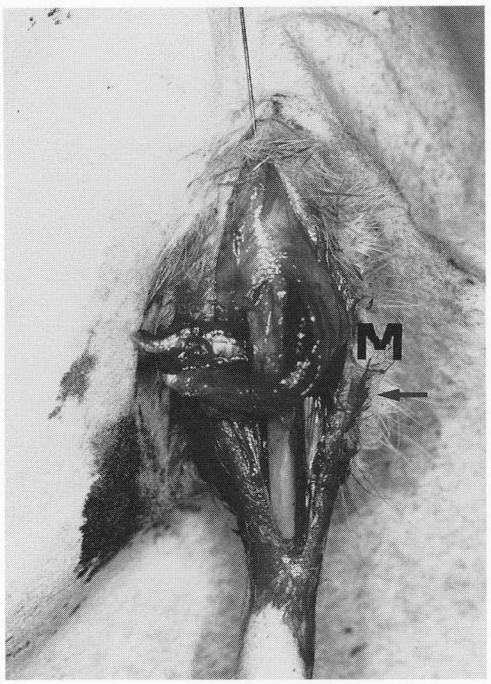


Fig. 1. The tibialis anterior muscle is interposed into a fracture gap of a rat tibial shaft. The proximal fracture fragment is covered with the muscle and sutured in place. (→: fracture gap, M: interposed muscle)

12, 24, 48, and 96 weeks after fracture. The fractured tibias were removed from the sacrificed rats in each time period and investigated by the standard histological and immunohistochemical techniques.

Immunohistochemistry

The removed tibias were fixed in 4% paraformaldehyde (PFA), then decalcified in ethylemediamine tetraaceticacid disodiumsalt (EDTA) solution. The decalcified tissues were then dehydrated with graded ethanol, embedded into paraffin, and sliced at 6μm thickness. Serial sections were stained with hematoxylin and eosin (H&E), or immunostained using the Vectastain ABC kit (Vector Laboratories, Burlingame, California). Endogenous peroxidase activity was removed by a 60 minutes incubation in methanol containing 0.3% hydrogen peroxide. Nonspecific immunoglobulin binding was blocked by incubating sections for 15 minutes in phosphate buffered saline (PBS) containing 5% normal goat serum. The proliferating cell nuclear antibody (PCNA), PC10, was purchased from Sigma Chemical Company (St. Louis, Missouri). The antibodies were applied to the specimens at dilutions of 1:800. The specimens were incubated overnight at 4 °C. Then the specimens were extensively washed and incubated with biotinylated goat anti-rabbit IgG followed avidin-enzyme complex.

Antibody binding was visualized by incubating the sections in 3, 3'-diaminobezidine solution (Sigma Chemical Company, St. Louis, Missouri) including hydrogen peroxide. Normal rabbit IgG (Lipshaw Immunon, Pittsburgh, Pennsylvania) at the same concentration was used as a negative control. All tissue sections were immunostained simultaneously. The incubation and development times were the same for all sections. To confirm PCNA localization and rule out false negative immunostaining, some slides were also stained with a higher antibody concentration of 1:400.

RESULTS

Macroscopic observations

The rats returned to normal activity within a few days postoperatively. No wound infections were found. Delayed union and gross instability between the fracture gap were found in all experimental fractures (n=48) early after surgery. In all rats with muscle interposition into the fracture site, a non-union developed and persisted for the complete two years observation period. The removed bone specimens showed large amounts of non-calcified connective tissue at the fracture site consistent with a true non-union. There was no interfragmentary fluid at the fracture site suggesting a pseudarthrosis.

Radiographic evaluation

Radiographs obtained one week after experimental fracture clearly demonstrated the fracture gap. Little or no callus was seen in both the non-union and control tibias(Fig.2A, B). Radiographs obtained in the control rats at two weeks after fracture revealed centripetally expanding subperiosteal circumferential fracture callus. However, in the non-union rats the radiographs at two postoperative weeks had a minimum volume of non-bridging fracture callus.

