Published in final edited form as:

Calcif Tissue Int. 2014 September ; 95(3): 282–291. doi:10.1007/s00223-014-9893-0.

Improved Trabecular Bone Structure of 20-Month-Old Male Spontaneously Hypertensive Rats

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Abstract

A few clinical studies have reported that elderly male participants with hypertensive disease frequently have higher BMD than the normotensive participants at several skeletal sites. The detailed mechanism is still unknown; therefore a study of bone structure and density using the hypertensive animal models could be informative. We used micro-computed tomography (μ CT) to quantitatively evaluate the tibial and 3rd lumbar vertebral bones in the 20-month-old male spontaneous hypertensive rat (SHR). The BMD, volume fraction, and the microarchitecture changes of the SHR were compared to those of same-age normotensive controls (Wistar-Kyoto rat, WKY). We found that in the very old (20-month) male rats, the trabecular bone fraction and microstructure were higher than those in the same-age normotensive controls. The observation of the association of hypertension with BMD and bone strength in hypertensive rats warrants further investigations of bone mass and strength in elderly males with hypertension.

Keywords

Trabecular Structure; Quantitative Micro-CT; Bone Strength; Hypertension; Spontaneous Hypertensive Rat; Aging

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The authors declare that they have no conflict of interest.

Introduction

High blood pressure is associated with a number of abnormalities in calcium metabolism [1], including an accelerated loss of bone mineral density (BMD) and elevated risk of osteoporosis and fragility fractures [2, 3]. A number of clinical studies have shown that antihypertensive drugs such as thiazide diuretics, beta-blockers, and angiotensin-converting enzyme (ACE) inhibitors are effective in ameliorating bone loss associated with hypertension, and even decreasing the risk of fracture [4–6]. Preclinical studies in the spontaneous hypertensive rat (SHR) and its normotensive control (Wistar-Kyoto rat, WKY) have consistently found a negative relationship between blood pressure and bone mass and quality. The SHR model has been found to exhibit abnormal calcium metabolism, increased bone turnover, and reduced trabecular bone mass [7–10]. In addition to histomorphometry studies, a micro-computed tomography (μ CT) study also revealed that bone microarchitecture variables were reduced more in SHR than in WKY [11]. Consistent with the epidemiologic data in humans, several forms of antihypertensive drugs have been shown to benefit bone in the spontaneous hypertensive rats [11–13], implying that the pathophysiology of hypertension-related bone loss in the SHR model may have some similarities to the bone loss in humans.

The association between hypertensive disease and the loss of bone mass and deterioration of trabecular structure has been studied in both human subjects and the SHR/WKY models over the past decades. Recent clinical studies that have evaluated elderly males over 65 years of age with hypertensive disease, have reported very different associations between hypertension and bone status. The investigators of the “Osteoporotic Fractures in Men Study” (MrOS) evaluated 5,995 male participants over 65 years of age using dual-energy x-ray absorptiometry (DEXA) [14, 15] and quantitative computed tomography (QCT) [16], and reported that hypertension was associated with higher bone mineral density (BMD) at several skeletal sites like femoral neck and lumbar spine. The specific mechanism behind this relationship remains unclear. Previous studies that have investigated bone strength in SHR and WKY rodent models have focused on relatively young adult animals that were between the ages of 8 to 26 weeks [7–11, 17]. A preclinical evaluation of bone structure in elderly male rats with hypertensive disease has not been performed.

The goal of our work was to investigate the relationships between hypertension, age, and skeletal status in young adult and elderly SHR and WKY rats.

Methods

Micro-computed tomography experiments were performed to investigate bone structure and strength in male spontaneously hypertensive rats (SHR) and its normotensive genetic control, the Wistar-Kyoto rat (WKY). Two cross-sectional studies were designed to evaluate differences between the hyper- and normo-tensive groups at 6.5-months and 20-months of age. Dynamic bone histomorphometry was performed for the 6.5-month time point.

