Accepted Article

J Musculoskelet Neuronal Interact 2025

Journal of Musculoskeletal and Neuronal Interactions JMNI

Original Article

Hindlimb Suspension and Subsequent Weight-Loading on Femoral Morphology, Knee Joint Alignment, and Patellar Tracking in Growing Rats

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Abstract

Objectives: Reduced weight-loading on the lower extremities during growth induces abnormal bone morphology and malalignment, but the effects of reloading on these abnormalities remain unknown. We aimed to determine the specific timeframes in which hindlimb suspension (HS) during growth induces abnormal femoral morphologies and knee malalignments present at skeletal maturity in rats. **Methods**: Four-week-old female rats were subjected to HS for one, two, or four weeks using the tail suspension method, followed by reloading. Age-matched rats were used as controls. At 12 weeks of age, femoral morphology and knee joint alignment were assessed using X-ray computed tomography. **Results**: Femoral anteversion angle, medial/lateral condylar height ratio (MC/LC), and patellar tilt angle were significantly larger, while trochlear angle, lateral condylar height (LC), and lateral trochlear inclination (LTI) were smaller in the groups that underwent HS for two or four weeks prior to reloading, compared with the control group. There were significant correlations between LTI and LC ($r = 0.57$), MC/LC ($r = -0.42$), and patellar tilt angle ($r = -0.49$). In the 4-week HS group, lateral patellar dislocation was observed in 3 of 8 animals. **Conclusion**: Insufficient weight-loading more than two weeks during growth may induce knee joint instability even after reloading.

Keywords: Femoral Morphology, Hindlimb Suspension, Knee Malalignment, Patellar Dislocation, Reloading

Introduction

Mechanical stress is considered essential during bone formation. Decreased mechanical stress, such as that experienced during weight unloading, bed rest, or space flight, results in bone loss and increased bone fragility[1,2]. Hindlimb suspension (HS), constituting an experimental reduction of mechanical stress, causes reductions in bone mass[3], mineral density[4], and strength properties[5] and alterations of bone microstructure[6]. However, few studies have examined how decreased mechanical stress affects bone morphologies and joint alignment.

Abnormalities in femoral morphology and knee joint alignment are associated with various musculoskeletal disorders of both the knee and hip, including patellofemoral instability, patellar dislocation, and osteoarthritis (OA)[7,8]. Alterations in bone morphology and alignment can induce the generation and accumulation of abnormal mechanical stress at joints, and consequently contribute to the development of musculoskeletal disorders. Indeed, abnormal femoral morphology is associated with abnormal locomotion patterns[9] and in concomitant with patellofemoral joint pathology in rat models[10]. Accordingly, preventing abnormal femoral morphology and knee joint alignment may have the potential to avert the onset of musculoskeletal diseases.

Several experimental models have demonstrated the ability to alter femoral morphologies and knee alignment in growing animals. For example, surgically induced patellar

The authors have no conflict of interest.

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Edited by: G. Lyritis
Accepted 22 September 2025

instability, achieved by lateral patellar dislocation[11-13] or the transection of the medial soft tissue restraints of the patella[14], altered femoral trochlear groove morphologies. Patellar dislocation has also been observed to cause knee malalignment, such as tibial tubercle lateralization[15]. HS in growing rats has been observed to induce various morphological abnormalities in the femur, such as increased femoral anteversion, outward rotation of the trochlear angle, and asymmetrical condyle growth, which are associated with knee disorders like patellofemoral instability and knee OA[16]. In addition, HS can cause knee joint malalignments, such as lateral tilt and lateral translocation of the patella, and increase the external rotation of the tibia, resulting in patellar dislocation in growing rats[10]. These findings suggest that insufficient loading on joints during growth may increase the risk of developing abnormalities in bone morphology and joint alignment. Previous studies have reported that reductions in bone volume and bone mineral density observed after two to four weeks of HS can be reversed by reloading[17-19]. However, few studies have explored the effects of reloading on HS-induced abnormalities in the knee joint, particularly femur morphologies and knee alignment which can lead to musculoskeletal disorders.

The objectives of this study were twofold: 1) to determine whether the abnormalities in femur morphologies and knee alignments induced by short-term HS in growing rats present after reloading until 12 weeks of age, and 2) to determine the periods of HS after which the developed abnormalities in femur morphologies and knee alignments present even after reloading.

To achieve these objectives, four-week-old immature rats were subjected to HS for one, two, or four weeks and then reloaded until 12 weeks old. After the experimental treatments, their femur morphologies and knee joint alignments were analyzed. We hypothesized that longer unloading periods would induce more pronounced abnormalities in bone morphologies and knee alignments, and that these abnormalities would not be reversed after reloading.

Materials and Methods

Animals

A total of 32 immature Wistar rats (female, four weeks of age, Japan SLC, Shizuoka, Japan) were used in this study. Weaning of rats is achieved at 3-4 weeks. Rats were randomly divided into two groups: the control (n = 8) and various periods of HS followed by reloading until 12 weeks of age (HS, n = 24). Rats in the HS group were further distributed among the 1wHS, 2wHS, and 4wHS groups (n = 8 per group) according to the period of HS (one, two, and four weeks, respectively). Both groups were maintained under the following conditions: room temperature of 20°C to 25°C, 12 h/12 h light/dark cycle, and unlimited food and water.

Hindlimb suspension (HS)

HS on rats was performed using the methods described in a previous study[16]. In brief, after being anesthetized with an intraperitoneal injection of a mixture of ketamine (40 mg/kg)/xylazine (5 mg/kg), a sterilized Kirschner wire was inserted into the proximal region of the tail disinfected with povidone-iodine. A looped stainless-steel chain was attached to both ends of the Kirschner wire. The chain was connected to a swivel at the top of a cylindrical cage (height 36 cm, diameter 32 cm) using clips. The length of the chain was adjusted to prevent contact between the hindlimbs and the floor, while allowing the forelimbs to maintain contact with the floor and move freely. Rats were monitored at least once every 2 days for infection or wire tolerance. No routine postoperative analgesia was used because tail K-wire placement is classified as a mild procedure at our institution, and systemic analgesics can confound bone-remodeling endpoints. Animals were observed twice daily for 48 h and then at least every 48 h for pain/distress (reduced activity or grooming, abnormal posture, piloerection, porphyrin staining, >10 % weight loss). A predefined rescue analgesia protocol was in place, but no animals required intervention. HS was maintained for one, two, and four weeks in the rats of the 1wHS, 2wHS, and 4wHS groups, respectively. A previous study reported that two or four weeks of HS induced morphological changes in the femur in growing rats[16], that the critical period for HS-induced bone morphological changes may be between one and four weeks. At the end of scheduled HS period, the HS device was removed and the rats were returned to a normal cage until they were 12 weeks old. In the control group, rats were housed in a normal cage until they were 12 weeks old. The experimental design is presented in Figure 1.

