Effects of Hormonal Conditions and Drugs on Both Muscle and Bone Strength Can Be Assessed in a Single Rat Test

T. S. KAASTAD,1,2 R. HUISKES,3 O. REIKERÅS,1 and L. NORDSLETEN1,2

1Institute for Surgical Research and Center of Orthopaedics, National Hospital, Oslo, Norway
2Department of Orthopaedics, Ullevaal Hospital, University of Oslo, Oslo, Norway
3Orthopaedic Research Lab, University of Nijmegen, Nijmegen, The Netherlands

Strength of both muscles and bone are important for fracture prevention in osteoporotic individuals. Therefore, drugs that are preclinically tested in animals for preventing or treating osteoporosis, and reducing fracture risk, should not only be checked for their effects on bone strength, but also for those on muscle strength. We developed a rat model to measure both in the same animal, using a single test. The model is based on an in vivo, ventral three-point bending test of the lower leg (Nordsletten L. and Ekeland A. J Orthop Res 11:299–304; 1993). This model was developed to test the contribution of triceps surae muscle contraction to the strength of the tibia. We hypothesized that this same test can be applied to determine bone and muscle strength independently, in an absolute sense. To investigate this possibility, the muscle contribution to bone stresses was estimated from mechanical analyses, based on direct assessment of muscle strength in a separate test. Sixteen mature female Wistar rats were used, half of which were ovariotomized. After 12 weeks, the rats were tested in vivo in three-point bending of the right lower leg during muscle contraction, and then the isolated triceps surae muscle strength in the left lower leg was measured separately, in another model. The rats were then killed, and the left nude shafts were tested mechanically in three-point bending in vitro to determine structural strength of the bone alone. Ultimate external bending moments of the in vivo and in vitro tests, maximal muscle force, and geometrical parameters formed the basis for the analysis. While contracting, the triceps surae loads the tibia in axial compression and bending. We found that the axial compressive stress on the bone due to muscle contraction was less than 2.5% of the bending stress this produced. This indicates that muscle contribution to lower leg strength is due almost entirely to the bending moment it produces, counteracting the external moment put on the leg by the testing device. Thus, the difference between the in vivo (lower leg) and in vitro (nude tibia) failure bending moments is approximately equal to the maximal muscle bending moment. This information can be applied to test the effects of hormonal conditions and drugs on both muscle and bone strength independently, in a single rat test, using the aforementioned procedure. (Bone 26:355–360; 2000) © 2000 by Elsevier Science Inc. All rights reserved.

Key Words: Fracture strength; Bone strength; Muscle strength; Rats; Osteoporosis; Test.

Introduction

With age, an increase in the incidence of hip fractures occurs parallel to a reduction in bone mass in both genders. Thus, osteoporosis is a primary research area with regard to prevention of fracture. However, treatment modalities for fracture prevention known to increase bone mineral density may also have effects on muscles, such as when giving vitamin D and calcium to patients in nursing homes to reduce the possibility of hip fracture. It is known that a lack of vitamin D reduces muscle strength. It has been suggested that the presence of soft tissues around bone reduces fracture risk, and that the active contraction of muscles in a limb enhances that effect. Muscle strength decreases with age, contributing to increased fracture risk and, through exercise, it is easy to increase muscle strength, whereas increasing bone mass in the elderly is much more difficult. Therefore, when drugs and other measures are tested with endpoint fracture, it is important to be able to determine whether the change in fracture rate is caused by an effect on bone, muscle, or a combination of both. This is difficult in human studies, but should be possible in animal experiments, where several models are in use for studies of risk factors and treatment of osteoporosis. The purpose of the current study was to find a method of discriminating between effects on bone and muscle using an in vivo fracture test in rats.

In an in vivo three-point bending test designed to assess fracture strength in the lower leg of rats during contraction of the triceps surae muscle, a higher ultimate bending moment was found than in tests of the nude contralateral tibia. This reinforcement was suspected to be caused by compressive axial-stress compensation in the bone by the muscle, as suggested by Nordin and Frankel. The muscle, according to their hypothesis, could be acting like prestressed wires in concrete columns. This increases compression, thereby transforming tensile to compressive stresses, increasing the structural strength of the bone, because concrete, like cortical bone, is stronger in compression than in tension.

Although this is a valid explanation in principle, whether it actually happens as such depends on the magnitude of the compressive stress generated by the muscle in the bone, as compared with the (tensile) bending stresses. Because the line of action of triceps surae force is remote from the tibial bone axis, it will produce not only axial compression, but also bending in the bone. If the axial stresses are negligible relative to the
bending stresses, the differences between the strengths of the lower leg and the nude tibia in the aforementioned tests are due not to axial-stress compensation, but rather to the bending moment the muscle produces. In this case, these differences could be used to estimate muscle strength. This hypothesis was investigated using mechanical analyses of the tests, in addition to independent measurements of maximal force in the triceps surae of rats.