Animals

All animals were purchased from Charles River Laboratories, Inc. (Wilmington, MA). Ten 6.5-month-old SHR (24-week-old) and the same number of WKY rats were acquired. For the 20 months of age, SHR (n=16) and WKY (n=12) rats were acquired at age 10-weeks and aged in the animal facility at Lawrence Berkeley National Laboratory. During the course of the study all animals were fed standard Purina rodent chow (LabDiet, St. Louis, MO) and water. The 6.5-month cohort were euthanized at 6.5 months of age (SHR: age= 6.49 ± 0.4 months, weight= 385.3 ± 32.9 grams; WKY: age= 6.49 ± 0.3 months, weight= 395.4 ± 20.6 grams), while animals in the elderly cohort died naturally or were euthanized at 20-months (SHR: age= 19.71 ± 1.5 months, weight= 374.1 ± 48.7 grams; WKY age= 20.44 ± 1.5 months, weight= 496.8 ± 31.8 grams). All elderly SHR animals exhibited signs of cardiac hypertrophy induced by hypertension confirmed with end-diastolic volume (EDV) measurement, determined using myocardial perfusion microPET imaging (Inveon, Siemens Molecular Solutions, Malvern, PA), and autopsy showing the heart-to-body weight ratio of the SHR group significantly higher than that of the WKY group [18]. Following death or euthanasia, the tibia and L3-vertebra were excised for subsequent μ CT and histomorphometry experiments.

All *in vivo* animal studies in this study were performed in accordance with the protocols approved by Institutional Animal Care and Use Committee (IACUC) from both University of California, San Francisco (UCSF) and Lawrence Berkeley National Laboratory (LBNL).

Micro-computed tomography (μ CT)

The proximal tibial metaphysis and vertebral body were imaged with an isotropic voxel size of 8 μ m using a micro-computed tomography system (μ CT 40, Scanco Medical AG, Brüttisellen, Switzerland). The following settings were used for the data acquisition: x-ray source voltage 55 kVp, tube current 144 μ A, pixel matrix 2048 $\times$ 2048, field of view 16.4 mm, 1000 projections over 180 degrees, and 200 second integration time. The acquisition settings for the diaphyseal tibia were the same as for the metaphyseal tibia and the vertebra except the doubled voxel size and the halved x-ray projection number. The metaphyseal region measured a span of 3-mm (375 slices) extending distally from the growth plate of the proximal tibia. The vertebral region measured a span of approximately 8.5-mm (870 slices) covering the region between the superior and inferior endplates of the L3 vertebra. Additional lower resolution acquisition (16- μ m voxel size) of the tibial diaphysis was performed to evaluate the cortical geometry. The diaphyseal scan covered a span of 1-mm (104 slices) and was located 1-mm proximal to the tibial-fibular junction.

All image segmentations, and bone index calculations were conducted using Image Processing Language software (IPL, Scanco Medical AG) installed on a dual-Alpha OpenVMS workstation (DS20E, Hewlett-Packard, Inc.) [19]. The cortical-trabecular compartment segmentation was performed using an automatic contouring procedure [20], while the mineral phase was segmented using a histogram-based adaptive threshold algorithm [21]. For the vertebra and tibial metaphysis, direct measures of bone volume fraction (BV/TV), trabecular thickness (Tb.Th), number (Tb.N), and separation (Tb.Sp), and cortical thickness (Ct.Th) were calculated [22–24]. In addition, three nonmetric descriptors

of the trabecular structure were calculated: structure model index (SMI) [25], connectivity density (Conn.D) [26], and the degree of anisotropy (DA) [27]. In order to assess the tissue mineral composition, attenuation values were calibrated by a hydroxyapatite (HA) phantom (QRM, Moehrendorf, Germany) that contained five different HA concentrations (0, 100, 200, 400 and 800 mgHA/cm³) [19]. Based on this calibration, tissue mineral densities of trabecular (Tb.TMD) and cortical bone (Ct.TMD) were calculated as the mean density values of all mineralized voxels for their respective compartments. All of these measurements and calculations were performed on the identical region of interest to ensure the validity of comparisons between the parameters. Partial volume effects were suppressed from these measurements by the exclusion of a two-voxel-thick layer at the bone/marrow boundaries. For the tibial diaphyseal region, average 2D histomorphometry parameters were calculated in the axial plane: average total area (Tt.Ar), cortical area (Ct.Ar), and cortical thickness (Ct.Th).