Tissue preparation

At the end of the experimental period, rats were sacrificed by exsanguination under anesthesia and their right knee joints (the femur-patellar-tibia complex) were harvested. The muscles in the lower leg and posterior thigh were roughly removed, while the anterior thigh muscles were preserved to avoid altering the patella position. The knee joints were fixed in 0.1 M phosphate-buffered 4% paraformaldehyde (pH 7.4) at 90° flexion for two days. Afterwards, the samples were stored in 0.05 M phosphate-buffered saline (pH 7.4) at 4°C until X-ray micro computed tomography (μCT) analysis.

X-ray micro-computed tomography (μCT)

Samples were scanned by μCT (inspeXio SMX-255CT FDP HR, Shimadzu, Kyoto, Japan) at a voxel size of 37 μm/voxel resolution (tube current: 70 μA; tube voltage: 200 KV). Scanned images were exported as Digital Imaging and Communications in Medicine (DICOM) files and used to measure femoral morphology and knee alignment. Three-

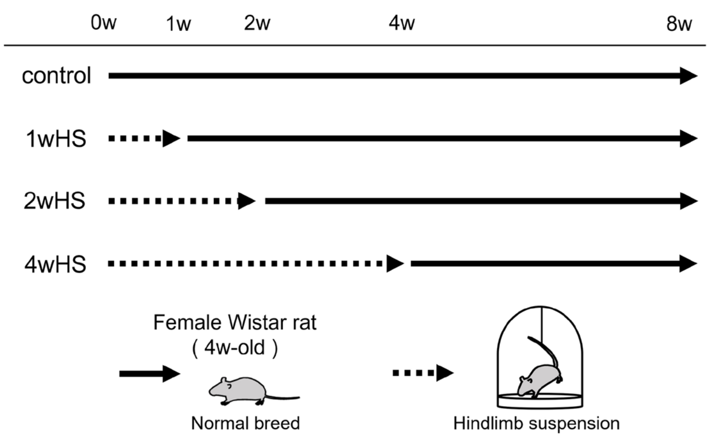

Figure 1. Experimental protocol. A total of 32 immature (4-week-old) Wistar rats were randomly divided into four groups: the control, hindlimb suspension (HS) for 1 week (1wHS), 2 weeks (2wHS), and 4 weeks (4wHS) groups (n = 8 each). Control and post-HS rats were housed in the standard cages until 12 weeks of age.

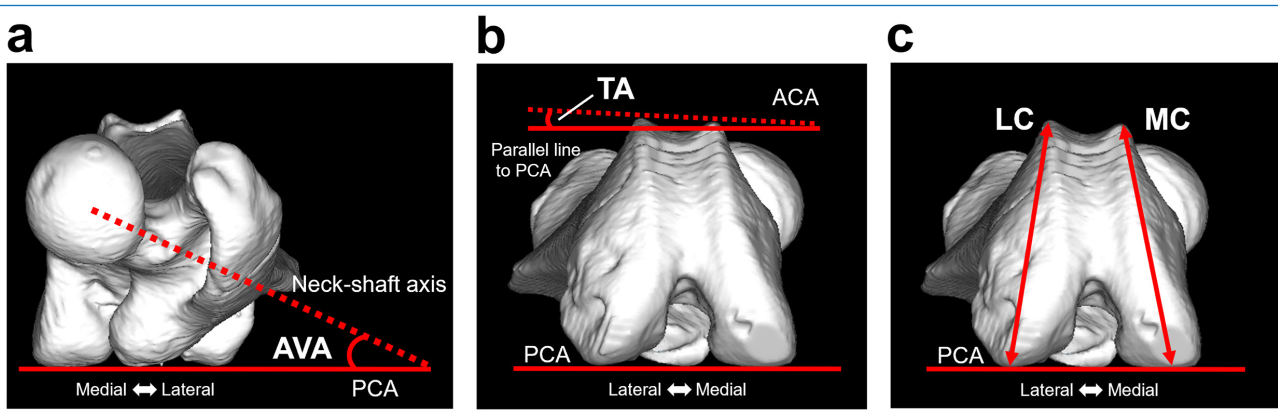

Figure 2. Measurements of the femoral morphology including the anteversion angle, trochlear angle (TA), medial condyle height (MC), and lateral condyle height (LC) were taken using three-dimensional reconstructed CT models. a: Anteversion angle. The angle between the neck shaft angle and the posterior condylar axis. b: TA. The angle between the anterior condylar axis and the posterior condylar axis. c: MC and LC. The distances between the anterior and posterior tips of the medial and lateral condyles, respectively. AVA: anteversion angle, PCA: posterior condyle axis, defined as the tangential line of the posterior tips of the femoral condyles on the horizontal plane of the bone axis. ACA: anterior condyle axis, defined as the tangential line of the anterior tips of the femoral condyles on the horizontal plane of the bone axis.

dimensional bone models of hindlimbs were reconstructed from the DICOM data using three-dimensional construction and measurement software (Zed View, LEXI Co., Inc, Tokyo, Japan). All indices of femoral morphology and knee alignment were determined using the 3D image analysis system (Zed Edit, LEXI). All assessments were performed in a blinded fashion by the same trained assessors throughout the study.

Femur morphological analysis

The horizontal femur plane was strictly defined as the plane orthogonal to the line connecting the most caudal point of the lesser trochanter and the midpoint between the medial and lateral condyles (i.e., the femoral axis). This horizontal femoral plane was used to measure all femoral morphology indices, such as anteversion angle (AVA), trochlear angle (TA), medial condyle height (MC) and lateral condyle height (LC), and lateral trochlear inclination (LTI).

