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Effects of surgical holes in mouse tibiae on bone formation induced by knee loading

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Abstract

Loads applied directly to the knee (knee loading) have recently been demonstrated to induce anabolic responses in femoral and tibial cortical bone. In order to examine the potential role of intramedullary pressure in generating those knee loading responses, we investigated the effects of drilling surgical holes that penetrated into the tibial medullary cavity and thereby modulated pressure alteration. Thirty-nine C57/BL/6 female mice in total were used with and without surgical holes, and the surgical holes were monitored with micro CT and histology. The left knee was loaded for 3 days, and the contralateral limb was treated as a sham-loaded control. Mice were sacrificed for bone histomorphometry 2 weeks after the last loading. Although the surgical hole induced bone formation in both loaded and non-loaded tibiae, due to regional and systemic acceleratory phenomenon the anabolic effect of knee loading was substantially diminished. Without the holes, knee loading significantly elevated cross-sectional cortical area, cortical thickness, mineralizing surface, mineral apposition rate, and bone formation rate on the periosteal surface. For example, the rate of bone formation was elevated 2.1 fold ($p < 0.001$; middle diaphysis - 50% site from the knee along the length of tibiae) and 2.7 fold ($p < 0.01$; distal diaphysis - 75% site). With the surgical holes, however, knee loading did not provide significant enhancement either at the 50% or 75% site in any of the histomorphometric measurements ($p > 0.05$). The results support the idea that alteration of intramedullary pressure is necessary for knee loading to induce bone formation in the diaphysis.

Keywords

loading; knee; bone formation; tibia; surgical hole; intramedullary pressure

Introduction

Mechanical loads can provoke remodeling of bone structure and prevention of bone loss [1–3]. Joint loading is one loading modality recently employed as a useful method for inducing bone formation in laboratory animals. Through lateral loads applied to the epiphysis, it has been shown to elevate anabolic responses in long bones including ulnae [4], tibiae [5], and femora [6]. Compared to ulna axial loading, for instance, joint loading (elbow loading) requires a smaller force (1/4 or less of the force required with ulna axial loading) and induces minute strain ($\sim 30 \mu\text{strain}$) at the site of bone formation [7]. Knee loading is one form of joint loading

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specific to a hindlimb, in which sideward loads are directed to a distal femoral epiphysis together with a proximal tibial epiphysis [5,6]. Since the loading site in the epiphysis and the region of bone formation in the diaphysis are structurally separated, biophysical and/or biochemical mediators likely connect these two locations [8].

Using the mouse hindlimb as a model system, we recently demonstrated that knee loading induces alteration of intramedullary pressure in the femoral bone cavity and that this alteration is synchronous to the loading frequency in Hz [9]. In separate loading studies, augmentation of intramedullary pressure through an external pressure source has also been shown to increase bone formation using *in vivo* turkey ulnae [10,11]. Similarly, a pressure gradient, elevated by venous ligation, was shown to increase interstitial fluid flow and this flow-mediated bone adaptation was considered to be independent of mechanical strain [12–14]. Alteration of intramedullary pressure is therefore likely to be a potential mediator for knee loading-driven bone formation [8]. Herein we examined the role of intramedullary pressure in knee loading using a surgical approach – drilling holes as a means of pressure interference.

Focusing on the middle and distal diaphysis in the tibia, the current study aimed to inquire about the role of intramedullary pressure in load-driven bone formation by drilling a penetrating surgical hole through the tibial shaft. We have previously demonstrated that knee loading enhances bone formation in the proximal diaphysis near the knee [5]. The section farther distant from the knee, however, has not yet been analyzed. The present study therefore addressed the following questions: do loads applied to the tibial proximal epiphysis enhance bone formation in the middle and distal diaphysis? If so, is the enhancement affected by the surgical hole that penetrates to the tibial bone cavity? From previous studies, it is known that surgical holes induce regional acceleratory phenomenon (RAP) and systemic acceleratory phenomenon as well as suppression of pressure changes in intramedullary cavities. First, the healing of a hole defect in femora and tibiae was accelerated through local and systemic tissue defensive reactions [15]. Second, in medullary nailing and total hip replacement a pressure release hole was shown to reduce build-up of intramedullary pressure [16,17]. We hypothesized that *RAP and systemic acceleratory phenomenon would induce bone formation regardless of knee loading, and that the overall induction would be stronger at the site closer to the hole*. We also hypothesized that *knee loading driven anabolic responses would be reduced by a surgical hole drilled through the tibial diaphysis*.