Radiographs obtained in the control rats four weeks after fracture revealed abundant callus at each end of the fracture fragments. However, there was no bridging fracture callus. At six weeks, fracture healing continued with further development of the fracture callus and obliteration of the fracture gap (Fig.2C). In contrast, the radiographs of non-union rats at six weeks revealed additional development of isolated fracture callus at each end of the fracture fragment, but there was no bridging fracture callus (Fig.2D). Radiographs of the non-union rats at twelve weeks after fracture had findings consistent with an established non-union. There was remodeling of isolated fracture callus found at the ends of both fracture fragments with no bridging callus. The radiographic appearance suggested hypertrophic non-union. At two years the non-union appeared radiographically atrophic.

Histological study

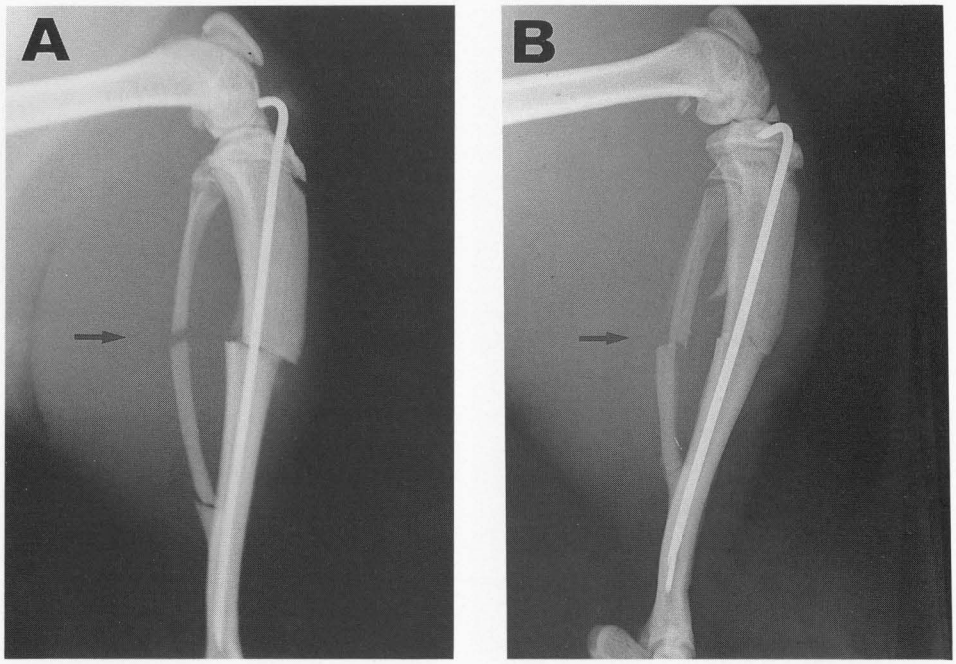
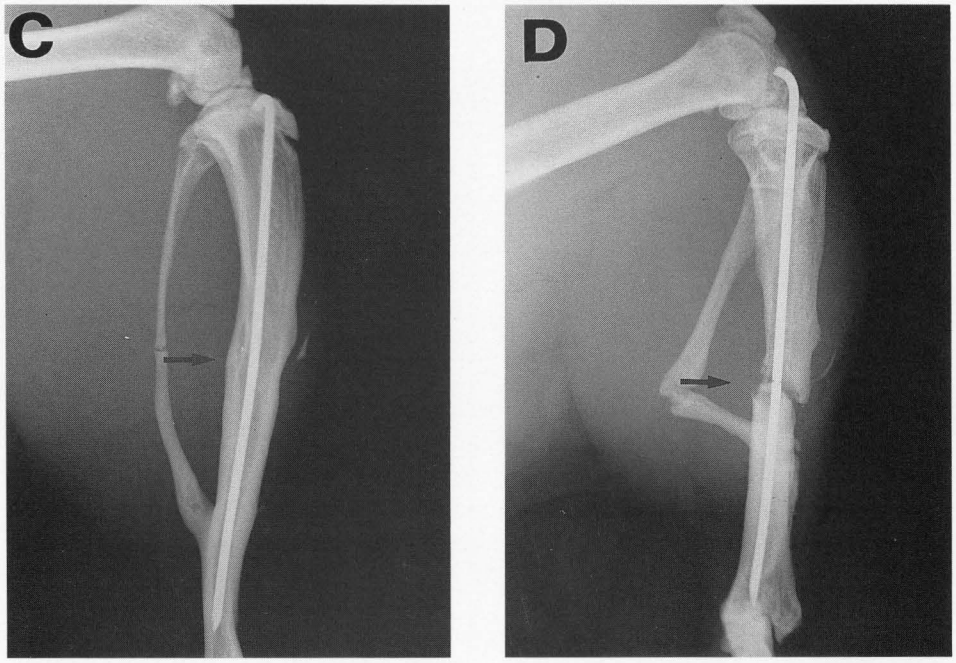