Materials and Methods

To compare the in vivo and in vitro ultimate bending moments and stresses in the lower leg in different kinds of individuals, we studied the results from testing of left and right lower legs of 16 mature female Wistar/Han/Mol rats (Møllegård, Copenhagen) with a mean weight of 235 g (range 219–249 g). To obtain a variety of bone properties among the test animals, half of the rats were ovariectomized (ovx) and the other half were sham-operated (sham), as previously described. All rats received normal chow (Ca 0.55%, P 0.50%, and vitamin D3 at 1500 IU/kg). To avoid the excessive weight gain seen in ovx rats, pair feeding was started after the operation. All animals had free access to tap water and were housed three or four in wire-top plastic cages (23.5 cm × 32.5 cm wide, 15 cm high) in a room with a light/dark cycle of 12/12 h. The experiment conformed to the Council of Animal Research’s Code for the Care and Use of Animals for Experimental Purposes.

After 12 weeks, the rats were anesthetized for in vivo testing of right lower leg strength during muscle contraction and also to measure the isolated triceps surae muscle strength in the left lower leg. The rats were then killed by an overdose of pentobarbital, both tibiae resected and cleaned of soft tissues, weighed to the nearest 0.1 mg (wet weight), lengths measured, and left nude shafts tested mechanically in three-point bending. Geometrical measurements were done proximal to the fracture site in the left tibia of each animal.

Mechanical Testing of the Tibia In Vivo

Mechanical testing was conducted as described earlier. The ischiatic nerve was connected to a nerve stimulator (Pulsar 6i, Frederick Haer, Brunswick, ME) through a bipolar electrode. A clamp was fixed to the distal lower leg with suspension under the foot, and the rat was placed in a modular test apparatus (Figure 1a). The lower leg was deflected ventrally in three-point bending until tibial fracture at a quasistatic rate of 0.095 rad/sec (5.43°/sec) during supramaximal stimulation of the ischiatic nerve (0.5 ms square pulses of 80 Hz with an amplitude of 6 V). The cam engaged the soft tissues dorsally to the upper part of the tibia. A fulcrum positioned anteriorly to the leg was the third point of force application. The pulley force, T, was measured with a load cell connected to a microcomputer via an amplifier. The load-deflection curve was recorded on-line using WomBenchMac (Strawberry Tree Inc., Sunnyvale, CA). The ultimate pulley moment (MT) was read directly from the computer recordings of the load-deflection curves.

Mechanical Testing of the Tibia In Vitro

The fracture position in the tibia tested in vivo was measured from the malleolar plane, and the corresponding position was marked on the contralateral tibia. In the in vitro tibia was placed in the test apparatus and loaded identically to the in vivo tibia, with the fulcrum positioned over the fracture mark (Figure 1b,c). The pulley load was measured as in the in vivo test (see earlier), and the ultimate pulley moment (MT) was read correspondingly. The ultimate pulley moment (MT) was read directly from the computer recordings of the load-deflection curves.

Evaluation of Ultimate Moments and Stresses

During the in vitro test, the tibia is loaded in three-point bending by the forces, P (from the cam), F (fulcrum), and R (distal fixation), as shown in the free-body diagram of Figure 2a. The ultimate bending moment (MT) at the fracture site, near the fulcrum, follows directly from the pulley moment (Figure 2b):
\[ M_t = bP = aT \]  

(1)

During in vivo testing, the muscle force, \( F_m \), is added, working at a distance, \( t \), from the fracture site (Figure 2c). As an effect, additional forces are applied to the tibia at the knee (\( J_a \)) and the ankle (\( J_a \)), as shown in the free-body diagram of Figure 2d. The precise magnitudes and orientations of these forces are unknown. Other forces, created in distal fixation and by the knee-flexion restraint due to the coat around the rat, also probably take place. As an effect, not only does a bending moment (\( M_{vt} \)) work at the fracture site, but also a compressive force (\( N \)), as shown in Figure 2e. If we assume that both hip and knee behave as frictionless hinges during the in vivo test, then equilibrium conditions for the femur dictate that \( J_a > F_m \). The externally applied moments (muscle moment, \( tF_m \), and pulley moment, \( aT \)) can be superpositioned in the plane as scalars. Hence, we took:

\[ N < F_m \text{ and } M_{vt} = aT - tF_m \]  

