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Perturbations of Periosteal Bone During Healing:
Effect of Non-Weight Bearing

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Abstract

Weight bearing (WB) is an important factor influencing bone remodelling. The present study evaluates the effects of weight bearing and non-weight bearing (S) (achieved by tail suspension and hindlimb elevation) on the healing of a fibular osteotomy in adult male rats. After 9, 18 or 36 days under WB or S conditions, periosteum near the callus formed at the osteotomy site was compared to periosteum of the contralateral fibula (which did not receive an osteotomy) or to periosteum of fibula of control animals which did not receive an osteotomy. Data show that periosteal bone healing is sensitive to alterations in WB and that the bone alterations over time can be complex. In the presence of an osteotomy under S conditions, bone formation is depressed and resorbing surface elevated compared to alterations in healing which occur under conditions of WB. Data support the accepted opinion that WB is essential for promotion of bone healing and increase our appreciation of the responsiveness of periosteal bone during healing.

Key Words: Bone, histomorphometry, fracture healing, weight bearing, suspension, osteotomy.

Introduction

The relationship of weight bearing (WB) to bone metabolism is clinically very important but not yet completely understood. A number of transitory, as well as prolonged conditions, including bed rest, microgravity experienced in space flight, disuse, and paralysis, are known to result in osteopenia [18]; in the transitory situation, return to WB usually corrects the osteopenia [5, 11, 15, 16, 17, 28].

Interest in the biologic impact of non-weight bearing has led to the development of a rat model which employs an inverted hindlimb elevation suspension cage described previously in detail [6, 13, 20, 21, 26, 27, 29]; this model has been used for a variety of studies, including employment as a ground-based control model for microgravity research in our laboratories and by other investigators [1, 2, 6, 7, 8, 24, 29] and for other types of bone and joint studies [20, 21]. The hindlimb elevation method allows a rat to be suspended by the tail in a preparation similar to human traction; a low-friction XY device is used which enables the animal to have unrestricted movement within the cage (Figure 1). This technique was utilized in the present study with a focus upon identifying changes in the periostium in and near a healing fibular osteotomy site.

The fibular fracture model was employed here because the fracture is stabilized by the tibia and thus does not require external or internal fixation [4]. We believe that this model is valid for investigating the healing process under conditions of relative stability. In addition, neither endosteum or periosteum are disrupted by rodding or plate/pin fixation. This absence of fixation requirements was advantageous in our previous use of the fibular osteotomy model in experiments on shuttle flight STS-29 [13].

Materials and Methods

Animal model

Experiments were approved by the Institutional Animal Care and Use Committee of Orthopaedic Hospital,
Los Angeles. Adult Long Evans out-dated breeders 9-11 months of age with an average weight of 519 ± 63 g were used following acclimation to the vivarium. Rats were randomly divided into two major groups: suspended (S) and weight bearing (WB), and sub-divided into osteotomy or non-osteotomy groupings. Three to six animals were available for histologic examination from each group. These four groups were each studied for three time periods: nine days, eighteen days and thirty-six days post-osteotomy.

Fibular osteotomy

Rats were anesthetized with an intraperitoneal injection of sodium pentobarbital, 0.1 ml/100 g body weight. After anesthesia was achieved, the lateral aspect of the right lower leg was prepared and surgical procedures carried out using aseptic technique. A 1 cm incision was made on the lateral aspect of the lower right leg above the fibula. Fascia was incised and muscle plains were bluntly dissected down to the fibula. A mid-shaft fibular osteotomy was performed with a mini compressed air oscillating saw with a 0.5 x 6 mm blade. Sterile saline was used for cooling and wound irrigation during the osteotomy. Fascia and skin were closed with 6.0 nylon interrupted sutures. Rats were observed until fully awake and either returned to their normal weight bearing activities or tail suspended as described below.

