Influence of antenatal vitamin D status on the morphometric and mechanical properties of mouse tibia

Yongtao Lu[1,2,3], Hanxing Zhu[4], Rukun Ma[2], Chengwei Wu[1,2,*]

[1]State Key Laboratory of Structural Analysis for Industrial Equipment, Dalian University of Technology, Dalian, China
[2]Department of Engineering Mechanics, Dalian University of Technology, Dalian, China
[3]INSIGNEO and Department of Mechanical Engineering, the University of Sheffield, Sheffield, UK
[4]School of Engineering, Cardiff University, Cardiff, UK

CORRESPONDING AUTHOR:

Chengwei Wu, PhD

Vice Dean of FVEM

Department of Engineering Mechanics,

Dalian University of Technology,

No. 2 Linggong Road,

116024, Dalian, China

Phone: +86 (0)411 84706353

Email: cwwu@dlut.edu.cn

Word count (Introduction through Discussion): XXX

Number of tables: 0

Number of figures: 5

Abstract

There are increasing evidences that a low level of antenatal vitamin D is linked to a smaller bone size in childhood as well as an increased bone fracture risk later in adulthood, independently of the later vitamin D status. In this study, the hypothesis that the antenatal vitamin D depletion results in reduced responses to mechanical loading and consequently reductions in bone stiffness and strength during both childhood and adulthood was tested using animal models. C57BL/6J mice were used to generate the vitamin D replete and deplete dams. Their 8-week-old and 16-week-old offspring were used and the left tibiae were mechanically loaded *in vivo* for two weeks. Then, both tibiae were dissected and scanned using a μCT imaging system. The μCT images were used for the calculations of tibial morphometric and mechanical parameters. It was found that for the 10-week-old offspring, the antenatal vitamin D replete marked increased the Tb.BV/TV, Tb.Th, Ct.Th, bone stiffness and failure load, decreased Tb.Sp and Ct.MA in the loaded tibiae, but only markedly increased Tb.Sp and Ct.MA in the non-loaded tibiae. For the 18-week-old offspring, the antenatal vitamin D status has a minimal effect on bone morphometric and mechanical parameters. These data implies that only during the childhood, antenatal vitamin D repletion results in increased responses to mechanical loading. This study emphasis the importance of enough vitamin D supply during pregnancy and physical activities during childhood for increasing the bone quality.

1. Introduction

Osteoporosis is a skeletal disorder characterized by increased bone fragility and fracture. Currently 1 in 3 women and 1 in 5 men aged 50-year-old and over experience an osteoporotic fracture. Hip fractures are associated with a 36% increased mortality in the first year after fracture [**Hendricks et al., 2015**]. Bone fragility fractures account for a significant cause of morbidity and mortality in the elderly [**Ensrud, 2013**].

To minimize the bone fracture risk, physical activity is widely discussed as a promising noninvasive preventive measure to maintain bone mass and structure in the elderly [**Kohrt et al., 1997; Kerr et al., 1996**]. In addition, vitamin D plays a significant role in bone growth and development. Previous studies have demonstrated that offspring from maternal vitamin D deficient mother has a decreased femur volume [**Loannou et al., 2012**], a decreased bone mineral content [**Viljakainen et al., 2010**] and a lower bone mass at birth [**Weiler et al., 2005**] compared to normal ones. Furthermore, the infants, who have reduced bone mineral contents at birth due to the maternal vitamin D depletion, also has a reduced bone quality in the periods of both childhood [**Javaid et al., 2006**] and adulthood [**Zhu et al., 2014**]. In contrast, infants with greater birth weights have higher bone mass and a reduced risk of hip fracture in the adult life [**Baird et al., 2011; Cooper et al., 2001**]. All these previous findings imply that the status of antenatal vitamin D has an impact on bone quality of offspring during childhood, and this effect may subsequently influence the bone quality during adulthood, the development of osteoporosis and the risk of bone fracture. However, there are no reported data on the combined effect of maternal vitamin D status and mechanical loading on the bone quality of offspring at the stages of both childhood and adulthood.