Anteversion angle

The AVA was defined as the angle between the posterior condylar axis and the neck-shaft axis (Figure 2a). The posterior condylar axis was defined as the line connecting the most caudal points of the medial and lateral condyles on the horizontal femur plane. The neck-shaft axis was defined as the line connecting the center of the sphere approximating the femur and the center of the narrowest point of the neck.

Trochlear angle

The TA was measured as the angle between the posterior condylar axis and the line tangential to the trochlear ridges of the medial and lateral condyles of the femur (Figure 2b).

Medial and lateral condyle height

The distances from the medial and lateral trochlear ridges to the most caudal points of the medial and lateral condyles were measured as the MC and LC heights, respectively. We then calculated the MC/LC ratio as an index of asymmetrical condyle growth (Figure 2c).

Lateral trochlear inclination

A projection plane perpendicular to the femoral axis was used to measure LTI (Figure 3a). LTI was measured as the angle between the femoral condyle axis and the lateral facet of the trochlear groove (Figure 3b).

Knee joint alignment

For the knee joint alignment measurement, a projection plane was defined as the femoral axial plane with the distal femoral axis inclined 30° in the posterior direction in the sagittal plane (Figure 3a). This projection plane was used for all knee joint alignment measurements, such as tibial tuberosity-trochlear groove (TT-TG) distance, bisect offset (BO), and patellar tilt angle (PTA).

Tibial tuberosity-trochlear groove distance

TT-TG distance was measured using two slices from the projection plane. The first slice passıing through the deepest point of the trochlear groove and the second slice passıing through the most prominent point of the tibial tubercle. These slices were overlaid and perpendicular lines were drawn from the deepest point of the trochlear groove or the most prominent point of the tibial tubercle to the posterior condyle axis (forming the TG and TT lines, respectively). The distance between the TG and TT lines was then measured as the TT-TG distance (Figure 3c).

Bisect offset

On the slice passing through the deepest point of the trochlear groove, two lines were drawn: 1) a line perpendicular to the posterior condyle axis through the deepest points of the trochlear groove and 2) a line connecting the most medial and lateral points of the patella (Figure 3d). Then, intersection of these two lines was determined. BO was calculated as the distance between most lateral point of the patella and the intersection of the two lines divided by the distance between the most medial and lateral points of the patella.

Patellar tilt angle

In the slice passing through the deepest point of the trochlear groove, the PTA was measured as the angle between the posterior condylar axis and the line connecting the most medial and lateral points of the patella (Figure 3e).

Lateral patellar dislocation

Lateral patellar dislocation was defined as a complete disappearance of the overlap between the patella and the trochlear groove.

Statistical Analysis

All data were expressed as means ± standard deviation (SD). Statistical analyses were performed using SPSS for Windows (ver. 28.0, IBM SPSS Statistics, Tokyo, Japan). For all parameters except LC and BO, we applied one way analysis of variance (ANOVA) after verifying data normality with the Shapiro–Wilk test and variance equality with the Levene test. If the results of the one-way ANOVA were significant, we applied Tukey's honest significant difference test to perform multiple comparisons. If equal variances could not be assumed, multiple comparisons were made using the Games-Howell method. For the LC and BO, the Kruskal Wallis test was applied followed by the Mann Whitney *U* with Bonferroni correction. Rats in the 4wHS group were subdivided by the presence or absence of patellar dislocation, and a Mann-Whitney *U* test was performed to check for any difference in knee joint alignment. Pearson's correlation coefficients were also obtained for the relationships between the LTI and other femur morphological indices (i.e., MC, LC, MC/LC, and PTA) using the pooled data. For all tests, P-values of less than 0.05 were considered statistically significant. To assist interpretation, the mean difference with the 95% confidence interval (CI) was also provided. We conducted a post hoc power analysis using G*Power 3.1 to confirm sufficient sample size for a one-way ANOVA on AVA data.