Suspension

Rats were manually restrained with a hospital bath towel, and a tail suspension device similar to human skin traction was applied to the proximal two-thirds of the tails in the following manner. Rat tails were washed with gauze and a warm, mild soap solution, rinsed thoroughly with warm water, cleaned with 70% ethanol and dried with a warm air blower. Tails were sprayed with tincture of Benzoin and again dried with a warm air blower. A 1 x 15 cm strip of orthopaedic traction tape was applied to the lateral sides of the proximal two-thirds of the tail. A spreader block was placed between the two sides of the traction tape to separate them and afford an attachment to an x-y suspension device. The traction tape and tail were wrapped with 2.5 cm bias cut stockinette and secured with a 1 x 3 cm piece of transpore tape. The rat was suspended from a low-friction x-y device permitting unrestricted movement within a plexiglass suspension cage with a floor space of 1444 cm². Tail suspension resulted in a head down tilt of approximately 30° (Fig. 1). Animals were checked daily to assure integrity of the suspension apparatus and to assess animal behavior.

Study termination

At the end of study periods, WB or S animals were euthanized (390 mg sodium pentobarbital/ml water by intraperitoneal injection), left and right fibulae and tibiae removed as units and fixed in 10% neutral buffered formalin. The fibula was separated from the tibia, dehydrated and embedded in methacrylate as previously described [8]. Five μm thick sections were cut longitudinally through the approximate midportion of the osteotomy site or intact fibula with tungsten carbide knives using a Reichert Jung 2050 SuperCut microtome and stained with Goldner’s stain to differentiate mature bone matrix and new osteoid [9]. Quantitative histomorphometry was performed utilizing a Zeiss microscope, camera lucida, and a Summagraphics Bit Pad One interfaced with an IBM-XT computer. Software used was "Morphom" (BioMed Stats, Inc., Tacoma, WA); histomor-
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Phometry data are expressed according to standardized nomenclature. Periosteal cortical bone features were assessed. Major data presented here were derived from examination of osteoid surface (OS/BS, percent of osteoid lining the bone, or boundary surface; ES/BS, percent eroded surface). Periosteal measurements at the osteotomy site consisted of 2-4 measurement fields (each 0.719 mm in length) immediately adjacent to the osteotomy site. Similar regions were measured in animals not receiving an osteotomy. In some specimens periosteal tissues were inadvertently removed during tissue procurement. This prevented collection of periosteal data in some animals in the intact, non-osteotomized S group at 36 days.

Statistical analyses

Standard statistical methods were used. Student's t-test for independent groups was used to evaluate body weight changes [20]. Bone histomorphometric data were analyzed with analyses of variance (SAS®, version 6.10) performed on the differences in %OS/BS and %ES/BS between right and left legs of the same animal, using weight bearing/suspended and number of days following osteotomy as factors (along with an interaction term, when needed). This was done separately for animals which had an osteotomy and those which did not have one. Analyses of variance were also performed on %OS/BS and %ES/BS using the factors: osteotomy/no osteotomy, weight bearing/suspended, and days (along with interactions, when necessary). This was done separately for right and left measurements. For all analyses, a p-value of < 0.05 was considered statistically significant. Data are expressed as mean ± s.e.m. (n).

Results

Body weight changes

At the 9 day time point, the S osteotomy group weighed significantly less than the WB osteotomy group (456.8 g ± 10.2 (5) vs 524.4 ± 23.6 (5)) (p = 0.01). At the 36 day time point there was no significant difference between mean weights for the WB osteotomy group and the WB group (629 g ± 25.6 (6) vs 632 g ± 41.8 (3), respectively). The S osteotomy group, however, weighed significantly less (564 g ± 18.4 (3) than the WB osteotomy group at study completion (p = 0.05). (Data on weights of the 18 day S osteotomy group and other groups are not available).

Qualitative morphologic features of the healing osteotomy

Weight bearing animals: At 9 days there was a well-defined callus at the osteotomy site. The callus consisted primarily of dense irregular connective tissue and contained both calcified and non-calcified cartilage. Minimal osteoid was noted. Periosteal reaction (new trabecular formation on the periosteum) was active at the osteotomy site but diminished toward the epiphyses. Angiogenesis was active in the regions of periosteal reaction but minimal in the callus proper.

Eighteen days of healing post-osteotomy showed calluses which were larger than calluses formed after 9 days, in relation to fibular diameter, and showed a decrease in dense irregular connective tissue with a concomitant increase in non-calcified and calcified cartilage. The osteoid in the periosteal reaction site was prominent. Polarized light microscopic examination showed the presence of both woven and mature bone. Periosteal reaction was increased and more advanced toward the epiphyses.