In order to test those hypotheses, we employed two groups of mice: one with a penetrating surgical hole on both left and right tibiae; and the other without holes. The left tibia was knee loaded, and the right tibia was treated as a sham-loaded control. The hole was positioned at a site 25% from the proximal end along the tibial length, and bone histomorphometry was conducted at the 50% site (middle diaphysis) and the 75% site (distal diaphysis). We measured alterations in cortical bone area and thickness, mineralizing surface, mineral apposition rate, and bone formation rate on periosteum and endosteum in the two groups with and without surgical holes.

Materials and Methods

Experimental design

Experimental procedures were approved by the Indiana University Animal Care and Use Committee and were in compliance with the Guiding Principles in the Care and Use of Animals endorsed by the American Physiological Society. Thirty-nine C57/BL/6 female mice, ~ 14 weeks of age (a body weight, ~ 20 g; Harlan Sprague-Dawley, Inc., Indianapolis, IN) were used. Four to five mice were housed per cage and they were fed with mouse chow and water *ad libitum*. The animals were allowed to acclimate for 2 weeks before the experiment and

divided into two groups: the first group without surgical holes ($n = 8$), and the second group with surgical holes ($n = 31$). The time line of the experiments is summarized in Fig. 1.

Surgical holes

A surgical hole which penetrated through the medial and posterior surfaces was drilled in the left and right tibiae [18,19]. The position was approximately 4 mm from the proximal tibial end (25% along the length of the tibia). The mice were first anesthetized with 2% isoflurane and the hindlimbs were shaved and sterilized with 10% providoneiodine solution. A 5-mm longitudinal skin incision was then made on the antero-medial side of the left hindlimb. A penetrating single surgical hole, 0.5 mm in diameter, was drilled with a new drill bit from the medial cortex to the opposite (posterior) cortex [18,19]. Note that the hole was aligned nearly perpendicular to the longitudinal tibial axis in the range of 70° to 90° . A plunge router attachment was used to maintain stability and consistency of the operation. Next, the muscles and skin were sutured and closed. After making the hole in the left tibia, the same procedure was applied to the right tibia. Antibiotic prophylaxis (Enrofloxacin, 5 $\mu\text{g/g}$) and analgesia (Morphine, 5 $\mu\text{g/g}$) were administered for the first two days postoperatively. These mice were also examined daily for abnormal behavior. Out of 31 mice with surgical holes, 5 mice were sacrificed without knee loading on the day of surgery for μCT imaging. Twenty-six mice were loaded from the fourth postoperative day using the procedure described below ($n = 10$ for bone histomorphometry; and $n = 16$ for μCT imaging).

Knee loading

The mouse was placed in an anesthetic induction chamber to cause sedation and then mask-anesthetized using 2% isoflurane. With our custom-made piezoelectric mechanical loader, knee loading was conducted as described previously [5,6]. In brief, loads were applied for 3 minutes per day for 3 consecutive days to the left knee in the lateral-medial direction. The loading frequency was 5 Hz, and the peak-to-peak force was 0.5 N (Figs. 2 and 3). The right hindlimb was used as a sham-loaded control, where the right knee was placed under the loading rod for 3 min in the same procedure used for the left knee without applying a voltage signal to the loader. After loading, the mouse was allowed normal cage activities. On days 2 and 6 after the last loading, the mice were given an intraperitoneal injection of calcein (Sigma, St. Louis, MO), a fluorochrome dye, at 30 $\mu\text{g/g}$ body mass.

Sample harvest

Eighteen animals were sacrificed 2 weeks after the last loading (3 weeks after the surgery in the second group) for bone histomorphometry. Twenty-one animals were sacrificed for μCT imaging ($n = 5$ on the operative day, $n = 7$ in 1 postoperative week, and $n = 9$ in 3 postoperative weeks). The left and right tibiae were harvested and cleaned of soft tissues. The distal and proximal ends were cleaved to allow infiltration of the fixatives containing 10% neutral buffered formalin. After 48 h in the fixatives bones were transferred to 70% alcohol for storage.