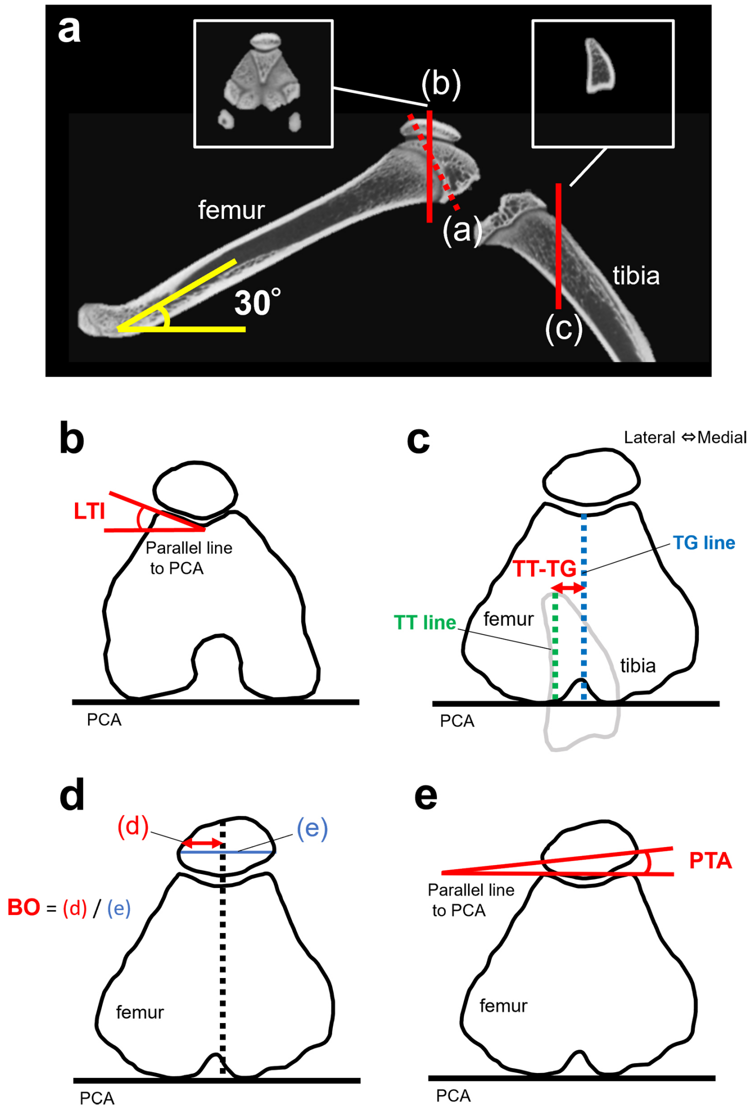

Figure 3. Measurements of knee alignment including lateral trochlear inclination (LTI), tibial tuberosity-trochlear groove distance (TT-TG), patellar tilt angle (PTA), and bisect offset (BO). a: Measurement planes. For the measurement of LTI, the plane passing through the posterior condylar axis and perpendicular to the femoral axis was used (plane (a): dotted line). For the measurement of the TT-TG, PTA, and BO, the plane perpendicular to the femoral axis was rotated 30° downwards along the proximal femur in the sagittal plane (plane (b, c): solid line). Two slices were obtained to measure the distal femoral morphology and alignment. A plane (b) passing through the center of patella and another plane (c) passing through the most prominent point of the tibial tubercle. b: LTI. In plane (a), the angle between the posterior femoral condyle axis (PCA) and the line tangential to the lateral trochlear facet was measured. c: TT-TG. Planes (b) and (c) from Figure 3A were overlayed and perpendicular lines were drawn from the deepest point of the trochlear groove or the most prominent point of the tibial tubercle to the posterior condyle axis (TG and TT lines, respectively) and the distance between the TG and TT lines was measured. d: BO. In plane (b), two straight lines were drawn: 1) a line perpendicular to the posterior condylar axis through the deepest part of the trochlear groove and 2) a line connecting the most medial and lateral points of the patella. The lateral length of the patella (double arrow, d) was calculated by dividing the patella width line (solid line, e) by a perpendicular line through the center of the posterior condylar line (dotted line), and the value was divided by the patella width. e: PTA. In plane (b), the angle between the posterior condylar axis and the line connecting the most medial and lateral points of the patella was measured.

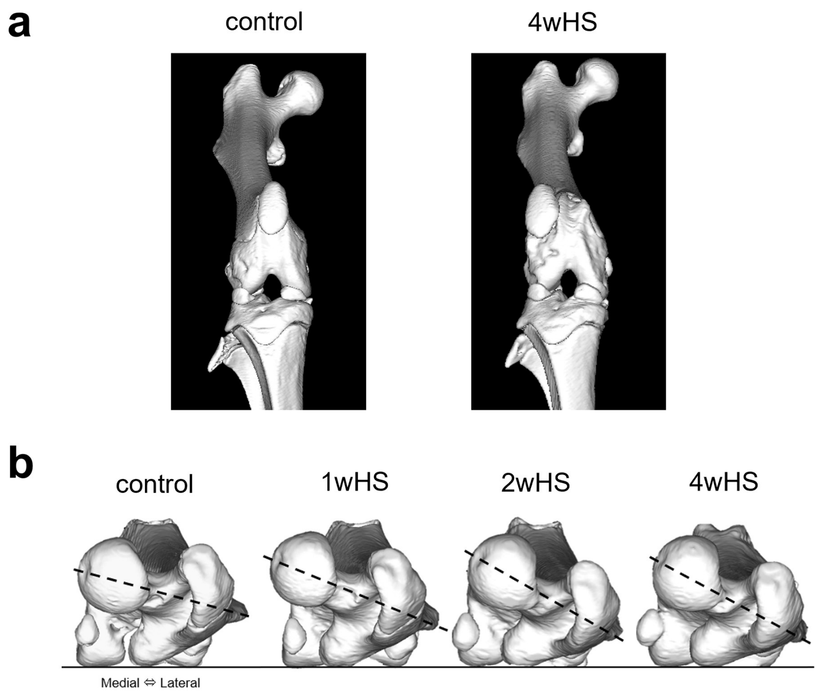

Figure 4. Changes in morphological parameters of the distal femur. Representative 3D computed tomography images. a: Images of knee joints from the control (left) and 4wHS (right) groups. Lateral patellar dislocation is evident in the 4wHS group. b: Femur images from the control, 1wHS, 2wHS, and 4wHS groups (view from proximal side). Dashed line: femoral neck axis. Solid line: posterior condylar axis.

Results

The 3D-μCT images show that lateral patellar dislocation had developed in 3 of the 8 animals in the 4wHS group, but not in any animals in the 1wHS and 2wHS groups. Representative μCT images of the front side of the knee joint in the control and 4wHS group are shown in Figure 4a.

Femur morphological analysis

Representative μCT images of the femur from proximal side are shown in Figure 4b. The AVAs were 30.6° ± 3.0° in the control group and 34.5° ± 3.6°, 35.8° ± 2.7°, and 39.4° ± 4.8° in the 1wHS, 2wHS, and 4wHS groups, respectively (Figure 5a). The AVAs were significantly larger in both the 2wHS and 4wHS groups compared to the control group ($P = 0.037$ with post-hoc Tukey test, 5.2 [0.2-10.2] ° and $P < 0.001$, 8.8 [3.8-13.7] °, respectively). There was no significant difference between the 2wHS and 4wHS groups ($P = 0.202$, 3.7 [-1.3-8.6] °, power > 0.99).

The TAs were 1.8° ± 0.7° in the control group and 0.2° ± 3.1°, -6.3° ± 2.4°, and -5.7° ± 6.1° in the 1wHS, 2wHS, and 4wHS groups, respectively (Figure 5b). The TA was significantly smaller, i.e., the trochlea had rotated outward, in both the 2wHS and 4wHS groups compared to the control ($P < 0.001$ with Games-Howell post-hoc test, 8.1 [5.3-10.9] ° and $P = 0.038$, 7.6 [0.5-14.7] °, respectively). There was no difference between the 2wHS and 4wHS groups ($P = 0.995$, 0.5 [-6.6-7.7] °).

The MCs were 7.0 mm ± 0.2 mm in the control group and 7.0 mm ± 0.2 mm, 7.1 mm ± 0.1 mm, and 7.0 mm ± 0.1 mm in the 1wHS, 2wHS, and 4wHS groups, respectively (Figure 5c). There were no significant differences among groups ($P = 0.333$ with ANOVA).