The aim of this study was to investigate the influence of antenatal vitamin D status on the mechanically loaded and non-loaded mouse tibiae during the periods of both continuing skeletal growth (childhood) and completed skeletal growth (adulthood) using *in vivo* mechanical loading, μCT imaging and finite element

analysis.

2. Materials and Method

2.1. Animals, *in vivo* mechanical loading, μCT imaging and image processing

C57BL/6J mice were used to generate vitamin D replete and deplete dams, who went on to deliver litters that remained on the maternal diet until weaning (**Fig.1**), mimicking the common situation of vitamin D exposure in humans. In brief, four-week-old C57BL/6J mice were purchased and housed in the same condition based on the standard procedures, which compiled with the UK Animals Act 1986 and were reviewed and approved by the Local Research Ethics Committee of the University of Sheffield. The mice were fed with a vitamin D supplemented diet (1000 units/kg) or vitamin D free diet from 4-week-old. At 10-week-old, female mice were mated with Vitamin D normal adult males. Dams remained on their respective diets throughout gestation until pup weaning. Offspring (n = 24) remained with their dams until weaning at day 22 whereupon they were weaned onto the Vitamin D replete diet.

A non-invasive method of *in vivo* tibia loading was used to examine the bone responses to mechanical loading. 8-week-old and 16-week-old offspring from both vitamin D replete and deplete groups were used (**Fig.1**). The ages were chosen to reflect the periods of either continuing or completed skeletal growth. Two-week (3 times/week) *in vivo* cyclic compressive loading was performed on the left tibia, while the contralateral non-loaded limb (right tibia) was served as an internal control for mechanical loading. A 10.5N dynamic load was superimposed onto a 0.5N preload at a rate of 160000 N/s. The *in vivo* loading protocol consisted of 40 trapezoidal-waveform load cycles (0.2 second hold at 11N) with a 10 second interval between each cycle. The peak load of 11N was selected because this is known to induce an osteogenic response in female C57BL/6J mice [**De Souza et al., 2005; Willie et al., 2013**]. After the mechanical loading, both left and right tibiae were dissected from the 10-week-old and 18-week-old offspring and were frozen at -20 ^{o}C (**Fig.1**). Based on the age of the offspring and the status of mechanical loading, the

tibiae were classified into 4 groups: loaded tibiae from 10-week-old offspring ('10week+11N'); non-loaded tibiae from 10-week-old offspring ('10week+0N'); loaded tibiae from 18-week-old offspring ('18week+11N'); non-loaded tibiae from 18-week-old offspring ('18week+0N') (**Fig.1**). There are 6 mice in each group.

The entire tibiae were scanned using a μCT imaging system (SkyScan desktop 1172) at the resolution of 10.0 μm, a voltage of 50 kV and a tube current of 200 μA. The μCT images were prepared for the morphometric and finite element analysis (**Fig.2**) (Amira 5.4.3). In brief, one left tibia from 10-week-old offspring was selected as the 'reference'. Its long (proximal-distal) axis was approximately aligned along the z-axis and the y-z plane passed through the central line of the articular surfaces of the medical and lateral condyles (**Fig. 2b**). To ensure that all the tibiae were aligned approximately in the same orientation, other left tibiae were aligned to the 'reference' tibia through the method of rigid registration. For the right tibiae, they were mirrored first, and then were aligned to the 'reference' through rigid registration (**Fig. 2d and e**). After transformation, all the image datasets were resampled using the Lanczos kernel, which is a low-pass filter considered to be the 'best compromise' among several simple filters [**Turkowski et al., 1990**].