(2)

The muscle force at fracture (\( F_m \)) was evaluated in a separate test (see later), the pulley moment (\( aT \)) was determined as described earlier, and the muscle moment arm (\( t \)) was estimated at 5 mm according to postmortem measurement of rat legs. Thus, the ultimate bending moment (\( M_{vt} \)) and the highest feasible compressive force (\( N = F_m \)) can be assessed. Assuming the Bernoulli beam-bending theory to be valid, the ultimate tensile stress in the in vitro test was calculated from:

\[ \sigma_{vi} = 0.5d_m M_{vi}/l \]  

(3)

where \( d_m \) is the anteroposterior periosteal diameter and \( l \) the area moment of inertia of the bone at the fracture site.\(^1\) Accordingly, the bending stress in the in vivo test was found from:

\[ \sigma_{vi} = 0.5d_m M_{vi}/l \]  

(4)

with \( M_{vt} \) as in equation 2. The potentially maximal axial stress compensation due to the force, \( N \), followed from:

\[ \sigma_a = F_m A \]  

(5)

where \( A \) is the cortical area of the bone at the fracture site (see later). If the axial stress, \( \sigma_a \), is negligible relative to the bending stress, \( \sigma_{vi} \), then the axial stress compensation plays no significant role, and our hypothesis is confirmed. In that case, the in vivo moment, \( M_{vt} \), at failure, calculated according to equation 2, must be equal to the in vitro failure moment, \( M_{vt} \). Hence, the difference between the in vivo and in vitro ultimate pulley moments (\( aT \)) must equal \( tF_m \). Because both \( t \) and \( F_m \) were measured in the experiments, our force analysis and its assumptions can be checked.

The anteroposterior (\( d_{1} \)) and mediolateral (\( d_{2} \)) periosteal diameters of the left tibia were measured using a sliding caliper just proximal to the fracture line. The diameter of the medullary canal (\( d_{3} \)) was measured by inserting cannules of increasing diameters with each one varying by 0.05 mm. The diameter of the largest cannula possible for insertion was taken as the medullary diameter. The cross section of the tibia was assumed to be elliptical in shape with a circular marrow canal. The area (\( A \)) was calculated from:

\[ A = \pi (d_1 d_2 - d_3^2)/4 \]  

(6)

The area moment of inertia (\( I \)) was calculated according to Bak and Jensen.\(^1\)

Figure 3. Mounting of the rat in the muscle-testing device (side view). The micrometer screws (a) could slide the table in two perpendicular directions. The ischiatic nerve was stimulated from a pulse generator (b). The load cell (c) was connected through a ring in the line of the leg muscle through the Achilles tendon, and the signal was amplified (d) before it was read.

**Assessment of Triceps Surae Force**

To evaluate the force of the triceps surae muscle during similar tetanic contraction as during in vivo three-point bending testing, we developed a new method that enabled us to measure maximal force through the Achilles tendon using a four-step procedure (Figure 3). First, the Achilles tendon was mobilized through a minimal incision distal to the leg at the insertion of the tendon to the calcaneus. The distal ends of the tendons were cleared from the surrounding tissues and mobilized along with a \( 3 \times 3 \times 3 \) mm\(^3\) block of the calcaneus containing the whole attachment of the tendon. Pins were then placed through the femoral and tibial condyles. Through a medial parapatellar incision, the plane between the quadriceps and adductor/hamstring muscles was dissected to reveal the upper attachment of the mediocollateral ligament on the femur. The same dissection procedure was performed to view the lateral side. A drill hole was made perpendicular to the femur from the upper mediocollateral ligament attachment site, parallel to the knee-joint line through the lateral femoral condyle, and a 1.5 mm Steinmann pin was inserted into the canal. The proximal tibia was dissected similarly, medially and laterally. After drilling, a 1.5 mm Steinmann pin was placed from behind the mediocollateral ligament parallel to the femoral pin at a distance of 5, 7, or 9 mm, with as little affection of the soft tissues as possible. After temporarily removing the pins inserted in the knee region, the third step involved placing the electrode on the sciatic nerve, as described previously.\(^2\) Finally, the fourth step was the actual measurement of the muscle force after the tibial and femoral pins were reinserted. The rat was placed face down on a plate, with a thickness of 2 cm, with the left thigh in a half-box construction of \( 4 \times 1.5 \times 1.5 \) cm\(^3\) mounted on one short end of the plate. The pins around the knee were placed in 7-mm-deep slots in the device, the tibial one fixed 12 mm from the end and the femoral pin in one of three slots 5, 7, or 9 mm proximal to the tibial one. The pins were secured by clamps on either side of the device. With the thigh and the knee immobilized in this manner, the lower leg and foot were free. The calcaneus block was inserted in the slot of a specially constructed ring that again was attached to a load cell, so the rest of the foot could hang vertically without affecting the transducer. The transducer was placed in line with the leg muscle direction through the Achilles tendon. A preload of 0.49 N was applied through adjustments with a micrometer screw (Figure 3a) after the rat was mounted. In pilot studies, this gave a muscle tension prior to stimulation that produced repeatable force curves. The rats were wrapped in a coat to avoid decrease of body temperature during testing. The electrodes were connected to an impulse generator (0.5 ms square pulses of 80 Hz with an amplitude of 6 V), and the stimulation was maintained for 20 sec.
deflection curve during three-point bending of the tibia in vitro. Differences were analyzed by Student’s paired t-test. Means and standard deviations were calculated for all results.