The LCs were 7.0 mm ± 0.2 mm in the control group and 6.9 mm ± 0.2 mm, 6.7 mm ± 0.1 mm, and 6.6 mm ± 0.2 mm in the 1wHS, 2wHS, and 4wHS groups, respectively (Figure 5d). The LCs were significantly smaller in both the 2wHS and 4wHS groups compared to control group ($P < 0.001$ and $P = 0.007$ with Mann-Whitney *U* test, respectively). There was no difference between the 2wHS and 4wHS groups ($P = 0.133$).

The MC/LC ratios were 1.01 ± 0.01 in the control group and 1.02 ± 0.02, 1.06 ± 0.02, and 1.06 ± 0.03 in the 1wHS, 2wHS, and 4wHS groups, respectively (Figure 5e).

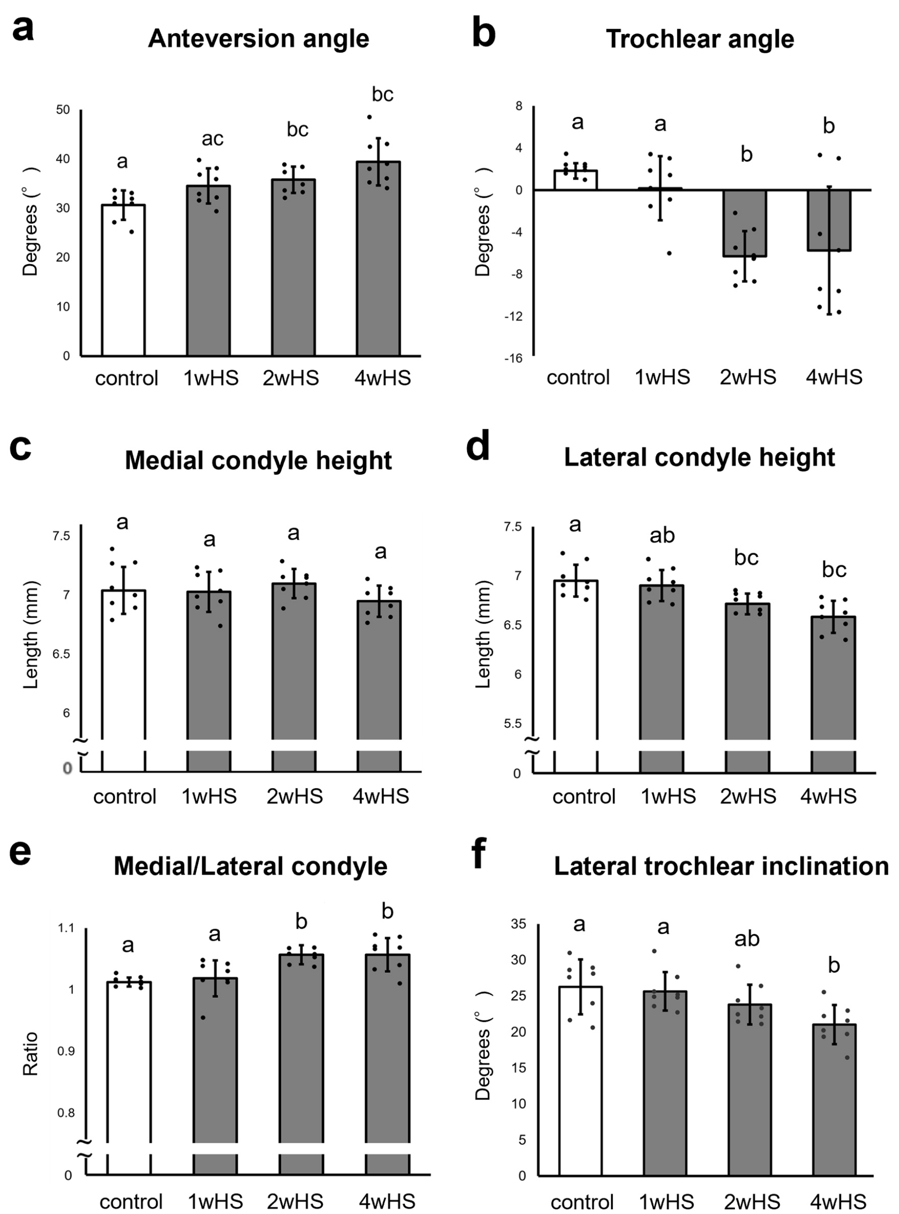

Figure 5. Changes in the morphological parameters of the femur. a: anteversion angle, b: trochlear angle, c: medial condylar height (MC), d: lateral condylar height (LC), e: MC/LC ratio, f: lateral trochlear inclination. n = 8 per group. Different letters indicate statistically significant differences between groups. Data are expressed as mean ± SD.

The MC/LC ratios were significantly higher in both the 2wHS and 4wHS groups compared to the control (both P = 0.002 with post-hoc Tukey test, 0.04 [0.01-0.07] each) and 1wHS groups (both P = 0.007 with post-hoc Tukey test, 0.04 [0.01-0.07] each).

The LTI angles were 26.3° ± 3.8° in the control group and 25.6° ± 2.7°, 23.8° ± 2.8°, and 21.0° ± 2.7° in the 1wHS, 2wHS, and 4wHS groups, respectively (Figure 5f). The LTI angle was significantly smaller in the 4wHS group compared to the control and 1wHS groups (P = 0.009 with post-hoc Tukey test, 5.2 [1.1-9.4] ° and P = 0.024, 4.6 [0.5-8.7] °, respectively). There was no difference between the 2wHS and 4wHS groups (P = 0.282).