2.2. The standard bone morphometric analysis

From the transformed μCT images, standard morphometric analysis on the trabecular and cortical bone regions were performed. For the analysis of trabecular bone, a region of 1.0 mm height, which is 0.2 mm below the proximal tibial growth plate, was chosen (**Fig.2f**). For the analysis of cortical bone, a region of 1.0 mm height in the tibial midshaft was chosen (**Fig.2f**). From the regions of interest, the following 3D bone morphometric parameters were generated: trabecular bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular separation (Tb.Sp) and trabecular thickness (Tb.Th), cortical thickness (Ct.Th) and cortical marrow area (Ct.MA).

2.3. The finite element analysis

Heterogeneous finite element models of mouse tibiae were generated from the transformed μCT images (**Fig. 2g**). The grayscale image dataset was smoothed with a Gaussian filter (convolution kernel [3 3 3], standard deviation = 0.65) and then

binarised into bone and background using a fixed single level threshold, i.e. 25.5% of maximal grayscale value (approximately 420 mg HA/ccm) [**Klinck et al., 2008**]. The single threshold method cannot completely segment tibia, because the entire lower limb was imaged and other bones, such as femur, were also present in the images. Therefore, the tibia and fibula were further manually segmented from other bones (Amira 5.4.3, FEI Visualization Sciences Group, France). The tibial-fibula joint and the tibial proximal growth plate region were manually filled to allow the transmission of loading. From the binarised tibia-fibula images, the μFE model was created by converting each bone voxel into hexahedron using an in-house developed Matlab code (Matlab 2015a, The Mathworks, Inc. USA) [**Chen et al., 2014**] (**Fig. 2**). The bone Young's modulus (E) for each bone voxel was calculated from the grayscale value of that bone voxel. In details, the Hydroxyapatite (HA)-equivalent bone tissue mineral density was calculated at each μCT image voxel using the relationship provided by the calibration phantom and then converted into Young's modulus using the established bone density-modulus relationship. In this study, the following exponential bone density-modulus relationship [**Easley et al., 2010; Yang et al., 2014**] was used:

$$\mathrm{E} = \begin{cases} 0.0104, & \rho < 0.4 \\ 1.127 \cdot 10^{-4}(1000 \cdot \rho)^{1.746} & 0.4 \le \rho \le 1.2 \\ 1.127 \cdot 10^{-4} 1200^{1.746} & \rho > 1.2 \end{cases}$$

where, E is the Young's modulus (unit in GPa) and ρ is the bone tissue mineral density (unit in g HA/cm^3).

The calculated image voxel-level moduli were mapped to the FE meshes using an in-house developed Matlab code. Poisson's ratio for each bone element is set to 0.3. The boundary condition applied in the FE models was chosen to mimic the experiment set-up used for the *in vivo* loading of mouse tibia. All nodes on the concave articular surface of the distal tibia were coupled to a distal RP and all degrees of freedom were fixed at the distal RP. The FE nodes at the contact surfaces on the tibial plateau were rigidly coupled to a proximal reference point (RP) and a uniaxial displacement of 0.05mm was applied (**Fig. 2g**). Totally, 48 tibial FE models (12 per group and both left and right tibiae) were generated. The models were solved on a

workstation (Intel Xeon E-5-2670. 2.60 GHz, 256 GB RAM). The tibial compressive stiffness was calculated as the total reaction forces divided by the applied displacement. The tibial failure load was estimated as the amount of force in order for the first principal strain to reach 7300 με or the third principal strain to reach 10300 με [**Bayraktar et al., 2004**]. During the calculation, the most proximal 1.2mm region and the most dital 0.6mm region were excluded to remove the influence of boundary conditions on the results.

2.4. Statistical analysis

ANOVA test was used to compare the parameters between the Vitamin D replete and the Vitamin D deficient groups. Analysis was performed using SPSS, and the probability of type I error was set to α=0.05, i.e., $p < 0.05$ was considered statistically significant.