Micro CT

Micro CT was performed using a desktop μCT -20 (Scanco Medical AG, Bassersdorf, Switzerland) [20]. The harvested tibia was placed in a plastic tube filled with 70% ethanol and centered in the gantry of the imaging device. A series of cross-sectional images were captured at 30- μm resolution. Three-dimensional reconstruction was performed and the images were analyzed using Scanco software (μCT v.1.4) (Figs. 3 & 4). Among the cross-sectional images, the largest opening of the surgical holes was defined as the size of the hole.

Bone histomorphometry

Bone histomorphometry was conducted as reported previously [5,6]. In brief, specimens were embedded in methyl methacrylate (Aldrich Chemical Co., Milwaukee, WI) and transverse sections were prepared at ~ 4 mm (25% site), ~ 8 mm (50% site), and ~ 12 mm (75% site) distant from the proximal end of the tibia. From fluorescent measurements such as total perimeter (B.Pm), single-labeled perimeter (sL.Pm), double-labeled perimeter (dL.Pm), and double-labeled area (dL.Ar), we derived mineralizing surface ($MS/BS = [1/2sL.Pm + dL.Pm]/B.Pm$ in %), mineral apposition rate ($MAR = dL.Ar/dL.Pm/4$ in $\mu m/day$), and bone formation rate ($BFR/BS = MAR \times MS/BS \times 365$ in $\mu m^3/\mu m^2$ per year). The relative parameters such as rMS/BS , $rMAR$, and $rBFR/BS$ were calculated as $([L\ C]/C \times 100 \text{ in } \%)$, where L = “loaded” and C = “control.”

We also measured total bone cross-sectional area (mm^2), bone medullary area (mm^2), and cortical thickness (mm). The cross-sectional cortical area was determined by subtracting the bone medullary area from the total bone cross-sectional area. The cortical thickness was defined as the mean distance between the endosteal surface and the periosteal surface on three tibial sides (medial, lateral, and posterior). The measurements were taken at the middle of each side, and the mean value was calculated from 2 independent measurements.

Statistical analysis

The data were expressed as mean $\pm$ SEM. Statistical significance among groups was examined using one-way ANOVA. For pair-wise comparisons a post-hoc test was conducted using Fisher’s “protected least significant difference.” A paired *t*-test was employed to evaluate statistical significance between the loaded samples and sham-loaded control. All comparisons were two-tailed and statistical significance was assumed for $p < 0.05$.

Results

Mice with surgical holes were less active for ~ 30 minutes after the surgery than mice with no operation. Their behavior recovered to nearly normal in about 2 hours. No infections were detected at the surgical site. During knee loading no bruising or other damage was detected at the loading site, and after loading mice did not show a weight loss or a diminished food intake.

Surgical holes

Micro CT images of the tibia with surgical holes clearly illustrate a partial removal of tibial cortex on the operative day (Fig. 4A & 4B). The original hole was 0.5 mm in diameter, and it penetrated through the medial and posterior cortices at a site 25% (~ 4 mm) distant from the proximal tibial end along the tibial length. The hole was open both in the control and loaded tibia one week after the surgery, and the size of cortical windows was 0.46 ± 0.01 mm ($n = 7$ in control) and 0.40 ± 0.01 mm ($n = 7$ in the loaded tibia) (Fig. 4C & 4D). In 3 weeks after the operation, callus formation was detected around the hole. The filling of the medullary cavity was detected, and the size of surgical holes became 0.26 ± 0.01 mm ($n = 9$ in control) and 0.22 ± 0.02 mm ($n = 9$ in the loaded tibia) (Fig. 4E & 4F).

Hole-induced bone formation without knee loading

In sham-loaded control (without knee loading), the surgical holes elevated the rate of bone formation both on the periosteal and endosteal surfaces at the 50% and 75% sites. First, the rate of bone formation in the group with surgical holes was significantly increased on the periosteal surface ($p < 0.001$ at 50%, and $p < 0.01$ at 75%) and the endosteal surface ($p < 0.05$ at 50%, and $p < 0.01$ at 75%) (Fig. 5A). The group with surgical holes exhibited a higher rate of bone formation than the group without surgical holes: 3.5 X (periosteum) and 2.0 X

(endosteum) at the 50% site; and 2.9 X (periosteum) and 1.6 X (endosteum) at the 75% site. Second, between the 50% and 75% sites on the periosteal surface, the rate of bone formation was higher at the 50% site than at the 75% site in the presence of surgical holes ($p < 0.001$) as well as in their absence ($p < 0.05$) (Fig. 5B). Regardless of surgical holes no statistical significance was detected between these two sites on endosteum.