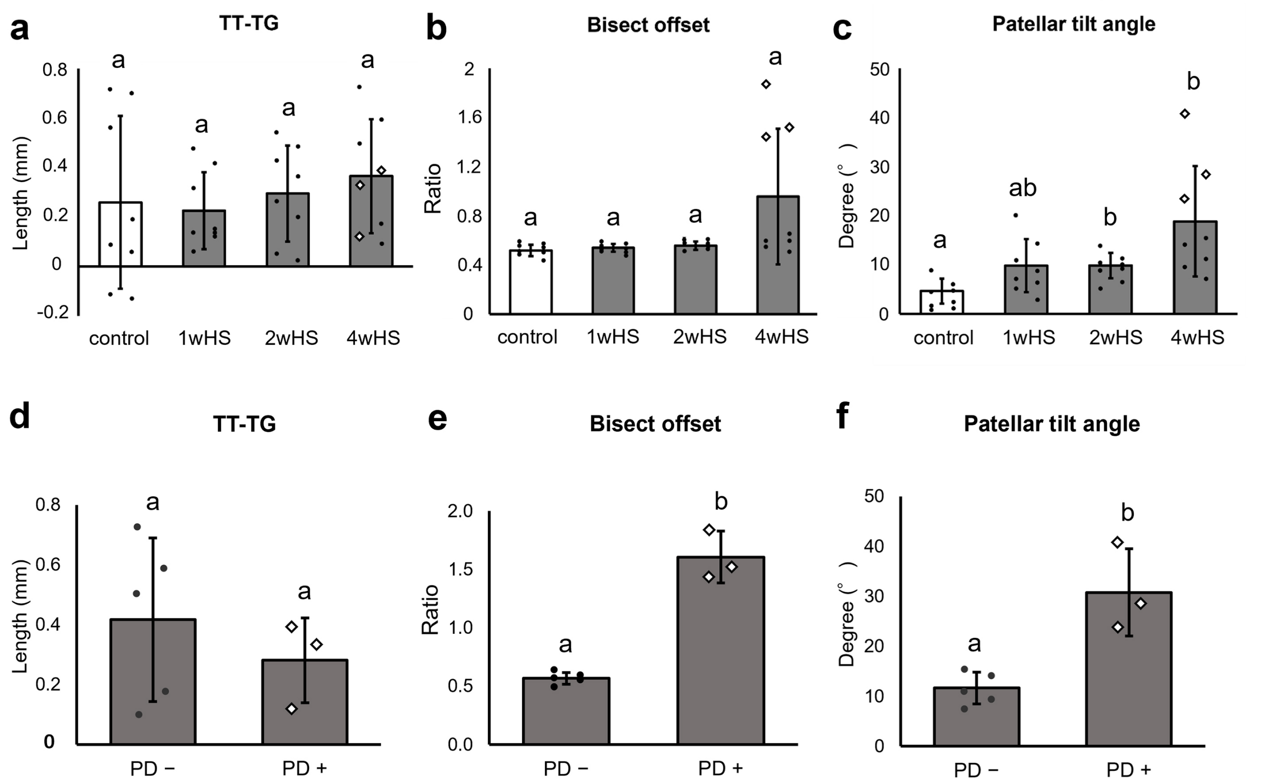

Figure 6. Changes in knee joint alignment including the tibial tuberosity-trochlear groove distance (TT-TG), bisect offset (BO), and patellar tilt angle (PTA). a-c: Comparison of knee alignment among groups. d-f: Comparison of knee alignment between individuals in the 4wHS group with and without lateral patellar dislocation. a, d: TT-TG, b, e: BO, and c, f: PTA. Different letters indicate statistically significant differences between groups. Data are expressed as mean ± SD. ●: without patellar dislocation. ◇: with patellar dislocation. PD+: with patellar dislocation. PD-: without patellar dislocation.

Knee alignment

The TT-TGs were 0.26 mm ± 0.35 mm in the control group and 0.23 mm ± 0.16 mm, 0.30 mm ± 0.19 mm, and 0.37 mm ± 0.23 mm in the 1wHS, 2wHS, and 4wHS groups, respectively (Figure 6a). There were no significant differences among groups (P = 0.698 with ANOVA).

The BOs were 0.52 ± 0.05 in the control group and 0.54 ± 0.03, 0.56 ± 0.03, and 0.96 ± 0.55 in the 1wHS, 2wHS, and 4wHS groups, respectively (Figure 6b). There were no significant differences among groups (P = 0.063 with Kruskal Wallis test).

The PTAs were 4.7° ± 2.5° in the control group and 9.8° ± 5.4°, 9.9° ± 2.6°, and 18.8° ± 11.2° in the 1wHS, 2wHS and 4wHS groups, respectively (Figure 6c). The PTAs were significantly larger in the 2wHS and 4wHS groups compared to the control group (P = 0.006 with Games-Howell post-hoc test, 5.2 [1.5-8.9] ° and P = 0.035, 14.2 [1.1-27.3] °, respectively).

A comparison of knee alignment between with and without patellar dislocation was performed for the 4wHS group (Figs. 6d-f). The TT-TGs in rats with and without patellar dislocation were 0.28 mm ± 0.14 mm and 0.42 mm ± 0.27 mm, respectively. There was no difference between the two groups (P = 0.571 with Mann-Whitney *U* test; Figure 6d).

The BOs in rats with and without patellar dislocation were 1.6± 0.22 and 0.57 ± 0.05, respectively. The BOs were significantly larger in rats with patellar dislocation compared to rats without dislocation (P = 0.013 with Mann-Whitney *U* test; Figure 6e).

The PTAs in rats with and without patellar dislocation were 30.79° ± 8.71° and 11.66° ± 3.15°, respectively. The PTAs were significantly larger in rats with patellar dislocation compared to rats without dislocation (P = 0.004 with Mann-Whitney *U* test; Figure 6f).

Supplementary Table 1 shows the mean ± SD and 95% CI for all outcome variables.