3. Results

The influence of antenatal vitamin D status on bone morphometric parameters is shown in **Figures 3 and 4**. The antenatal vitamin D depletion significantly reduced Tb.BV/TV only in the loaded tibiae of 10-week-old offspring, i.e., in the group of '10week+11N' (**Fig.3a**). The antenatal vitamin D status has minimal effect on Tb.N (**Fig.3b**). The antenatal vitamin D depletion significantly reduced the Tb.Th only in the loaded tibiae of 10-week-old offspring (**Fig.3c**). The antenatal vitamin D depletion significantly increased Tb.Sp only in both mechanical loaded and non-loaded tibiae of 10-week-old offspring (**Fig.3d**). For the cortical bone, the antenatal vitamin D depletion significantly reduced Ct.Th only in the loaded tibiae of 10-week-old offspring (**Fig.4a**). The antenatal vitamin D depletion significantly increased Ct.MA in both mechanical loaded and non-loaded tibiae of 10-week-old offspring and in the loaded tibiae of 18-week-old offspring (**Fig.4b**).

The influence of antenatal vitamin D status on bone mechanical parameters is shown in **Figure 5**. It is shown that the antenatal vitamin D depletion significantly reduced tibial stiffness and failure load only in the loaded tibiae of 10-week-old

offspring.

4. Discussion and conclusion

The aim of this study was to test the hypothesis that antenatal vitamin D status may be responsible for altered bone responses to postnatal mechanical loading, may have a major influence on bone quality (bone volume ratio, stiffness, strength, etc.), and thus may act as a determinant for later bone quality.

First, in this study, it is revealed that, in response to mechanical loading, the effect of antenatal vitamin D status on bone parameters is different during the period of continuing skeletal growth and during the period of completed skeletal growth. During the period of continuing skeletal growth, in response to mechanical loading, Tb.BV/TV, Tb.Th, Ct.Th, bone stiffness and failure load were markedly increased, Tb.Sp and Ct.MA were markedly decreased in the loaded tibia of offspring from antenatal vitamin D replete dams. During the period of completed skeletal growth, in response to mechanical loading, only Ct.MA was significantly increased in the loaded tibiae of offspring from antenatal vitamin D replete dams. This is in agreement with previous studies that the aged skeleton is less responsive to the mechanical activity [**Birkhold et al., 2014; Holguin et al., 2014; Lynch et al., 2011**], but for the first time, the combined effect of vitamin D status and mechanical loading on bone quality in young and aged mice was investigated in the present study. The data revealed that mechanical loading that results in bone gain in younger individuals are not able to elicit the same beneficial responses in older individuals. Such observation is of general importance to any preventive training concept that aims at maintaining bone structure in aged individuals.

Second, it is revealed that the influence of antenatal vitamin D status on bone parameters was different in the mechanically loaded and non-loaded tibiae. In the mechanically loaded tibiae, Tb.BV/TV, Tb.Th, Ct.Th, bone stiffness and failure load were increased in the 10-week-old offspring from antenatal vitamin D replete dams. In the non-loaded tibiae, the antenatal vitamin D status had a minimal influence on

bone morphometric and mechanical parameters. These data imply that vitamin D status alone cannot make alternations to bone quality. Only after the application of cyclic mechanical loading, more bone appositions were stimulated in the offspring from antenatal vitamin D replete dams compared to those from the antenatal vitamin D deficient dams.

In summary, it is revealed that the influence of antenatal vitamin D status on the bone of offspring was only during the period of continuing skeletal growth when the mechanical loading was applied. These data reveal that for increasing the bone quality (bone volume ratio, bone strength, etc.), it is important for the dams to have enough vitamin D during pregnancy and for the offspring to do enough physical exercise during childhood.

Conflict of interest statement

The authors declare that there are no financial or personal relationships with other persons or organizations that might inappropriately influence this work.