Effects of knee loading on alteration in cortical area and thickness with and without surgical holes

Knee loading elevated cortical area and cortical thickness for the mice without surgical holes, but the enhancement was diminished for the mice with surgical holes (Fig. 6). Without surgical holes knee loading increased the cross-sectional cortical area from $0.52 \pm 0.01 \text{ mm}^2$ (control) to $0.57 \pm 0.01 \text{ mm}^2$ (loading) at the 50% site ($p < 0.01$), and from $0.56 \pm 0.02 \text{ mm}^2$ (control) to $0.63 \pm 0.01 \text{ mm}^2$ (loading) at the 75% site ($p < 0.05$). Similarly, in the group with no surgical holes knee loading elevated cortical thickness from $0.183 \pm 0.004 \text{ mm}$ (control) to $0.203 \pm 0.004 \text{ mm}$ (loading) at the 50% site ($p < 0.01$) and from $0.223 \pm 0.004 \text{ mm}$ (control) to $0.252 \pm 0.006 \text{ mm}$ (loading) at the 75% site ($p < 0.01$). However, in the tibiae with surgical holes no significant alteration by knee loading was observed in cortical area at either site ($p = 0.097$ at 50%; and $p = 0.098$ at 75%). Furthermore, in the mice with surgical holes knee loading did not show any statistically significant increase in cortical thickness at the 50% site ($p = 0.062$) or the 75% site ($p = 0.390$).

Differential role of knee loading in bone formation with and without surgical holes

Load-driven bone formation was stimulated in the group with no surgical holes in the middle and distal diaphysis in the tibia (Table 1). On the periosteal surface at the 50% site, the increase was 1.6 times (MS/BS with $p < 0.05$), 1.4 times (MAR with $p < 0.05$), and 2.1 times (BFR/BS with $p < 0.001$). Similarly, the loading elevated these parameters at the 75% site on the periosteal surface (2.0 X for MS/BS with $p < 0.01$, 1.4 X for MAR with $p < 0.001$, and 2.7 X for BFR/BS with $p < 0.01$). On the endosteal surface of mice without surgical holes, bone formation was stimulated by knee loading at the 50% and 75% sites but a statistically significant alteration was observed only at the 75% site. Three morphometric parameters at the 75% site were 1.3 fold (MS/BS with $p < 0.05$), 1.2 fold (MAR with $p < 0.01$), and 1.5 fold (BFR/BS with $p < 0.01$) higher than sham-loaded control.

Compared to the group without surgical holes, the observed effects of knee loading in the group with surgical holes were less prominent. At the 50% and 75% sites with surgical holes, no statistical difference regarding bone formation was detected between the loaded and sham-loaded tibiae on either the periosteal or endosteal surface (Table 1).

The differential effects of knee loading were also observed as percent change (with and without surgical holes) in rMS/BS, rMAR, and rBFR/BS (Fig. 7). Compared to the group without surgical holes, loading for the group with surgical holes presented a lower value of rMS/BS, rMAR, and rBFR/BS (all $p < 0.01$) at the 50% site, and a smaller increase in rMS/BS ($p < 0.01$), rMAR ($p < 0.001$), and rBFR/BS ($p < 0.001$) at the 75% site. Unlike the periosteal surface, no discriminatory effects of surgical holes were observed on the endosteal surface.

Discussion

The present study describes anabolic responses with knee loading in the middle and distal diaphysis, and the abated effects of knee loading with a single surgical hole which penetrated into the tibial medullary cavity. First, without the holes the observed histomorphometric parameters consistently exhibited load-driven bone formation. Together with a previous study which focused on the outcome in the proximal diaphysis [5], the present study reveals that knee

loading is effective in enhancing bone formation throughout the tibia. Second, we employed a surgical hole and impeded alterations of intramedullary pressure under knee loading. We observed that anabolic responses attributable to knee loading were decreased by the surgical hole located between the loading site and the distal cortical shaft. Although the holes were narrowed during the 3-week experiment in the loaded and control tibiae, the open window into the medullary cavity was visible through μ CT images.