Fig. 2. The radiographs of fracture repair in the control rats and the rats developing non-union. (→: fracture gap)

2A: Formation of early fracture callus after one week in a control rat tibia.

2B: Isolated slight fracture callus formation on one side only after one week in a non-union rat tibia.



2C: This radiograph shows isolated abundant fracture callus bridging the fracture fragments in a control rat six weeks after fracture.

2D: This radiograph shows isolated abundant fracture callus limited to each end of the fracture fragments with no bridging callus in a non-union rat six weeks after fracture.

In the control rats, one week after fracture, intramembranous bone was observed at both sides of the fracture site (Fig. 3A). However, in non-union rat one week after fracture, there was no intramembranous bone or hematoma at the fracture site where muscle had been interposed. Only slight intramembranous bone was observed at regions that were not covered by the interposed muscle (Fig. 3B). Cell nuclei were visualized in the interposed muscle which indicated that the muscle was alive. Little or no cartilage was observed in the vicinity of the fracture site in both the control and non-union groups.

Histological analysis was performed on specimens harvested from both the control and non-union rat three weeks after fracture. In the control rats fracture callus was observed with both subperiosteal bone formation and endochondral ossification at the interface between subperiosteal bone and cartilage (Fig. 3C). Chondrocytes at the fracture site were increased in size with a diminished extracellular matrix.

In contrast, in the non-union rats, bridging soft calls was not observed, and small isolated regions of cartilage were observed only where the bone was not covered by muscle. At three weeks after fracture almost all interposed muscle at the fracture site had been replaced by fibrous tissue. At six weeks in the non-union group, the fracture gap was completely occupied with fibrous or fibrocartilagnous tissue (Fig. 3D).

Distinct changes in localization of PCNA throughout cartilage formation

The association between cartilage formation and non-union development was investigated with serial immunohistological staining using antibodies against PCNA. PCNA is associated with cell proliferation. Detection of PCNA activity is a good technique for the determination of cellular activity. The fracture callus was immunohistochemically stained at one, two, and three weeks after fracture in specimens harvested from both the control and non-union rats.

In the control rats, PCNA expression was identified in almost all the fracture site chondrocytes at one and two weeks (Fig. 4A). However, in the control rats,