Acknowledgements

This work was funded by the National Natural Science Foundation of China (11702057, 11772086), and the Open Fund from the State Key Laboratory of Structural Analysis for Industrial Equipment (GZ1611), Dalian University of Technology. Dr. Steph Borg (University of Sheffield) is well acknowledged for providing the μCT images for all the analysis performed in this study.

References

1. Hendricks G, et al., A look behind the scenes: The risk and pathogenesis of primary osteoporosis. Nature Reviews Rheumatology 2015, 11: 462-474.
2. Loannou C, et al., The effect of maternal vitamin D concentration on fetal bone. Journal of Clinical Endocrinology and Metabolism 2012, 97: E2070-2077.
3. Viljakainen et al., Maternal vitamin D status determines bone variables in the

newborn. Journal of Clinical Endocrinology and Metabolism 2010, 95: 1749-1757.

4. Weiler et al., Vitamin D deficiency and whole-body and femur bone mass relative to weight in healthy newborns. CMAJ 2005, 172: 757-761.
5. Javaid et al., Maternal vitamin D status during pregnancy and childhood bone mass at age 9 years: A longitudinal study. Lancet 2006, 367: 36-43.
6. Zhu et al., Maternal vitamin D status during pregnancy and bone mass in offspring at 20 years of age: A prospective cohort study. Journal of Bone and Mineral Research 2014, 29: 1088-1095.
7. Baird et al., Does birthweight predict bone mass in adulthood? A systematic review and meta-analysis. Osteoporosis Int. 2011, 22: 1323 -1334.
8. Cooper et al., Maternal height, childhood growth and risk of hip fracture in later life: A longitudinal study. Osteoporosis Int. 2001, 12: 623-629.
9. De Souza et al., Non-invasive axial loading of mouse tibiae increases cortical bone formation and modifies trabecular organization: a new model to study cortical and cancellous compartments in a single loaded element. Bone, 2005, 37: 810-818.
10. Willie et al., Diminished response to in vivo mechanical loading in trabecular and not cortical bone in adulthood of female C57Bl/6 mice coincides with a reduction in deformation to load. Bone, 2013, 55: 335-346.
11. Kerr D, et al., Exercise effects on bone mass in postmenopausal women are site-specific and load-dependent. J Bone Miner Res 1996; 11: 218-25.
12. Kohrt WM, et al., Effects of exercise involving predominantly either joint-reaction or ground-reaction forces on bone mineral density in older women. J Bone Miner Res 1997; 12: 1253-61.
13. Ensrud KE. Epidemiology of fracture risk with advancing age. J Gerontol A Biol Sci Med Sci 2013; 68: 1236-42.
14. Birkhold et al., The influence of age on adaptive bone formation and bone resorption. Biomaterials 2014; 35: 9290-301.
15. Holguin et al., Aging diminishes lamellar and woven bone formation induced by

tibial compression in adult C57BL/6. Bone 2014; 65: 83-91.
16. Lynch et al., Tibial compression is anabolic in the adult mouse skeleton despite reduced responsiveness with aging. Bone 2011; 49:439-46.
17. Turkowski et al., Filters for common resampling tasks. In: Glassner AS, editor. Graphics gems 1. Academic Press; 1990, 147-65.
18. Klinck et al., 2008. Radiation effects on bone architecture in mice and rats resulting from in vivo micro-computed tomography scanning. MEP, 30, 888-895.