The round hole used in this study has been previously employed by others to investigate the healing of local bone defects [18,19]. When a surgical hole was employed to diffuse intramedullary pressure in the present study, RAP and systemic acceleratory phenomenon were observed in the tibial diaphysis [15]. In sham-loaded controls we detected elevated bone formation rate by the surgical holes, and the elevation was greater at the 50% site near the hole than the 75% site away from it. In spite of these acceleratory phenomena, the histomorphometric measurements unambiguously exhibited decreased efficacy of knee loading due to the surgical holes. In the presence of surgical holes, knee loading did not provide any statistically significant alterations in MS/BS, MAR, or BFR/BS. In contrast, without the surgical holes all morphometric measurements supported knee-loading driven anabolic effects at the 50% and 75% sites.

The results here do not directly demonstrate the role of intramedullary pressure behind knee loading driven bone formation. However, together with previous biomechanics studies using fluorescence recovery after photobleaching (FRAP) [21] and fiber optical pressure sensors [9,12,13] they allow us to consider a certain biophysical model. FRAP data are consistent with load-driven fluid flow, while pressure data support alterations of intramedullary pressure in response to mechanical loading. In the model, cyclic deformation of the epiphysis alters pressure in the medullary cavity and the pressure gradient induces fluid flow in the diaphysis [22–25]. In the presence of surgical holes, it is expected that the gradient is not adequately established because of incomplete pressure sealing. During the course of those experiments, we observed a tendency for the holes in the loaded tibiae to become smaller with knee loading than the holes in controls. This observation indicates that knee loading might be effective in stimulating the healing of bone defects, but further analyses are necessary to evaluate the effects of knee loading on wound healing. It should be noted that it might be easier to transmit a pressure signal over a cartilaginous growth zone than over an ossified growth zone due to its lower mechanical impedance.

We previously reported that knee loading is more effective at the periosteal surface than the endosteal surface in tibiae and femora of C57/BL/6 mice [5,6]. The differential sensitivity between the periosteal and endosteal surfaces was also observed in the present study regardless of surgical holes. There might be a possibility that load-induced fluid flow could be stronger on the periosteal surface than the endosteal surface depending on the structure of lacunocanicular networks and the distribution of interstitial fluid in bone. Further biophysical analyses are certainly warranted. Besides differences in the micro architecture of the periosteum and endosteum, a local cellular and molecular environment might contribute to the observed differential sensitivity. For instance, osteoblast specific factor 2 (periostin) is preferentially expressed on periosteum and considered to play a role in the recruitment and attachment of osteoblast precursors in the periosteum [26]. Note that other mouse strains may respond differently to the same loading modality [27–29].

In summary, we demonstrate that knee loading is an effective means to enhance bone formation in the middle and distal diaphysis in the mouse tibia, and its efficacy is interfered with by a surgical hole in the medullary cavity. Note that knee loading herein induces approximately 30 μ strain at the site of bone formation and the number of loading cycles per day is 900 for 3 days [5,30]. Further studies are required to extend our knowledge on the interplay of mechanical

stimulation with knee loading to joint and bone tissues. Nevertheless, our knee loading research platform may contribute to the future development of biophysical treatments and promotion of bone health.

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Figure 1.
Time line of the study. μ CT = micro computer tomography.

Figure 2.

Knee loading without a surgical hole. (A) Left knee without a surgical hole on a loading table for knee loading. (B) Schematic diagram of the piezoelectric mechanical loader used in this study. Two locations (50% and 70% sites from a tibial proximal end along the length of a tibia) were used for bone histomorphometry. (C) Cross-section of the control tibia at the 50% site. Bar = 200 μ m in C to F. (D) Cross-section of the loaded tibia at the 50% site. (E) Cross-section of the control tibia at 75% site. (F) Cross-section of the loaded tibia at 75% site. Note that “med” = medial surface; “lat” = lateral surface; and “post” = posterior surface.

Figure 3.

