

Signs of irreversible architectural changes occur early in the development of experimental osteoporosis as assessed by in vivo micro-CT

G. M. Campbell · H. R. Buie · S. K. Boyd

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Abstract

Summary Using in vivo micro-computed tomography, we assessed bone loss in the rat during the first twelve weeks after ovariectomy when structural changes were most rapid. Significant changes to the trabecular architecture were observed, including irreversible changes reflected by reduction in connectivity after only two weeks. This highlights that topological changes to the structure occur early in this experimental model of osteoporosis.

Introduction The purpose of this study was to establish a longitudinal time course of bone loss in the ovariectomized (OVX) rat model during the initial twelve-week period where structural changes are most rapid, and to identify when irreversible changes occur using in vivo micro-computed tomography (micro-CT).

Methods The proximal tibiae of OVX (N=10) and sham (N=10) operated mature female Wistar rats were micro-CT scanned every two weeks from week 0 to week 12, excluding week 10. Changes in architecture were quantified using direct three-dimensional techniques and serum osteocalcin and CTX-I was measured at weeks 0, 6 and 12. Biomechanical properties were determined from femoral three-point bending and L-4 vertebral compression at the end of the protocol. ANOVA and paired *t*-tests were used to analyze the longitudinal and endpoint data, respectively.

Results All of the measured architectural parameters changed significantly over the study in the OVX group, including irreversible changes reflected by connectivity density after two weeks. Osteocalcin concentration was elevated in the OVX group. Moderate changes in the mechanical properties of the femora midshaft and vertebrae were observed.

Conclusions Changes to the bone architecture and mechanics within twelve weeks after OVX highlight the importance of early diagnosis and treatment of osteoporosis.

Keywords Biomarkers of bone turnover · Biomechanics · Micro-computed tomography · Osteoporosis · Ovariectomy rat · Tibial metaphysis

Introduction

Osteoporosis is a skeletal disease in which bone mass declines and trabecular architecture deteriorates, leading to increased bone fragility and risk of spontaneous fracture [1]. While measurement of bone mineral density using planar techniques such as dual energy x-ray absorptiometry (DXA) is a common method for the assessment of bone loss, the quantification of three-dimensional (3D) architecture provides additional information about bone quality and fracture risk [2]. Micro-computed tomography (micro-CT) is a non-destructive 3D imaging method that can be employed to examine bone microarchitecture both in vivo and ex vivo at high isotropic resolutions (i.e., 10 μ m) and provide insight into bone adaptation. Traditional cross-sectional animal studies of bone loss, in which subjects are sacrificed at each time point and measurements are averaged over the group, can mask trends in bone properties due to

G. M. Campbell · H. R. Buie · S. K. Boyd (✉)
Department of Mechanical and Manufacturing Engineering,
University of Calgary,
2500 University Drive, N.W.,
Calgary, Alberta T2N 1N4, Canada
e-mail: skboyd@ucalgary.ca

G. M. Campbell · H. R. Buie · S. K. Boyd
Roger Jackson Centre for Health and Wellness Research,
University of Calgary,
Calgary, Canada

inter-subject variability. Longitudinal *in vivo* studies on the other hand, in which a subject is scanned repeatedly over time, reduce the number of subjects required and increase statistical power to detect changes in architecture [3–5].

The ovariectomized (OVX) rat is a well established model of osteoporosis. OVX in rats induces a rapid reduction in bone volume due to estrogen deficiency that occurs more rapidly than in post-menopausal humans [6]. A long term *in vivo* micro-CT study (over six months) has identified the first twelve weeks post OVX as a critical phase of bone loss where the largest changes in trabecular architecture occur [5]. A more detailed description of short-term bone loss is required to provide insight into the changes in bone architecture immediately following OVX and to identify when irreversible bone loss takes place that may indicate when interventions should be initiated. We define irreversible changes as losses of connections in the trabecular network, as opposed to general thinning of structures. Evaluation of the mechanical properties of the bone is needed to assess any increased risk of fracture caused by OVX.

The purpose of this study was to establish a detailed longitudinal time course of bone loss in the OVX rat model in the initial twelve-week period where the rate of structural changes are the highest [5]. The architectural changes in the trabecular region were assessed over time, and bone turnover (resorption and formation) characteristics were studied using biomarkers. The biomechanics of the femur and L-4 vertebra were determined at the end of the protocol. It was hypothesized that irreversible changes in bone micro-architecture as well as reductions in mechanical properties would occur within the first twelve weeks after OVX.