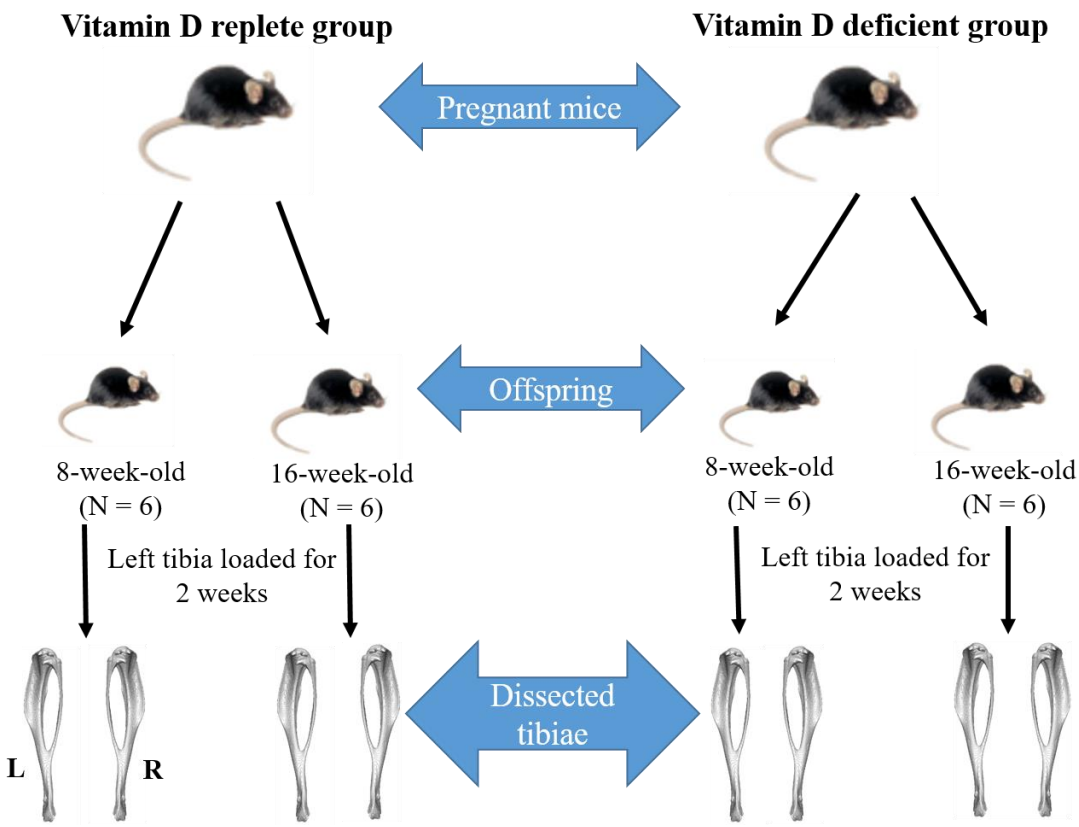

Figure 1. Experimental design: the offspring of 8-week-old and 16-week-old from vitamin D replete and deficient mom groups were used for investigations. First, the left tibiae were mechanically loaded *in vivo* for 2 weeks. Then both tibiae were dissected and went for bone morphometric and finite element analysis.