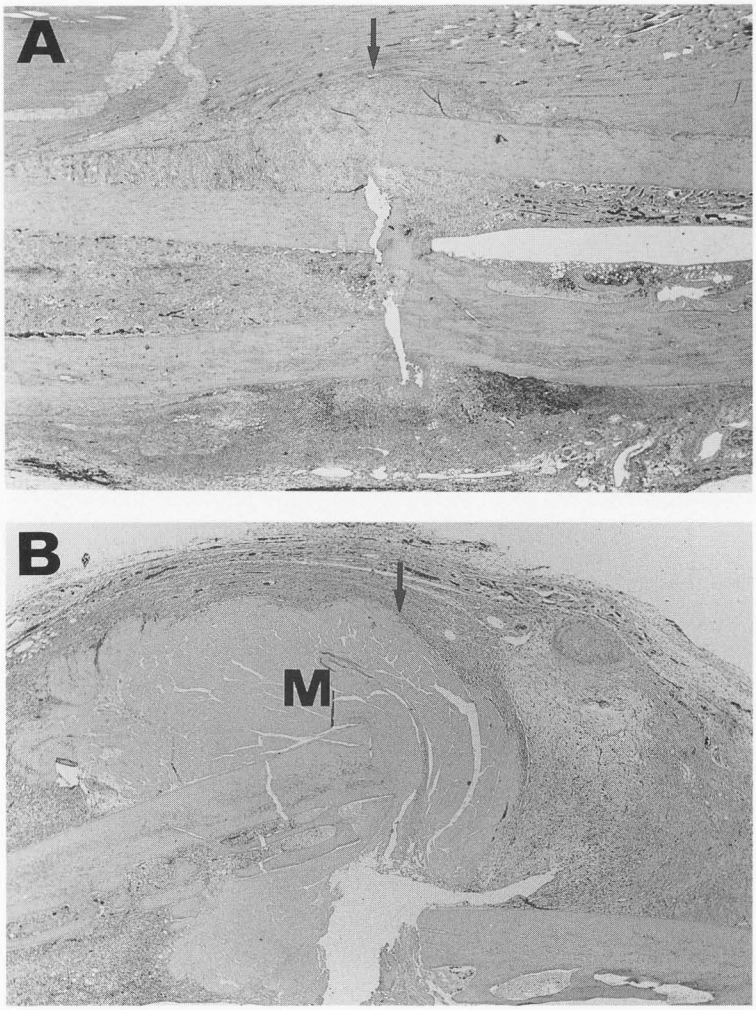
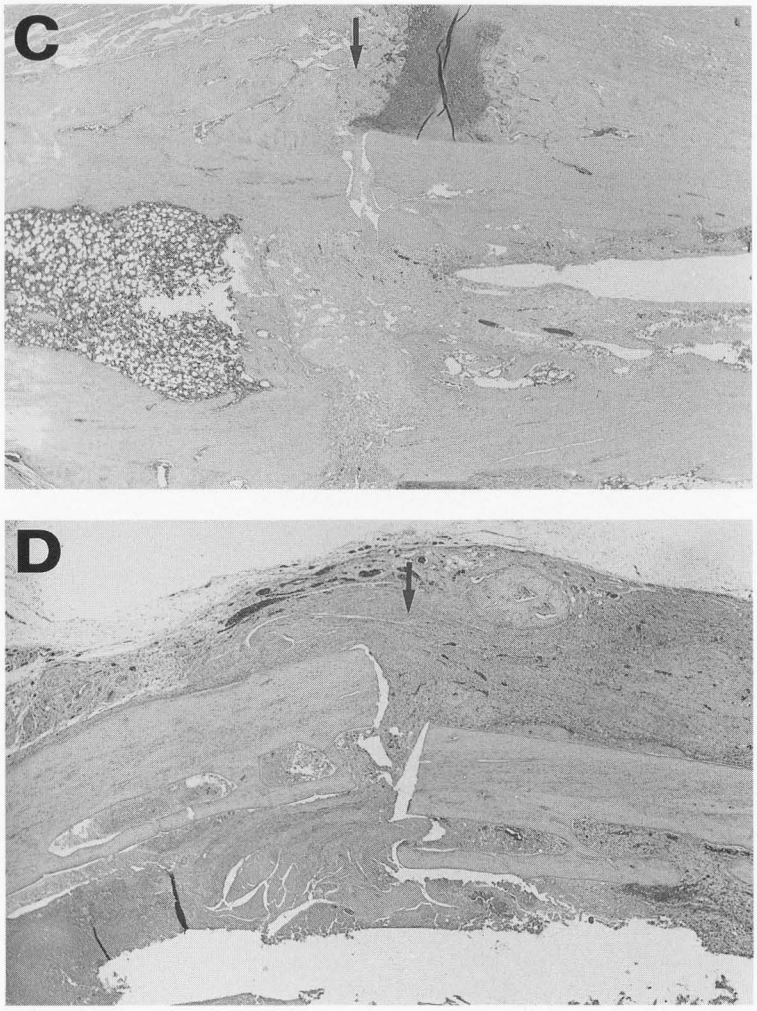