Finite element analysis

Apparent elastic biomechanical properties were determined for each vertebral specimen using linear finite element (FE) analysis. The binary μ CT image was converted to a mesh of isotropic brick elements using a voxel conversion technique [28] and each element was assigned an elastic modulus of 18 GPa and a Poisson's ratio of 0.3 [29, 30]. A uniaxial compression test in the axial direction (superior-inferior) was performed with an applied strain of 1%. An iterative solver (Scanco FE Software v1.12, Scanco Medical) was used to compute reaction forces at the superior and inferior ends of the vertebrae for the prescribed boundary conditions. Cortical and trabecular elements were assigned different material labels, with identical material properties, to enable the calculation of load distribution between the two compartments. The FE model computations were performed at the UCSF/QB3 Shared Computing Facility – a mixed-architecture Linux grid comprised of 4500-processor nodes.

Histomorphometry

Dynamic bone histomorphometry was conducted in the 6.5-month SHR and WKY groups. At 26 weeks of age, each animal received a subcutaneous injection of Alizarin Red S solution (Sigma Aldrich, St. Louis, MO) consisting of 2.5 mg/ml in pH 7.2 PBS buffer with 10 mg per 500 grams of rat's weight. Seven days later the animals were injected with the same concentration of calcein (Sigma Aldrich, St. Louis, MO) with 5 mg per 500 grams of the rat's weight. The animals were euthanized 4 days after the second injection. The right proximal tibiae were excised, dehydrated, and embedded undecalcified in methyl methacrylate. Sections were either stained in tetrachrome, or left unstained sections were used for assessing fluorochrome labeling and dynamic changes in bone. Histomorphometry was performed using a semi-automatic image analysis system (Bioquant Image Analysis Corp., Nashville, TN). Bone area and perimeter were measured at a magnification of 25x, and indices of bone formation and cell surfaces at 250x. Bone measurements included the percent of trabecular bone surface covered by osteoblasts (Ob.S/BS) and osteoclasts (Oc.S/BS), single- and double-labeled perimeters and inter-label width (Ir.L.Wi). The raw data were used to calculate mineralizing surface (MS/BS = 1/2 single label surface + double label surface), mineral apposition rate (MAR), and bone formation rate (BFR/BS = MS/BS $\times$

MAR) according to the ASBMR guidelines for bone histomorphometry [31–34]. Toluidine Blue and von Kossa stains were used to identify osteoclast surface and bone resorption based on published references [35, 36].

Statistics

The means and standard deviations for each μ CT-derived and histomorphometric parameters were calculated for the SHR and WKY groups at each age. A 2-by-3 (6.5-month or 20-month; SHR or WKY; weight < 0.5 standard deviation (SD) of mean weight, weight within 0.5 SD of mean weight, or weight > 0.5 SD of mean weight) two-way analysis of variance (two-way ANOVA) was used to evaluate significant differences between the two age (6.5-month and 20-month) groups, or between hyper- and normo-tensive groups, taking into account of the effect of intragroup-weight change. Another 2-by-2 two-way ANOVA (6.5-month or 20-month; SHR or WKY) was also applied to show the overall significance of the effect from hypertension and aging. A two-tailed *t*-test was used to test for the bone histological differences between the two age groups, and between hyper- and normo-tensive groups. All statistical analyses were performed using OriginPro (Northampton, MA), with the level for significance at $p < 0.05$.

Results

Summarized statistics for the μ CT analyses are shown in Table 1 (tibia) and Table 2 (vertebrae) by age for both hyper- and normo-tensive groups. Representative images from the proximal tibia are illustrated in Figure 1.

Hyper- vs. normo-tensive bone structures

In the 6.5-month study groups, moderate differences were found in both cortical and trabecular bones. While no statistically significant difference was found in BV/TV, Tb.Sp and Tb.Th were lower in the SHR group than those in the WKY group (–12.6% and –16.4%, respectively, $p < 0.05$), indicative of a finer microstructure in the 6.5-month WKY group than that in the SHR group. The degree of anisotropy (DA) was greater in the SHR group than that in the WKY group (+8.6%, $p < 0.05$), which implied that a certain orientation of trabecular bone started losing more bone in the 6.5-month SHR group in comparison to the 6.5-month WKY group. No statistically significant difference was noted at the diaphysis (see Table 1).