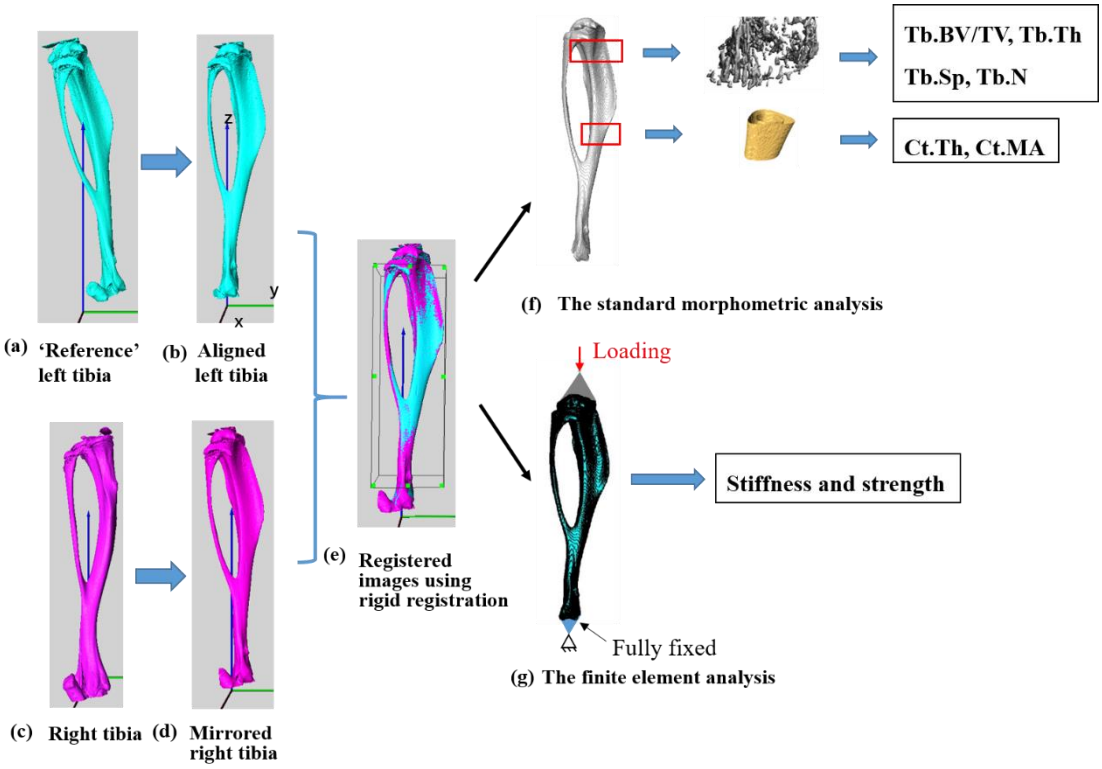

Figure 2. The schematic procedure for the tibial morphometric and finite element analysis. All the tibiae were re-orientated first. The morphometric analysis was performed on the proximal trabecular part (trabecular bone volume fraction, thickness, separation and number were calculated) and tibial midshaft (cortex thickness and marrow area were calculated). The finite element analysis was performed on the entire tibia.