Fig. 3. Histological appearances of fracture healing in the control rats and the development of the non-union stained with Hematoxylin and eosin.

(→: fracture gap, M: interposed muscle)

3A: Histological appearance of early fracture callus in a control rat tibia after one week.

3B: Histological appearance of the non-union rat tibia after one week. Intramembranous bone or cartilage formation is absent at the fracture end covered with the muscle. Slight intramembranous bone and cartilage formation is present at the uncovered fracture site.



3C: Histological appearance of maturing fracture callus in a control rat tibia after three weeks.

3D: Histological appearance of the non-union rat tibia after six weeks. There is no bridging callus and the interposed muscle has been replaced by fibrous cells.

only faint PCNA-immunopositive products were identified on the mature chondrocytes three weeks after fracture (data not shown).

In the non-union rats, intense PCNA staining was identified only in the immature chondrocytes found in the fracture region not covered by muscle. However, PCNA immunostaining was abruptly lost in chondrocytes two weeks after fracture (Fig. 4D). Yet, there was PCNA-immunopositive staining in the fibroblast like cells that were adjacent to the cartilaginous tissue. In the non-union rats, PCNA expression was no longer identified in specimens harvested three weeks after fracture.

DISCUSSION

Previous clinical and experimental studies have identified many factors that influence bone healing. For example, fracture instability, infection, soft-tissue interposition, distraction of fracture fragments, poor vascularity and soft-tissue damage have been demonstrated as significant factors leading to the development of a fracture non-union.[1,13,17,19,20,22] Constitutional factors such as old age, starvation, and chronic illness have also been identified as influential factors towards abnormal or delayed fracture healing.[7,8,11]

The non-union model described in the present investigation is a modified one of the technique originally described by Altner et al.[1] Previously published reports have shown that a reliable non-union in the dog can be created by interposing the ulnaris lateralis muscle into the site of an ulnar shaft segmental osteotomy. This canine non-union model has been thoroughly studied and the results in the present study are consistent with the previously described radiological, histological and biological findings at the canine non-union.

Altner et al.[1] has described a technique that an osteotomy is performed in the dog's ulnar bone and a three to five millimeter segment of the bone tissue was removed. The authors modified Altner's technique by performing a closed fracture of the bone without removing any bone tissue to create a fracture gap. The authors prevented normal fracture healing strictly by interposing muscle into the fracture site without artificially creating a fracture gap. Altner's technique was