In the 20-month study groups, the trabecular bone comparisons in the tibia between the SHR and WKY groups showed a reverse pattern. Trabecular bone fraction (BV/TV) was 28.7% greater in the SHR group than that in the WKY group. This greater volumetric density was manifested by denser trabecular network (+19.1% greater Tb.N; +26.3% narrower in Tb.Sp; +127.8% greater Conn.D, $p < 0.05$). In contrast, the metaphyseal cortex was lower in the 20-month SHR group than that in the 20-month WKY group (+18.4% narrower in Ct.Th, $p < 0.05$). There was no statistically significant difference in trabecular tissue mineral density (Tb.TMD). At the mid-diaphysis, the SHR group showed greater total bone thickness and cortical area, and a statistically significant greater cortical thickness (Ct.Th, +16.6%, $p < 0.05$) than those in the WKY group.

At the lumbar spine, the 6.5-month SHR group had lower trabecular bone mass and architecture than those in the 6.5-month WKY group. Trabecular BV/TV and Tb.N were lower (−9.4% and −9.3%, respectively, $p<0.05$) in the SHR group, while Tb.Sp was 9.9% higher than those in the WKY group ($p<0.05$). The apparent modulus was significantly lower (−20.4%, $p<0.05$), while the stiffness was significantly higher (+15.3%, $p<0.05$) in the 6.5-month SHR group than those in the 6.5-month WKY group (see Table 2),

However, smaller bone structural differences were found (BV/TV, Tb.N, Tb.Sp, and Tb.Th) in the vertebral trabecular bone between the 20-month SHR and WKY groups. In the contrary, Ct.TMD was slightly higher (+0.2%, $p<0.05$) in the SHR group than that in the WKY group at 20-months. Stiffness was also higher (+38.3%, $p<0.05$) in the 20-month SHR group than that in the 20-month WKY group.

Dynamic bone histomorphometry

Histomorphometric analysis of the proximal tibial metaphysis in the 6.5-month rats did not detect significant differences in the trabecular structure (Figure 2). In addition, no statistically significant differences were observed in the inter-label width (Ir.L.Wi) and seven-day mineral apposition rate (MAR) between the SHR and WKY groups by dynamic histomorphometry. However, the percent coverage of surface osteoclasts was significantly lower in the SHR group than that in the WKY group (−31.8%, $p<0.05$), while the mineralizing surface percentage was significantly higher (MS/BS, +45.5%, $p<0.05$). Hence, the overall bone formation rate per unit bone surface area was on average +36.3% greater in the SHR group than that in the WKY group.

Discussion

From the perspective of bone indices shown in our findings, a weaker bone structure could be inferred from the increased value of Tb.Sp, SMI, DA, and the decreased value of TMD, BV/TV, Tb.Th, Tb.N, and Conn.D in the 20-month-old SHR animals. However, in the 20-month old proximal tibial metaphysis -- contradictory to previous studies (8~26 weeks) indicating that hypertension will lead to lower bone strength [7–10] -- most of the trabecular bone indices we measured such as BV/TV, Tb.N, Tb.Sp, and Conn.D, showed an inverse trend, which meant old-male hypertensive rats showed better trabecular bone structure compared to the same-age normotensive controls. In addition, we compared the differences between the 20-month SHR and WKY groups to the differences between the 6.5-month SHR and WKY groups (see Table 1: $6M_{SHR-WKY}$ & $20M_{SHR-WKY}$), and we found that the differences between the two groups for trabecular bone indices (except DA) were greater in the 20-month old group than those in the 6.5-month old group. The weight-dependent bone microstructure change further indicated the better bone structure in the 20-month SHR group. The BV/TV, Tb.N, and Tb.Sp all showed finer and more compact bone structure in the 20-month SHR group even when the average weight of the 20-month-old SHR rats was over 120-g lighter than that of the 20-month-old WKY rats. On the other hand, from the perspective of aging (from 6.5 to 20 months), the proximal tibia of SHR again seemed less affected by trabecular bone loss (except DA) due to aging (see Table 1: WKY_{20M-6M} & SHR_{20M-6M}).

The same trend could also be seen by our histomorphometry data (see Figure 2), although there was no clear morphological difference yet in the 6.5-month SHR and WKY groups, the osteoclast covered percentage of proximal tibia was already 31.8% lower in the SHR group than that in the WKY group while retaining the same surface cover percentage of osteoblast cells (WKY: 6.86%; SHR: 6.87%). Furthermore, in the SHR rats, the overall osteoblast function was higher, which resulted in higher mineralizing surface despite their numbers/surface remained the same. Through calculating the mineral surface coverage (MS/BS) and the mineral apposition rate (MAR), the bone formation rate per bone surface unit in the SHR group was already 36% faster than in the WKY group, and it may have led to bone structural differences in the 20-month-old SHR and WKY rats by the long-term accumulation.