Knee loading with a surgical hole. (A) Left knee with a surgical hole on a loading table for knee loading. (B) Locations of a loading site, a surgical site and two sites for bone histomorphometry. (C) Cross-section of the control tibia at the 50% site. Bar = 200 μ m in C to F. (D) Cross-section of the loaded tibia at the 50% site. (E) Cross-section of the control tibia at 75% site. (F) Cross-section of the loaded tibia at 75% site. Note that “med” = medial surface; “lat” = lateral surface; and “post” = posterior surface.

Figure 4.

Surgical holes in the tibia. (A) Micro CT image of the hole in the medial surface on the surgery day. Bar = 500 μ m in A to D. (B) Micro CT image of the hole in the posterior surface on the surgery day. (C) Micro CT image of the control tibia in 1 week after the surgery. (D) Micro CT image of the loaded tibia in 1 week after the surgery. (E) Cross-section of the holes in the control tibia, 3 weeks after the surgery (without knee loading). Bar = 200 μ m in E and F. (F) Cross-section of the holes in the loaded tibia, 3 weeks after surgery (with knee loading).

Figure 5.

Comparison ($\mu\text{m}^3/\mu\text{m}^2/\text{year}$) with and without surgical holes in periosteum and endosteum in sham-loaded control. The results are expressed as mean $\pm$ SEM, and the asterisks show statistical significance at $p < 0.05$ (single), $p < 0.01$ (double) and $p < 0.001$ (triple), based on Fisher's protected least significant difference at $\alpha = 0.05$. (A) Comparison between mice with and without surgical holes. (B) Comparison between the 50% and 75% sites.

Figure 6.

Alteration in cross-sectional area and cortical thickness in response to knee loading. The results are expressed as mean $\pm$ SEM for samples with and without surgical holes. The single and the double asterisks indicate $p < 0.05$ and $p < 0.01$, respectively. (A) Cortical cross-sectional area (mm^2) at the 50% site. (B) Cortical thickness (mm) at the 50% site. (C) Cortical cross-sectional area (mm^2) at 75% site. (D) Cortical thickness (mm) at 75% site.

Figure 7.

Alteration in histomorphometric parameters on the periosteal and endosteal surfaces in response to knee loading. The results are expressed as mean $\pm$ SEM, and the double and triple asterisks indicate $p < 0.01$ and $p < 0.001$, respectively, based on Fisher's protected least significant difference at $\alpha = 0.05$. (A) Relative MS/BS (%difference from control). (B) Relative MAR (%difference from control). (C) Relative BFR/BS (%difference from control).

Table 1

Bone formation in both the periosteum and endosteum, with and without surgical holes

		MS/BS (%)	p value	MAR (µm/day)	p value	BFR/BS (µm ³ /µm ² /year)	p value
50% site	without hole	12.21±1.20		0.30±0.03		12.92±1.35	
Periosteum	control	18.97±1.93	<0.05	0.41±0.04	<0.05	26.73±1.68	<0.001
	knee-loading	9.69±0.93		0.27±0.02		9.63±1.24	
Endosteum	control	11.43±1.02	NS	0.32±0.02	NS	13.21±1.26	NS
75% site	knee-loading						
Periosteum	without hole	11.15±1.40		0.20±0.01		8.13±1.30	
	control	21.79±2.51	<0.01	0.27±0.01	<0.001	21.64±2.78	<0.01
Endosteum	knee-loading	12.34±1.07		0.20±0.01		9.05±0.95	
	control	16.23±1.13	<0.05	0.23±0.01	<0.01	13.92±1.18	<0.01
50% site	with hole						
Periosteum	control	61.58±4.28		0.20±0.01		45.39±3.49	
	knee-loading	69.90±6.40	NS	0.22±0.01	NS	55.82±5.95	NS
Endosteum	control	27.12±4.71		0.19±0.01		19.25±3.42	
	knee-loading	32.96±4.46	NS	0.22±0.01	NS	26.91±4.22	NS
75% site	with hole						
Periosteum	control	36.54±5.34		0.18±0.01		23.96±3.86	
	knee-loading	43.52±6.56	NS	0.19±0.01	NS	31.44±5.65	NS
Endosteum	control	24.19±1.86		0.16±0.01		14.13±1.20	
	knee-loading	27.80±1.64	NS	0.17±0.01	NS	17.81±1.48	NS

NS indicates that *p* value exceeds 0.05.