After 36 days, calluses approached bridging. Cartilage and osteoid were increased and there was a

![Figure 2. Light microscopic photomicrographs comparing 18 day WB vs S animals with histologic views of the cortex. Note the abundant periosteal reaction present in WB animals (Fig. 2A) but absent in S animals (Fig. 2B) at this time; osteotomy site is to the left of Figures 2A and 2B. Goldner's stain. Photo width = 1.25 mm.](image)
Figure 3. Changes in % osteoid surface (OS/BS) at the 9, 18 and 36 day time points. A: right fibula; B: left fibula. Data are mean ± s.e.m.; n = 4-6 animals/sampling point. W = WB (weight bearing); S = suspension; WOs = WB with osteotomy of the right fibula; SOs = suspension with osteotomy of the right fibula. See text for analysis of variance evaluation.

Figure 4. Changes in % total periosteal surface involved in resorption (ES/BS) at the 9, 18 and 36 day time points. A: right fibula; B: left fibula. Data are mean ± s.e.m.; n = 4-6 animals/sampling point. W = WB (weight bearing); S = suspension; WOs = WB with osteotomy of the right fibula; SOs = suspension with osteotomy of the right fibula. See text for analysis of variance evaluation.
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Table 1. Mean osteoid widths (in μm) in right fibulae.

<table>
<thead>
<tr>
<th></th>
<th>9 days</th>
<th>18 days</th>
<th>36 days</th>
</tr>
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<tbody>
<tr>
<td>Right WOs</td>
<td>6.0±0.5 (6)</td>
<td>6.4±0.9 (6)</td>
<td>5.7±0.4 (6)</td>
</tr>
<tr>
<td>Right SOs</td>
<td>2.6±0.6 (6) *</td>
<td>4.9±0.7 (3)</td>
<td>3.1±0.8 (4)**</td>
</tr>
</tbody>
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*p < 0.001, **p = 0.004 versus WB Os

decrease in the relative amount of dense irregular connective tissue. Periosteal reaction was present, but decreased compared to 18 days of healing. Angiogenesis was increased within the callus. Resorption and remodelling was present at the cortical ends of the fibular osteotomy site.

Suspended animals: Fibulae from the 9 day suspended (S) rats with osteotomy had smaller calluses in which dense irregular connective tissue was prominent; cartilage was minimal. Osteoid was difficult to locate and the periosteal reaction diminished compared to WB 9 day fibulae. Angiogenesis was noted particularly in areas of periosteal reaction.

The 18 day S callus showed a delay in healing when compared to calluses formed in the 9 or 18 day WB groups. The callus size increase was minimal when compared to the 9 day S group. Both dense irregular connective tissue and cartilage were present, but osteoid was difficult to detect. Periosteal reaction was greatly diminished in 18 day S animals vs 18 day WB animals as shown in Figure 2.

At 36 days, callus formation still lagged behind that seen in WB animals. Non-calciﬁed and calcified cartilage, as well as osteoid, were increased in comparison to the 18 day S callus but decreased when compared to the 18 day WB callus. Periosteal reaction, resorption and remodelling were also similar to that seen at 18 day WB healing.

Quantitative histologic features of the periosteum in the healing osteotomy

Figures 3 and 4 summarize two major indices of bone formation (%OS/BS, percent osteoid surface) and the total resorbing surface (%ES/BS) quantified along the cortical periosteum in the sampling site of the osteotomy. In these ﬁgures, "right fibula" data are derived from right ﬁbula with or without an osteotomy in animals either WB or S; "left fibula" data are derived from the contralateral ﬁbula of these same animals.

WB or S, osteotomy or no osteotomy, and time effects: Analysis of variance ﬁndings: Bone formation (evaluated in terms of %OS/BS) from the right ﬁbula showed a signiﬁcant effect of S (p = 0.03), osteotomy (p = 0.0004) and time (p = 0.0001). Significant interactions were present in the data for both osteotomy or no osteotomy vs time (p = 0.0001) and for a three-factor interaction (p = 0.04).

As shown in Figure 3A, the elevation in bone formation seen under normal WB conditions following osteotomy is severely depressed under conditions of S following osteotomy.