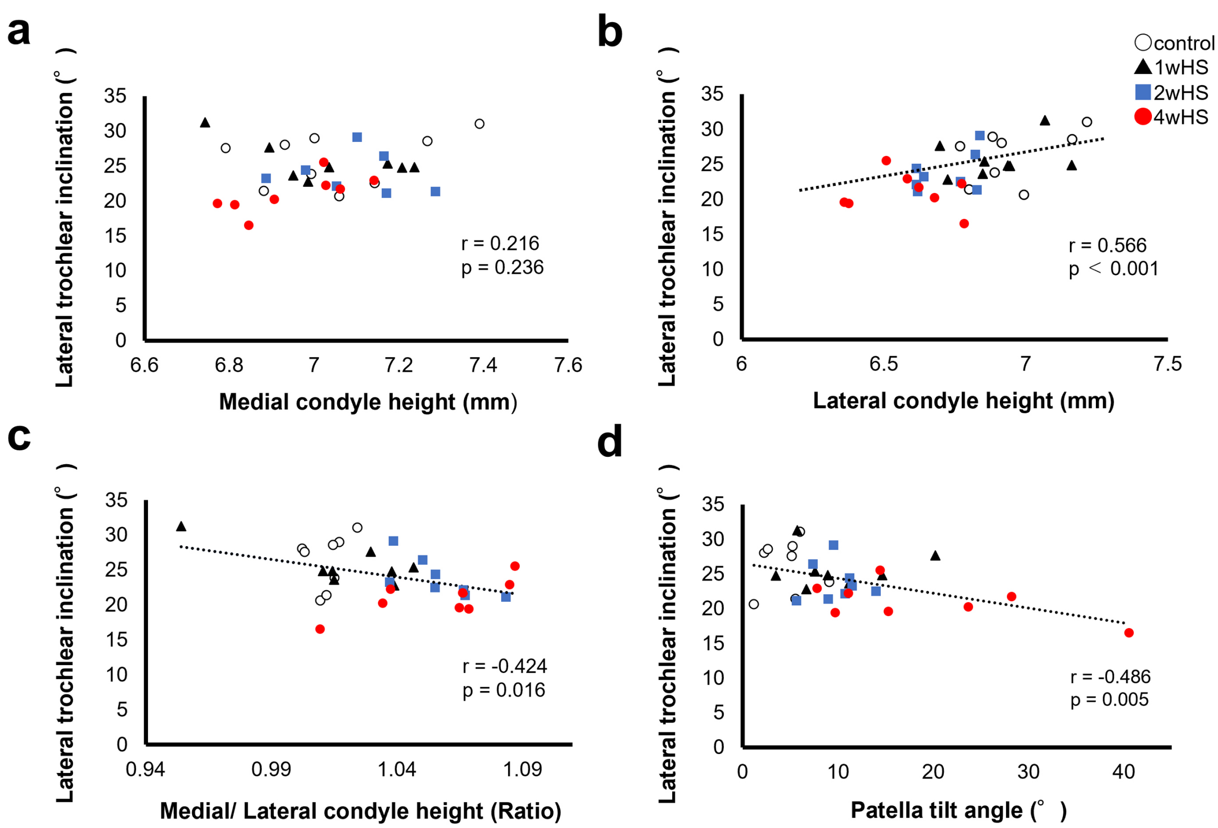

Figure 7. Correlations between the lateral trochlear inclination (LTI) and medial condylar height (MC), lateral condylar height (LC), medial/lateral condylar height ratio (MC/LC), and patellar tilt angle (PTA) in each group. a: Correlation between LTI and MC, b: correlation between LTI and LC, c: correlation between LTI and MC/LC, and d: correlation between the LTI and patellar tilt angle. Moderate correlations ($0.4 < |r| \leq 0.7$) exist between LTI and LC, between LTI and MC/LC, and between LTI and PTA. No significant correlation was found between LTI and MC.

Correlation analysis

The correlation analyses between the LTI and the morphological parameters of the distal femur are shown in Figure 7. A moderate correlation ($0.4 < r \leq 0.7$) was found between the LTI and LC (Figure 7b). Moderate negative correlations ($-0.7 < r \leq -0.4$) were found between the LTI and MC/LC ratio and between the LTI and PTA (Figures 7c–d). No significant correlation was found between the LTI and MC (Figure 7a).

Discussion

HS during development in growing rats affects the morphology of femoral bones and knee joint alignment, but these changes have not as yet been examined after subsequent reloading. This study determined the duration of HS during growth (at four weeks of age) at which changes in femoral bone morphology and knee joint alignment persisted until the time of musculoskeletal maturation (12 weeks of age). In the one week HS group, there were no significant differences in any of the parameters compared to the control group. However, in the two and/or four weeks HS groups, the AVA, MC/LC ratio, and PTA were significantly higher than in the control group, while the TA and LC were significantly lower. The increase in AVA and decrease in TA were consistent with previous observations in growing rats after more than two weeks of HS without reloading[16]. Therefore, we speculate that the changes in AVA and TA in the two and four week HS groups were induced by unloading rather than reloading. On the other hand, PTA increased significantly after reloading following two and four weeks of HS in this study. However, PTA does not change after HS alone for the same period[10], suggesting that the change in PTA may have occurred after reloading rather than HS. Taken together, these results suggest that changes in bone morphology and patellofemoral alignment can be induced by joint unloading or subsequent reloading during growth, and can even persist until musculoskeletal maturation at 12 weeks of age.

Higher AVA and condylar asymmetry, as observed in the 2wHS and 4wHS groups, have been reported as anatomical factors associated with patellofemoral pain syndrome and patellar instability[20-22]. In the present study, lateral patellar dislocation was only observed in the 4wHS group, but there were no significant differences in any of the measures of femoral bone morphology between the 2wHS and 4wHS groups. This indicates that the induction of patellar dislocation cannot be explained solely by joint instability resulting from changes in femoral bone morphology. The involvement of other factors associated with patellar dislocation, such as vastus medial/vastus lateralis muscle imbalance[23,24] and weakness of ligaments and soft tissues[25], is unknown and requires further investigation.

Increases in the TT-TG, BO, and PTA, which are indicators of knee joint alignment, have also been associated with lateral patellar dislocation[21,26-28]. In this study, however, there were no significant differences in TT-TG between the control group and any HS group, including the 4wHS group in which patellar dislocation was observed. In our previous study, TT-TG increased significantly with lateral patellar dislocation after eight weeks of HS, but not after four weeks[10]. This suggests that the weight reloading following the four weeks of HS in the 4wHS group may have prevented an increase in TT-TG that would have occurred with continued HS. As for TT-TG, the mean values of BO did not differ among groups. However, when comparing groups with or without lateral patellar dislocation, BO was significantly higher in the group with dislocations. Therefore, BO may reflect the presence of patellar dislocation. Of the three indices of malalignment, only PTA significantly increased in the 2wHS and 4wHS groups compared to the control group. This may have been because the PTA is more susceptible to distal femoral morphological changes than the other alignment indicators, such as TT-TG and BO, because the patella makes contact with the trochlear groove. Indeed, a moderate correlation was observed between the LTI and PTA. Correlation analyses also indicate that the LTI may be influenced by asymmetrical growth of the femoral condyles (LC > MC), especially when the growth of the lateral, not medial, condyle is inhibited.