The relatively better bone strength indices could also be found in the mid-shaft diaphyseal tibia and the 3rd lumbar vertebra bone sites in the 20-month-old SHR rats. In 6.5-month old group, there was no significant structural difference between the SHR and WKY rats in the mid-shaft tibia, but a significant difference was measured in the cortical thickness between the 20-month-old SHR and WKY rats (*see* Table 1: Diaphysis). Besides, although the 6.5-month old SHR of the L3-vertebra in our study showed lower bone densities and worse bone microarchitectures in eight out of ten bone strength indices than the 6.5-month old WKY, we found that the bone loss and deterioration in the SHR cohort were also all less than those in the WKY cohort during the 13.5-month period of aging (except for DA, *see* Table 2: WKY_{20M-6M} & SHR_{20M-6M}). We also note that in the 6.5-month old group, the cortical thickness (Ct.Th) was higher at the tibial metaphysis whereas it is lower at the vertebrae in the SHR cohort than those in the WKY cohort. It is common for biological effects to manifest differently across skeletal sites. Good examples would be habitually weight-bearing bones vs. relatively unloaded bones, skeletal sites that are primarily composed of cortical bone vs. sites that are primarily composed of trabecular bone, and sites where the marrow is predominantly yellow marrow vs. sites that have a high amount of hematopoietic marrow. In our case, the comparison of the 6.5-month-old SHR and WKY rats at two different anatomic sites revealed that the thickness differences of trabecular and cortical bones between the 6.5-month-old SHR and WKY rats were greater in the tibial metaphysis, the weight bearing bone (Tb: -16.4%, Ct: +11.7%) than they were in the vertebrae, the relatively unloaded bone (Tb: +1.0%, Ct: -4.5%). This implied that the bone loss (thinner Tb.Th) from hypertension is prone to happening at the weight-bearing bone (e.g., trabecular bone) first in the young SHR rats, and thicker Ct.Th seemed a compensated reaction for losing trabecular bones. However, we need others robust evidence to support this conclusion, which is beyond the scope of this manuscript.

There is a discrepancy between μ CT analyses (higher Tb.Th and lower Tb.Sp) and bone histomorphometry (no significant difference) for trabecular structure in the 6.5-month-old SHR rats in comparison to the 6.5-month-old WKY rats. First while the proximal tibia was evaluated for both μ CT and histomorphometry, μ CT measures trabecular structure using direct 3D methods that are not based on model assumptions (i.e., the histomorphometry analysis here used “rod” stereological calculations for Tb.Dm, Tb.N, and Tb.Sp). And, in fact, the structure type in the tibia of our model was moderately intermediate, exhibiting a

mixture of rods and plates (SMI between 1.0 and 2.0). Furthermore, with μ CT, we evaluated the large majority of the tibial metaphysis, whereas the histomorphometry studies evaluated only a single section of bone from each sample.

In this study, the 20-month SHR group showed higher values in many bone structure indices, especially in the tibia. However, some parameters, like Tb.Th and DA, of the proximal tibia site were still better in the 20-month WKY group than those in the 20-month SHR group. These obscured the value of the bone structure index used as a surrogate to predict the overall mechanical properties of bone strength, toughness, or the resistance to osteoporosis. In 2005, Mitra et al. [37] evaluated seven trabecular bone strength indices that we used here (SMI, BV/TV, Tb.Sp, Tb.N, DA, Tb.Th, and Conn.D) as a measure of ultimate mechanical bone strength. The correlation coefficient showed that Tb.Th and DA were the two least significant indices from the perspective of ultimate mechanical strength. In addition, another group also reported a simulation that for the same amount of bone volume loss (BV/TV reduction), the trabecular thinning (decrease in Tb.Th) led to a lower loss of strength than the trabecular loss (decrease in Tb.N) [38]. Moreover, our investigation of overall mechanical properties of the vertebra site using linear FEA, also revealed that the 20-month old SHR had almost 40% greater stiffness than the WKY (*see* Table 2). In summary, although some bone indices showed the structure was weaker in the 20-month old SHR than the control, it seemed the pathway of bone loss and bone deterioration in the SHR, at least for the trabecular bones, was less than the same age WKY for retaining better mechanical properties, and we found this phenomenon could only be seen in the very old hypertensive animals.