Statistical Evaluation

From the transducer, the signals were amplified to a mechanical printer as well as on-line in WorkBenchMac. In this way a curve for the time development of muscle force was obtained (Figure 3). For each rat, the force at fracture ($F_m$) in the in vivo three-point leg-bending test was estimated from this curve, considering the time lapse between muscle stimulation and fracture.

Results

Despite pair feeding, the ovx rats gained more weight than the sham rats ($p < 0.05$). Tibial weights and lengths were similar, whereas tibial shaft diameter, area, and area moment of inertia were increased in ovx compared with sham animals ($p < 0.05$). The ultimate moments and stresses on the tibia were similar in both groups, except for in vivo stress, which was higher in sham animals ($p < 0.05$). Tibial weights and lengths were similar, whereas tibial shaft diameter, area, and area moment of inertia were increased in ovx compared with sham animals ($p < 0.05$). The ultimate moments and stresses on the tibia were similar in both groups, except for in vivo stress, which was higher in sham animals ($p < 0.01$). Because our study concerned a comparison between collateral legs in the same animal, the differences and similarities in the data for both groups were irrelevant for testing the hypothesis, and therefore the groups were treated as a whole.

During in vivo fracture testing the pulley moment rose sharply when the stimulation of the ischiatic nerve was started, and then continued to rise steadily toward its ultimate value. On the other hand, the pulley moment in in vitro bending tests were performed. Two issues relative to the results are of particular interest: (1) with regard to interpretation of this experimental model in earlier reports, and (2) concerning the prospects for using the test to assess both muscle and bone strength independently.

Relative to the first issue, we found that, although muscle contraction was found to generate axial compression, its compensating effect for bending stress on the tensile side of the bone was no more than 2.5% at most. Thus, the protective muscle effect of the bone assumed earlier, based on suggestions from Nordin and Frankel, was not realized in a practical sense. Therefore, the interpretation of this test was erroneous. The muscle does not cause an increase in bone-bending moment, but only of the external pulley moment. We found that muscle contraction counteracts the external pulley moment in this in vivo, ventral, three-point bending test. If, however, the external moment were dorsally directed in this model, the contracting muscle would assist in breaking the tibia, making it seem weaker instead of stronger, as in the ventral test. The notion that a strengthening effect of the muscle would occur was originally derived from the results shown in Figure 4, comparing in vivo with in vitro bending moment vs. deflection curves. It is evident in retrospect, however, that muscle contraction produces an immediately increased pulley moment (Figure 7). This appears in the curve because the test is displacement controlled, implying that the machine, through the pulley moment, is forced to maintain a constant rotation rate. To accomplish this, the machine must increase its force against that of the muscle.

It must be appreciated that the force analysis was not mathematically rigorous, as assumptions were made about forces that could not be measured. The mechanical consequences of the clamping of the foot of the rat were uncertain. Also uncertain were the reaction forces working on the proximal tibia, produced in the knee and at the contact between the knee and the coat that partly prevented it from flexing. In addition, the estimate of the value of the muscle-bending moment relative to the fracture site could not be very accurate. The muscle force was measured in a separate test, not during the in vivo fracture test. The fracture site was not always exactly the same, and the estimate of the muscle-force moment arm, based on assumptions about the force line of action, could not have been very precise. Because the assumed strengthening effects of the muscle did not occur, the in vivo and in vitro ultimate bending moments of the tibial diaphysis should be approximately equal in all rats. This was indeed

Figure 4. (a) Load-deflection curve with the three-point bending test in vivo where the arrow marks the point of start of the triceps surae contraction when the pulley moment rises sharply. (b) Typical load-deflection curve during three-point bending of the tibia in vitro.