Several factors have been proposed as leading to trochlear dysplasia (trochlear flattening and femoral condyle asymmetry) associated with patellar instability and patellar dislocation. Kang et al.[29] noted that femoral trochlear morphology is (1) genetically predetermined, (2) a result of mechanical stimuli during intrauterine or childhood growth, or (3) influenced by a combination of genetics and mechanical stimuli. Their study observed increased anteversion angles due to femoral rotational osteotomy changes in femoral trochlear shapes in growing rabbits[29]. Similarly, Yang et al.[13] reported that insufficient patellar stress induced by patellectomy or surgically induced patellar dislocation resulted in pulley dysplasia in growing rats. Consistent with these reports, the results of the present study support the possibility that external influences (e.g., reduced mechanical loading) can lead to trochlear dysplasia.

In this study, the effects of different periods of HS starting from 4 weeks of age on the knee joint at 12 weeks of age. Therefore, the reloading period was heterogeneous, i.e., longer HS periods had shorter reloading periods and shorter HS periods had longer reloading periods. How does this heterogeneity in reloading period affect the persistence (or recovery) of the observed deformities? Ireland et al. reported that age at onset of walking in infancy is associated with variations in proximal femur shape in older age[30]. This suggests that hip morphology characteristics during infancy may not be affected by prolonged reloading. Assuming that the effects of reloading on the knee joint are comparable to that of the hip joint, the effects of heterogeneity in reloading period on bone morphology and alignment may be negligible or at least not significant. The effects of heterogeneity in the reloading period on the knee joint need to be further examined.

Limitations

This study was subject to several limitations. First, the observed changes in bone morphology and alignment may have been induced by postural factors in addition to joint unloading, as rats experiencing HS tended to be placed in postures of hip flexion and internal rotation. Second, atrophy of the periarticular muscles of the hip and knee may have altered bone morphology and alignment. Muscle atrophy was not assessed in this study, but atrophic changes have been observed in hindlimb muscles after HS in a same model[16, 31]. Third, the duration of reloading was short (4–7 weeks) and the long-term effects were not assessed. Future studies should clarify whether changes in bone morphology and alignment observed in rats after HS for two or four weeks would be reversed by longer periods of reloading (more than seven weeks). Fourth, our results show that the indices of femoral morphology and knee alignment were significantly affected by two and/or four weeks of HS, but not one week, in four-week-old rats at 12 weeks of age. However, these results cannot be extrapolated to an exact timeframe in humans because this study was conducted using a rat model. Fifth, dynamic patellar tracking was not performed to evaluate lateral patellar dislocation.

Conclusions

In this study, we determined the specific timeframes in which HS during growth induces abnormal femoral morphologies and knee malalignments present at skeletal maturity in rats. Our results showed that HS for 4 weeks induced morphological abnormalities of the femur, patellofemoral malalignments, and patellar dislocation at 12 weeks of age. These findings suggest that appropriate mechanical loading during early life may be prevent the development of bone morphology abnormalities and knee malalignment, and consequently prevent musculoskeletal disorders in the future.

Ethics approval

All experimental procedures were approved by the Committee on Animal Experimentation at Hiroshima International University (Approval number: AE20-025). All animals were handled in accordance with fundamental guidelines for proper conduct of animal experiment and related activities in Japan.

Authors' Contributions

M.K., A.I., and J.O. conceived and designed research; M.K. and A.I. performed experiments, analyzed data, prepared figures, and drafted manuscript; A.K. and J.O. edited and revised manuscript; M.K., A.I., A.K., and J.O. interpreted results of experiments and approved final version of manuscript.

Funding

This study was supported by the Japan Society for the Promotion of Science (KAKENHI Grant no. 21H03346).

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Supplementary Table 1. Mean ± standard deviation and 95 % confidence interval for each outcome variable.

Femoral anteversion angle (°)					
Group	Sample No	Mean	SD	95% CI	
				Lower	Upper
Cont	8	30.63	2.97	28.14	33.11
1wHS	8	34.51	3.57	31.53	37.50
2wHS	8	35.77	2.69	33.52	38.03
4wHS	8	39.42	4.80	35.41	43.44
Trochlear angle (°)					
Group	Sample No	Mean	SD	95% CI	
				Lower	Upper
Cont	8	1.83	0.72	1.23	2.44
1wHS	8	0.18	3.06	-2.37	2.74
2wHS	8	-6.28	2.39	-8.28	-4.28
4wHS	8	-5.73	6.06	-10.80	-6.62
Medial condyle height (mm)					
Group	Sample No	Mean	SD	95% CI	
				Lower	Upper
Cont	8	7.04	0.20	6.87	7.21
1wHS	8	7.03	0.17	6.89	7.17
2wHS	8	7.10	0.12	6.99	7.20
4wHS	8	6.95	0.13	6.84	7.06
Lateral condyle height (mm)					
Group	Sample No	Mean	SD	95% CI	
				Lower	Upper
Cont	8	6.95	0.16	6.82	7.09
1wHS	8	6.90	0.16	6.77	7.03
2wHS	8	6.72	0.11	6.62	6.81
4wHS	8	6.58	0.16	6.45	6.72
Medial/Lateral condyle ratio					
Group	Sample No	Mean	SD	95% CI	
				Lower	Upper
Cont	8	1.012	0.007	1.006	1.018
1wHS	8	1.018	0.015	0.994	1.043
2wHS	8	1.056	0.015	1.044	1.070
4wHS	8	1.056	0.027	1.034	1.079
Lateral trochlear inclination (°)					
Group	Sample No	Mean	SD	95% CI	
				Lower	Upper
Cont	8	26.25	3.81	23.07	29.44
1wHS	8	25.64	2.67	23.41	27.89
2wHS	8	23.80	2.76	21.49	26.11
4wHS	8	21.03	2.71	18.77	23.30
SD: standard deviation, CI: confidence interval					