To the best of our knowledge, there has not been an animal study that has investigated differences in bone quality up to end-stage (up to 20 months) hypertension-induced hypertrophy resulting in heart failure. In 1997, Liang et al. found higher trabecular bone area, higher trabecular bone number, and lower trabecular separation at the proximal tibial metaphysis in 25-week old female SHRs [17], and they doubted the elevated trabecular bone mass in the SHR group was related to the hyperparathyroidism. It was because the higher serum level of PTH was frequently found in the SHR model, and the elevated PTH was reported increasing the bone loss rate [9, 39]. Furthermore, the higher level of PTH was also reported increasing the bone loss in the cortical site and producing more bone in the trabecular site, so-called “cortical steal” [40]. Our data here also showed a decrease in cortical thickness and an elevated number of trabeculae in the SHR, the idea of “cortical steal” seemed likely to describe the “improved” trabecular bone strength in the 20-month old SHR’s tibial metaphysis. However, primary blood samples did not show any significant difference in the PTH (data not shown here), thus further research for understanding how and why the old-male SHR showed a better trabecular bone structure is still needed.

On the other hand, a higher level of angiotensin II (Ang II) in the renin-angiotensin system (RAS) is known to lead to both high blood pressure and elevated osteoclast activities [41, 42]. It has been reported that higher levels of Angiotensin II and hypertensive disease lead to increased production of reactive oxygen species (ROS) and the elevated oxidative stress in the human serum and also in the rodent model [43, 44]. Moreover, oxidative stress and low circulating levels of anti-oxidants have been proven to be associated with reduced BMD and

even osteoporosis [45–47]. On the other hand, recent clinical studies in elderly subjects have demonstrated that high serum uric acid (UA) levels are associated with higher BMD at several skeletal sites [48, 49]. It has been suggested that the positive correlation between uric acid levels and BMD can be explained by the anti-oxidative ability of hyperuricemia. Most importantly, in both human and pre-clinical studies, high uric acid levels have been consistently been linked with the presence of hypertensive disease [50–54]. We hypothesize that higher serum uric acid levels in the 20-month old male SHR was higher than the level in the same-age normotensive rats (20-month old WKY) and also higher than the mid-age control (6.5-month old SHR). The better anti-oxidative ability in the old SHR relieved or even compensated the bone loss and structural deteriorations that caused by aging and the hypertensive disease.

In summary, we observed the 20-month-old hypertensive rats had greater trabecular bone mass, volume and apparent strength than the age-matched control rats, and the bone mass increased from age 6.5 months to 20 months in the hypertensive rats. Since hypertension is associated with the increased BMD in elderly men, the etiology of the gain in the bone mass and apparent strength with age in this hypertensive rat strain requires further investigation.

Acknowledgments

The authors would like to thanks Dr. Kathleen Brennan (LBNL) and Ms. Stephanie Murphy (UCSF, Radiology) for handling the serum test and harvesting the bone samples. The study was supported in part by the National Institutes of Health of the U. S. Department of Health and Human Services under Grant R01 EB007219 (GTG) and R01 AG17762 (SM), and R01 EB012965 (YS), and by the Director, Office of Science, Office of Biological and Environmental Research of the U. S. Department of Energy under contract DE-AC02-05CH11231.

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Figure 1.

Representative 2D images (median by BV/TV) from the 6.5 months (A, B) and 20 months (C, D) of tibial metaphysis region. The control animal WKY is on the left (A, C) and the SHR is on the right (B, D).

Figure 2. Bone histomorphometry indices for the proximal tibia of 28-week SHR (n=10) and WKY (n=10) male rats. The asterisks (*) indicate where statistically significant differences (t-test, p<0.05) were found between the two cohorts.

Table 1

Summary μ CT results for the proximal tibial metaphysis and diaphysis of 6.5-month (6M) and 20-month (20M) SHR and WKY male rats.