Figure 5. The typical triceps surae muscle load curve during 20 sec of stimulation through the ischiatic nerve.

Discussion

An established in vivo rat model designed to evaluate the effects of physical activity, hormonal deficiencies, and drugs on lower leg strength in three-point bending during muscle contraction, was analyzed in this study. A new test was developed to measure the triceps surae contractile force in each rat separately, and mechanical analyses of the in vivo and in vitro three-point bending tests were performed. Two issues relative to the results are of particular interest: (1) with regard to interpretation of this experimental model in earlier reports, and (2) concerning the prospects for using the test to assess both muscle and bone strength independently.

Relative to the first issue, we found that, although muscle contraction was found to generate axial compression, its compensating effect for bending stress on the tensile side of the bone was no more than 2.5% at most. Thus, the protective muscle effect of the bone assumed earlier, based on suggestions from Nordin and Frankel, was not realized in a practical sense. Therefore, the interpretation of this test was erroneous. The muscle does not cause an increase in bone-bending moment, but only of the external pulley moment. We found that muscle contraction counteracts the external pulley moment in this in vivo, ventral, three-point bending test. If, however, the external moment were dorsally directed in this model, the contracting muscle would assist in breaking the tibia, making it seem weaker instead of stronger, as in the ventral test. The notion that a strengthening effect of the muscle would occur was originally derived from the results shown in Figure 4, comparing in vivo with in vitro bending moment vs. deflection curves. It is evident in retrospect, however, that muscle contraction produces an immediately increased pulley moment (Figure 7). This appears in the curve because the test is displacement controlled, implying that the machine, through the pulley moment, is forced to maintain a constant rotation rate. To accomplish this, the machine must increase its force against that of the muscle.

It must be appreciated that the force analysis was not mathematically rigorous, as assumptions were made about forces that could not be measured. The mechanical consequences of the clamping of the foot of the rat were uncertain. Also uncertain were the reaction forces working on the proximal tibia, produced in the knee and at the contact between the knee and the coat that partly prevented it from flexing. In addition, the estimate of the value of the muscle-bending moment relative to the fracture site could not be very accurate. The muscle force was measured in a separate test, not during the in vivo fracture test. The fracture site was not always exactly the same, and the estimate of the muscle-force moment arm, based on assumptions about the force line of action, could not have been very precise. Because the assumed strengthening effects of the muscle did not occur, the in vivo and in vitro ultimate bending moments of the tibial diaphysis should be approximately equal in all rats. This was indeed
found in the force analysis of the tests, which was a vital contribution to their validity.

In the analysis, sham and ovx rats were combined into one group. The overall results, however, would not have been different for each group separately and, as the analysis was based purely on comparisons between in vivo and in vitro measurements in one and the same animal, the use of different groups only strengthened the generality of our findings.

It was serendipitously found that the triceps muscle could not break the tibia by itself, but it could possibly develop microcracks or permanent yield deformations when contracting maximally in isolation. The maximal bending moment the muscle produced in the in vivo fracture test was about one third of the ultimate failure moment, indicating a safety factor of about 3. This is much lower than the safety factors of 5–7.5 reported for mammals in physiological function studies.\(^2,12\)

Hence, the development of yield damage is likely if the bending moments on the bone are not compensated by antagonistic muscles.

Relative to the second issue just examined, we found that our hypothesis was confirmed, and that the difference between the in vivo (with muscle stimulation) and in vitro (nude tibia) external pulley moments at fracture can be used to estimate maximal muscle force. Based on this background, bone and muscle strength can be assessed separately using this model. As shown in Figure 7, however, one does not have to break the bone to determine the maximal bending moment of the muscle, as it already appears in the curve long before the pulley moment reaches its ultimate value. This means that muscle and bone strength could even be measured separately on the same leg (and thus also on both legs) by first measuring the muscle moment in a test with muscle stimulation, but without fracture, followed by a test without stimulation, but with fracture. If the latter could be accomplished reliably on the in situ leg, instead of the nude tibia, then a relatively simple model for bilateral assessment of both muscle and bone strength could evolve.

**Acknowledgments:** The authors thank Harald Steen and Per Ludvigsen (Biomechanical Laboratory of the Center of Orthopaedics of the National Hospital) for their contribution to the development of the muscle test. We also thank Vera Halvorsen (Department of Orthopaedics, Ullevaal Hospital) and Ihsan Basaran (Department of Plastic Surgery, Hacettepe University Hospital, Ankara, Turkey) for their contributions during testing.

**References**


Date Received: April 6, 1999
Date Revised: November 16, 1999
Date Accepted: December 9, 1999