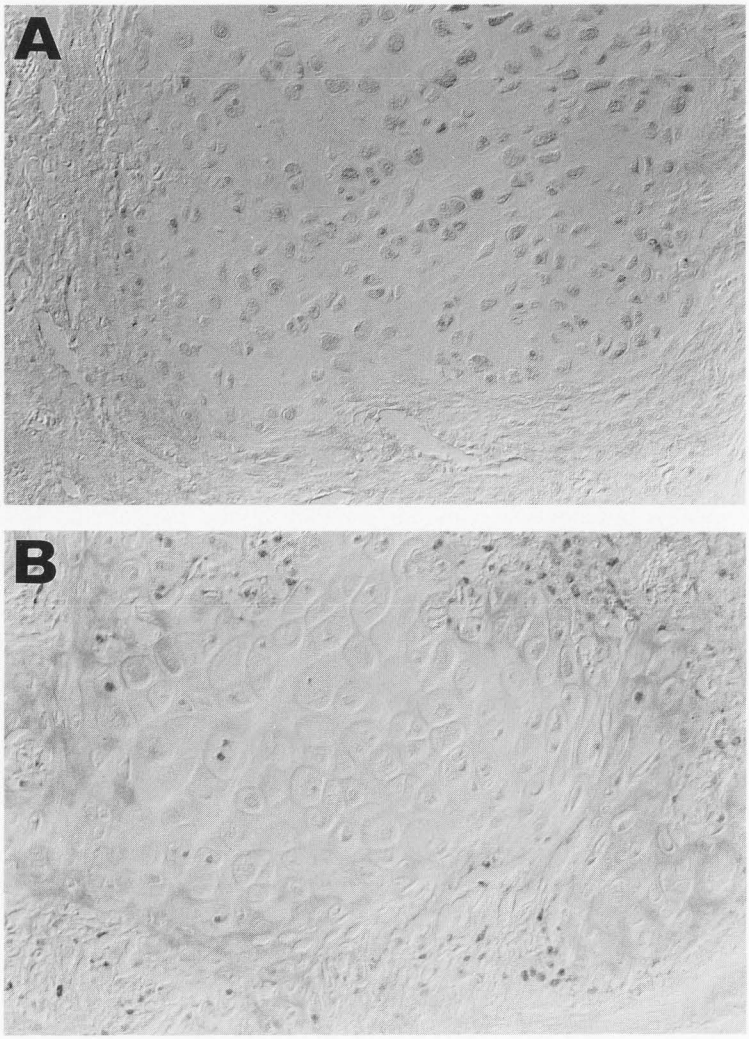


Fig. 4. Immunosataining was performed with anti-PCNA antibody in both the control and non-union rat two weeks after fracture.

4A: Chondrocytes stained intensely with PCNA.

4B: No staining with this antibody was observed in cartilage of the non-union fracture model. PCNA-immunopositive staining is observed in fibroblast-like cells surrounding a cartilagenous tissue.

called an osteotomy model due to the artificially creation of the fracture gap. The author have described a non-union model in the present study accurately mimics the clinical events that produce a non-union.

We have tried to create a reliable technique to produce a non-union that accurately reproduces the biological events in human non-union. This accurate non-union model will be used to investigate techniques to promote fracture healing in humans. An optimum application of this animal model is the investigation of the influence of growth factors like bone morphogenic proteins (BMPs) on fracture healing and healing of a non-union.

Non-unions have been described as atrophic or hypertrophic, but both types demonstrate soft tissues without mineralization at the fracture gap.[5] Histologically, our non-union model showed fibrous proliferation filling the fracture gap within three weeks after the fracture. This histological character is compatible with the non-union histology described by Milgram.[14]

Immunohistological staining against PCNA was performed to determine when the primary fracture healing process ceased. The authors performed immunohistological staining against PCNA on both the control and non-union specimens. Jingushi et al.[10] reported that immature and mature chondrocytes are PCNA-immunopositive during early fracture repair.

In the control rats the normal progression of fracture healing is characterized with the change of the intensity of PCNA-immunopositive staining observed in specimens harvested at intervals over a three weeks period. The PCNA-immunopositive staining of the control rats was consistent with the rat fracture model described by Jingushi et al.[10] In the control rats the concentration of chondrocytes decreased with the progression of endochondral ossification. However, in the non-union rats chondrocytes population decreased within two weeks despite the absence of endochondral ossification. The early disappearance of chondrocytes without endochondral ossification may be a significant etiological factor in the development of a non-union. The loss of the chondrocytes implies the termination of the fracture repair for more than one year. The method of non-union production presented in this paper successfully

produced a non-union in all the rats of the non-union group.

The authors have presented a reliable technique to produce non-union in the tibial bone of the rat. This animal model is biologically similar to a human non-union. The animal model described in this paper is technically simple, reproducible, and dose not require periosteal stripping, surgical osteotomy, or resection of bone to produce an artificial bone gap. The non-union produced in the present study resembled the usual clinical non-union observed in humans.

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