Bone resorption (as evaluated in terms of %ES/BS) data from the right ﬁbula showed a signiﬁcant effect of osteotomy or no osteotomy (p = 0.03) and a signiﬁcant interaction of osteotomy with time (p = 0.001). A signiﬁcant three-factor interaction of osteotomy or no osteotomy, WB or S and time was also present (p = 0.0001). As shown in Figure 4A, total resorbing surface by 36 days is highly elevated in the S osteotomy group.

WB or S, osteotomy or no osteotomy, and time effects: Analysis of variance evaluation of "right" minus "left" differences (Figures 3B and 4B): Evaluation of bone formation data showed a trend towards significance of WB or S (p = 0.0505), a signiﬁcant effect of time (p = 0.0001) and no signiﬁcant interaction between these two variables (p = 0.064). Fibula without osteotomy but subjected to S showed no signiﬁcant interactions.

Total resorbing surface data from the osteotomy sites showed signiﬁcance for time (p = 0.002) and for the interaction of WB or S and time (p = 0.002). There was a trend towards signiﬁcance for ﬁbula with no osteotomy subjected to S (p = 0.058) and also for time (p = 0.068) however, there was no signiﬁcant interaction effect.

Discussion

This study shows that osteotomy healing in the ﬁbula of suspended animals is markedly different and signiﬁcantly delayed compared to healing under normal WB conditions. The ﬁbula is not the principal weight bearing strut of the lower leg; the magnitude of forces transmitted through it during weight bearing are not known. It is probable, however, that ground reaction forces and muscular contraction forces generated during weight bearing activities are transmitted through the ﬁbula and through its developing callus. It is important to remember, however, that healing processes in major weight bearing bones (such as the tibial or femoral shaft) may differ temporally from the sequence of events reported here for the ﬁbula. In addition, methods such as those developed by Bonnarens and Einhorn [4] employ reaming of the medullary canal and subsequent support of the fracture site by rodding or pinning. Such methods are probably complicated by endosteal changes associated with the reaming which are then superimposed upon the
fracture healing process. One should also note that the endosteal and periosteal changes identified here could have been significantly masked if an osteotomy/fracture model with plating and pin placement (which in themselves disrupt the periosteum) had been used.

Normal fracture healing has been well described and characterized in previous work by a number of investigators [3, 12, 19, 23]. The present study contributes quantitative normative data on fibular osteotomy healing associated with our methodology and provides quantitative data on bone remodelling patterns in the normal and in the healing fibular periosteum under normal WB or under S conditions.

Data presented here show that periosteal remodelling is altered and delayed during healing of an osteotomy under hindlimb suspension conditions. At 9 and 18 days bone resorative surface showed little alteration in fibulae subjected to both S and osteotomy. This is in marked contrast to the 9 and 36 day depression of osteoid surface seen in osteotomized S rats. Bikle et al. [2] have recently shown with molecular techniques that there is an altered pattern of gene expression for osteocalcin, alkaline phosphatase, IGF-I and the IGF-I receptor during microgravity and hindlimb suspension. S exerts an inhibitory effect on these key indices of osteoblast differentiation and function. As the authors note, however, their data are pooled from both endosteal and periosteal compartments.

Delayed callus remodelling and altered remodelling of the nearby periosteal cortex were significant findings in our present study. These data support the accepted opinion that WB is essential for appropriate callus formation and early chondrogenesis and osteogenesis. Under hindlimb suspension conditions, the course of events of chondrogenesis and osteogenesis are delayed but appear normal in morphology. The periosteal reaction and its associated neo-angiogenesis are also delayed. Given an extended period of time, the healing process would probably go to completion even under the suspension condition.

Lack of weight bearing and immobilization are well recognized as risk factors for bone loss and osteopenia (see McKenna for review [18]). In addition to the contribution from these factors in our study, metabolic and physiologic adaptations to the "head-down" suspension model were also present [1] but were not monitored in the present study. Previous work with this model has shown that hindlimb suspension results in reduced tibial metaphyseal trabecular bone volume [14, 29], decreased femoral bone density [14] and decreased periosteal and trabecular bone formation in the tibial metaphysis [7, 8, 22, 24, 29]. O'Connor documented that epiphyseal trabecular bone also responds to suspension but to a lesser degree than is seen in the tibial metaphysis [20]. The present work shows that the model can also be utilized in fracture healing studies and it continues to serve as a ground-based control system for microgravity studies and as a model valuable for general hind limb unweighting studies.