Tibia	WKY 6M	SHR 6M	WKY 20M		SHR 20M		p for factor A (SHR or WKY)	p for factor B (6m or 20m)	p for Interaction of factor A & B
N	11	11	12	14					
Rat Weight (g)	395.36 ± 20.63	385.27 ± 32.88	496.83 ± 31.78 [†]		374.07 ± 48.73*		9.98E-08	8.73E-05	2.60E-06
Proximal Metaphysis									
BV/TV (%)	0.27 ± 0.05	0.27 ± 0.01	0.14 ± 0.02 [†]	0.18 ± 0.03* [†]	0.18 ± 0.03* [†]	0.18 ± 0.03* [†]	2.06E-02	6.44E-15	0.060
Tb.N (mm ⁻¹)	5.14 ± 0.74	5.50 ± 0.35	2.43 ± 0.34 [†]	3.14 ± 0.36* [†]	3.14 ± 0.36* [†]	3.14 ± 0.36* [†]	3.31E-04	<1.0E-15	0.208
Tb.Sp (mm)	0.19 ± 0.03	0.16 ± 0.01*	0.41 ± 0.07 [†]	0.31 ± 0.04* [†]	0.31 ± 0.04* [†]	0.31 ± 0.04* [†]	5.42E-06	<1.0E-15	0.001
Tb.Th (µm)	73.20 ± 10.86	61.22 ± 2.01*	81.42 ± 6.58	73.43 ± 3.48* [†]	73.43 ± 3.48* [†]	73.43 ± 3.48* [†]	3.49E-06	2.32E-06	0.296
Tb.TMD (mgHA/cm ³)	888.68 ± 18.49	879.41 ± 8.32	941.24 ± 8.96 [†]	946.23 ± 10.62 [†]	946.23 ± 10.62 [†]	946.23 ± 10.62 [†]	0.546	<1.0E-15	0.049
Conn.D (mm ⁻³)	163.64 ± 40.41	215.43 ± 35.47*	22.96 ± 7.53 [†]	52.29 ± 13.53* [†]	52.29 ± 13.53* [†]	52.29 ± 13.53* [†]	5.10E-06	<1.0E-15	0.158
SMI	1.00 ± 0.29	1.09 ± 0.08	1.59 ± 0.20 [†]	1.24 ± 0.42*	1.24 ± 0.42*	1.24 ± 0.42*	0.123	5.81E-05	0.012
D.A.	1.94 ± 0.08	2.11 ± 0.03*	1.83 ± 0.13	2.17 ± 0.23*	2.17 ± 0.23*	2.17 ± 0.23*	5.09E-07	0.598	0.059
Ct.Th (mm)	0.15 ± 0.01	0.17 ± 0.01*	0.22 ± 0.02 [†]	0.18 ± 0.03*	0.18 ± 0.03*	0.18 ± 0.03*	0.074	1.34E-07	2.64E-05
Ct.TMD (mgHA/cm ³)	952.91 ± 11.64	969.00 ± 11.09*	1058.43 ± 13.49 [†]	1036.98 ± 24.86* [†]	1036.98 ± 24.86* [†]	1036.98 ± 24.86* [†]	0.589	<1.0E-15	4.15E-04
Diaphysis									
Tt. Ar (mm ²)	6.29 ± 0.53	6.12 ± 0.36	7.07 ± 0.43 [†]	7.65 ± 0.35* [†] , w [†]	7.65 ± 0.35* [†] , w [†]	7.65 ± 0.35* [†] , w [†]	0.064	2.33E-13	0.003
Ct. Ar (mm ²)	4.61 ± 0.45	4.46 ± 0.26	4.98 ± 0.25 [†]	5.88 ± 0.28* [†]	5.88 ± 0.28* [†]	5.88 ± 0.28* [†]	4.26E-05	6.84E-14	5.58E-07
Ct. Th (mm)	0.64 ± 0.05	0.65 ± 0.03	0.56 ± 0.06	0.65 ± 0.06*, w [†]	0.65 ± 0.06*, w [†]	0.65 ± 0.06*, w [†]	0.002	0.013	0.006
Ct.TMD (mgHA/cm ³)	1169.46 ± 11.96	1162.69 ± 11.87	1255.81 ± 9.48	1254.87 ± 5.96 w [†]	1254.87 ± 5.96 w [†]	1254.87 ± 5.96 w [†]	0.170	<1.0E-15	0.300

The statistical differences (2-by-3 two-way ANOVA, $p < 0.05$) taking into account of the intragroup weight change (3 groups) between strain-matched age groups and between age-matched SHR and WKY groups are reported, with an asterisk (*) indicating statistically significant age-matched SHR/WKY differences; with a palatal clicks symbol ([†]) indicating statistically significant strain-matched 6M/20M differences, considering and separating the intragroup effect of weight through a "w" sign (w* or w[†]). The p-values (2-by-2 two-way ANOVA, $p < 0.05$) showed statistical significances for factors: hypertension, age, and their interaction affected bone metrics. Factor A = hypertension (SHR or WKY); Factor B = age (6.5 or 20 months)

Table 2

Summary μ CT results for the L-3 vertebra of 6.5-month (6M) and 20-month (20M) SHR and WKY male rats.