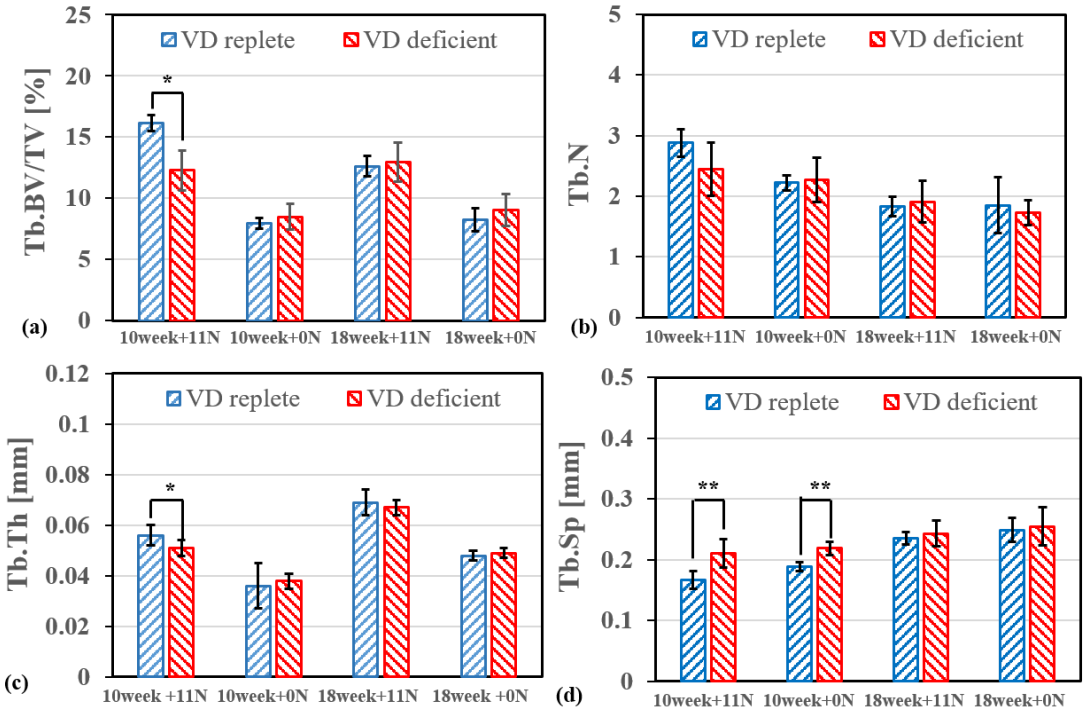

Figure 3. Influence of antenatal vitamin D depletion on trabecular bone parameters. Data are presented as mean values ± standard deviation (* p<0.05, ** p<0.01).

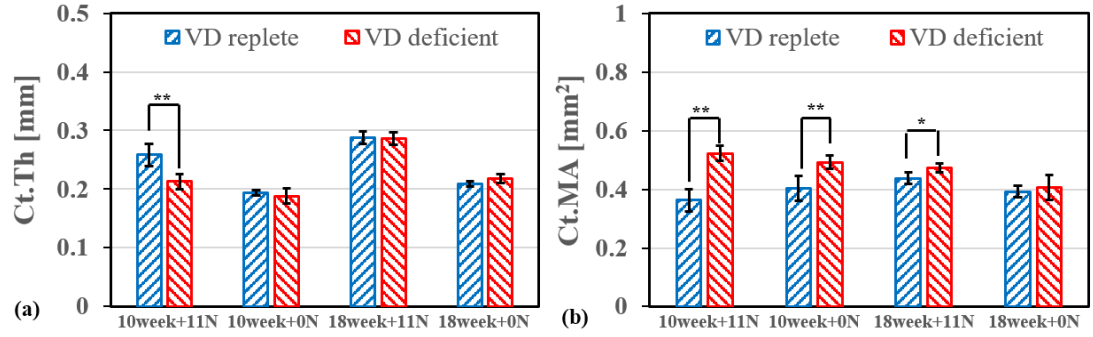

Figure 4. Influence of antenatal vitamin D depletion on cortical bone parameters. Data are presented as mean values ± standard deviation (* p<0.05, ** p<0.01)

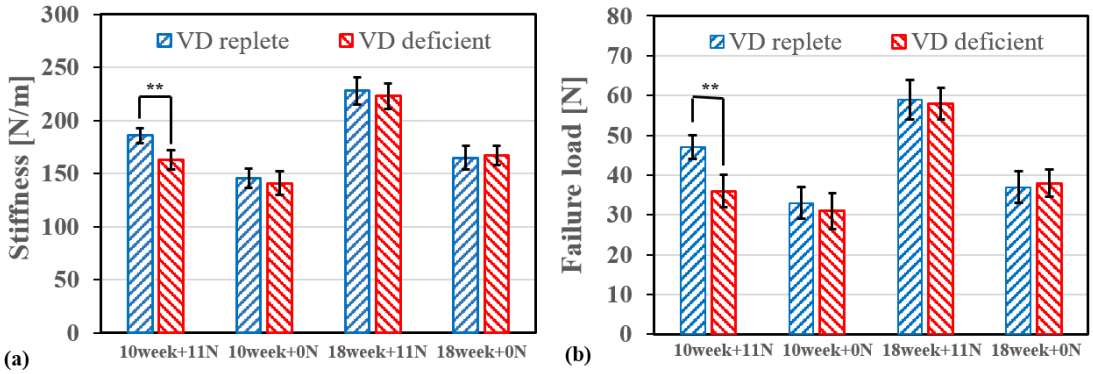

Figure 5. Influence of antenatal vitamin D depletion on the mechanical behavior of mouse tibia. Data are presented as mean values ± standard deviation (* $p<0.05$, ** $p<0.01$)