Another factor present in our study was a weight decrease in the suspended osteotomy animals as compared to the WB osteotomy group. Other investigators have seen such a weight loss with suspension. O'Connor [20] noted that suspension produced an average weight loss of 18 grams during the first week, followed by a rebound to normal. Weight loss during S probably reflects a physiologic adaptation to the S model; effects of such adaptation on the concurrent healing process in an osteotomy site are not known. Future studies which focus on physiological changes of S may benefit from pair feeding regimes; pair feeding, however, may not be practical when the model is used in tandem with microgravity studies since animals in orbit have historically been fed ad lib.

In summary, our data on the healing of an osteotomy of the mid-fibula subjected to hindlimb suspension suggest that the initial effect of suspension is most readily seen in respect to a depression in osteoid surface. These data support the accepted opinion that WB is essential for promotion of bone healing and increase our appreciation of the responsiveness of the periosteal bone populations during fracture healing.

Acknowledgement

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References

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Discussion with Reviewers

W.S.S. Jee: Were these out-dated breeders 9-11 months acclimated before they were studied? When was the last pregnancy for each one?

Authors: Rats were acclimated for 7 days prior to initiation of study or prior to surgery. We do not have written records from the breeder on the time of the last pregnancy for each one. It is our understanding from the supplier, however, that these rats are bred for 6 months and then placed into the retirement colonies. Since the animals utilized here were 9-11 months of age, the approximate time since last lactation was probably at least 3 months.
This was an important point to raise, since the dynamic response of the female rat skeleton to pregnancy and lactation is quite marked [30].

**W.S.S. Jee:** Why was fluorescent labelling not used to evaluate bone formation parameters: osteoblastic recruitment and activity? What was the appearance of the fibula in the pretreatment control? Could you have quantitated the periosteal responses?

**S.C. Miller:** As the authors consider their use of OS/BS as a formation indicator, the question arises if the osteoid seams were thicker or thinner. If so, this would imply either differences in the mineral appositional rates or osteoid maturation times. Unfortunately, this could not be determined without some fluorochrome markers.

**Authors:** This study was performed as part of the initial methodological preparation for studies of fibular healing in an experiment on shuttle flight STS-29 [13]; we knew that the astronauts would not be injecting tetracycline labels for that study; hence, labelling was not included in this study. For future work, it would be critical. The appearance of the fibula in pretreatment controls was normal. In our laboratory, periosteal responses have been best quantified by cross-sections of the long bone of interest at a defined sampling site, such as the tibio-fibular junction for the tibial studies (unpublished results). Since this study was focused upon callus morphology examined in longitudinal section, these measurements were not done. The measurements of the periosteal reaction, however, tell us what has happened to the cortical surface near the healing site. Osteoid width was measured. Data are given in Table 1.

**W.S.S. Jee:** I noticed there is a great deal of porosity in the cortex in Figures 2a and 2b. Does the control exhibit it too?

**Authors:** There appears to be increased porosity in the suspended animals, but we have not yet morphologically assessed this.

**S.C. Miller:** Why did the authors select osteoid as an indicator of bone formation rather than osteoblast surface?

**Authors:** Some of our control specimens had the periosteum inadvertently removed during tissue harvest, thus limiting the application of this particular measurement. We did perform measurements of percent osteoid surface covered by osteoblasts, and it generally followed the same trend as the osteoid surface data presented here.

**S.C. Miller:** Another issue which the authors may consider is the issue of increased glucocorticoid production in the suspended animals. It is well known that steroid levels in rats increase with confinement and this may be at least partially responsible for some of the early effects, perhaps including the initial weight loss.

**Authors:** Although the suspension model is very useful, understanding the physiological changes occurring during initiation and adaptation of the head down tilt is very complex. Certainly, additional studies to clarify the physiologic adaptations are important, and we hope that investigators who find this model interesting can help unravel these changes. Unfortunately, we do not have any data from this study on glucocorticoid levels. Previous work (Halloran et al. [31]) with the model showed that plasma corticosterone levels were not different in loaded and suspended rats. Such studies may point to the effect of local bone loading sensor effects rather than systemic glucocorticoid changes.

**Additional References**