L-3 Vertebra	WKY 6M	SHR 6M	WKY 20M		SHR 20M		p: factor A (SHR or WKY)	p: factor B (6m or 20m)	p: Interaction of factor A & B
	10	11	6	16	16	16			
Rat Weight (g)	396.90 ± 21.07	385.27 ± 32.88	490.33 ± 30.51⁺	372.00 ± 49.86[*]			5.56E-06	0.002	1.05E-04
BV/TV (%)	0.32 ± 0.03	0.29 ± 0.02 [*]	0.27 ± 0.03 ⁺	0.26 ± 0.02 ⁺			0.006	2.09E-06	0.240
Tb.N (mm ⁻¹)	4.88 ± 0.18	4.43 ± 0.15 [*]	3.86 ± 0.21 ⁺	3.98 ± 0.21 ⁺			0.010	8.77E-15	3.34E-05
Tb.Sp (mm)	0.18 ± 0.01	0.20 ± 0.01 [*]	0.24 ± 0.02 ⁺	0.23 ± 0.02 ⁺			0.677	1.87E-11	1.90E-04
Tb.Th (μm)	70.52 ± 3.93	71.21 ± 2.90	74.88 ± 4.07	76.87 ± 2.53 ⁺			0.205	2.21E-05	0.535
Tb.TMD (mgHA/cm ³)	962.90 ± 11.63	951.85 ± 8.12 [*]	983.14 ± 12.87 ⁺	985.18 ± 10.72w ^{*,+}			0.200	2.01E-09	0.066
Conn.D (mm ⁻³)	174.96 ± 12.83	168.08 ± 14.72	80.11 ± 8.15 ⁺	110.89 ± 10.77 ^{*,+}			0.004	<1.0E-15	2.35E-05
SMI	-0.24 ± 0.25	0.14 ± 0.13 [*]	-0.14 ± 0.18	0.52 ± 0.19 ^{*,+}			4.29E-10	5.39E-04	0.034
D.A.	1.90 ± 0.04	1.85 ± 0.03 [*]	1.81 ± 0.05 ⁺	1.79 ± 0.07 ⁺			0.065	7.51E-05	0.385
Ct.Th (mm)	0.25 ± 0.01	0.24 ± 0.01	0.23 ± 0.02 ^{+, w⁺}	0.28 ± 0.02 ^{*,+}			0.001	0.352	3.50E-06
Ct.TMD (mgHA/cm ³)	1021.09 ± 13.56	999.63 ± 6.26 [*]	1041.41 ± 15.07 ⁺	1043.82 ± 10.90 ⁺			0.013	7.87E-11	0.002
<i>Biomechanical Properties- FEA</i>									
Apparent Modulus (GPa)	4.48 ± 0.56	3.57 ± 0.55 [*]	3.35 ± 0.20 ⁺	3.41 ± 0.40			0.008	1.22E-04	0.002
Stiffness (kN/mm)	8.75 ± 1.03	10.09 ± 0.80 [*]	6.92 ± 0.80 ⁺	9.57 ± 0.79 [*]			1.14E-08	1.41E-04	0.023

The statistical differences (2-by-3 two-way ANOVA, p<0.05) taking into account of the intragroup weight change (3 groups) between strain-matched age groups and between age-matched SHR and WKY groups are reported, with an asterisk (*) indicating statistically significant age-matched SHR/WKY differences; with a palatal clicks symbol ($\dagger$) indicating statistically significant strain-matched 6M/20M differences, considering and separating the intragroup effect of weight through a "w" sign (w* or w $\dagger$). The p-values (2-by-2 two-way ANOVA, p<0.05) showed statistical significances for factors: hypertension, age, and their interaction affected bone metrics. Factor A = hypertension (SHR or WKY); Factor B = age (6.5 or 20 months)