Methods

Animals

Twenty female retired breeder Wistar rats (Charles River, Canada) between the ages of six and eight months were housed at 25°C with a 12:12-hour light-dark cycle in groups of two per cage and were given standard food (Prolab 2500 Rodent 5P14) and water *ad libitum*. Rats were subjected to either OVX (N=10), or sham (N=10) operations. All animal experimentation was conducted in accordance with accepted standards of humane animal care, and approved by the Health Sciences Animal Care Committee at the University of Calgary.

In vivo micro computed-tomography

The right proximal tibial metaphysis was micro-CT scanned *in vivo* (vivaCT 40, Scanco Medical, Switzerland) every

two weeks from week 0 (time of operation) to week 12, excluding week 10 (six measurements per animal). The rats were anesthetized (1.75 v/v % isoflourane) and maintained on anesthetic for the duration of each measurement (approximately 20 minutes per rat). The mass of the animal was recorded at every time point. After the final measurement, the rats were euthanized by CO₂ asphyxiation. A volume of interest 2.65 mm thick (212 slices) was acquired, 1mm distal to and extending away from the growth plate with an isotropic voxel size of 12.5 µm (55 kV, 109 µA, 200 ms integration time, 1024 projections on 360°, 2048 CCD detector array, cone-beam reconstruction). The radiation dose from the micro-CT scanner was estimated to be 338 mGy CTDI_{air} for each scan and this protocol has no detectable effect on bone architecture [7, 8]. The scanner was calibrated weekly using hydroxyapatite (HA) phantoms with densities of 0, 100, 200, 400, and 800 mg HA/cm³. Over the course of the study, the calibration slope change was less than 0.5%.

Dual energy x-ray absorptiometry

Rats were whole body scanned at the end of the micro-CT scan protocol using dual energy x-ray absorptiometry (DXA) (Hologic, Discovery, QDR series, USA). The DXA machine was calibrated with an anthropomorphic spine phantom of 1.00 g HA/cm² and 53.94 g HA areal density and content, respectively. Measurements of areal bone mineral density (BMD) and bone mineral content (BMC) were determined for sham and OVX animals from the endpoint scans.

Morphological analysis

A semi-automated contouring method was used to isolate the trabecular region in the images obtained from the micro-CT [9]. The resulting grayscale datasets were Gaussian filtered ($\sigma=1.2$, $\text{supp}=2$) and globally thresholded (27% of maximal grayscale value) to form binary images on which the analysis of the architecture was performed. The cortical compartment was obtained by subtracting the trabecular region from the image. Direct 3D measurement methods were used to assess bone volume ratio (BV/TV), bone surface ratio (BS/BV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), trabecular number (Tb.N), connectivity density (Conn.D), structural model index (SMI), and cortical thickness (Ct.Th) at each time point [10–13].

Biomarkers of bone turnover

At week 0, 6 and 12, 300 µL of blood was extracted from each rat. The serum was separated and extracted using a centrifuge and subsequently stored at -20°C. An enzyme-

linked immuno-sorbent assay (ELISA) kit was used to measure the serum levels of osteocalcin (Biomedical Technologies, Inc, Stoughton, MA) to assess bone formation, and CTX-I (RatLats ELISA, Nordic Bioscience Herlev, Denmark) to assess bone resorption [14].

Mechanical testing

Femora and L-4 vertebrae were dissected, stored in saline soaked gauze, and frozen at -20°C . Prior to testing, the bones were thawed at room temperature for two hours and scanned at a resolution of $82\text{ }\mu\text{m}$ for the femora and $12.5\text{ }\mu\text{m}$ for the vertebrae. All samples were removed from the gauze prior to testing. The femora were tested in three-point bending using a materials testing machine (MTS Corp., Minneapolis, MN). Femora were placed posterior side up on supports spanning 16 mm and the load was applied halfway between the supports. The samples were pre-loaded to 5 N and held for 20 seconds before being loaded to failure at a deformation rate of 2 mm/min and the load-deflection curve was recorded. The ultimate load was taken as the maximum force on the curve and the extrinsic stiffness was determined from the slope of the linear portion. The work to yield and failure were calculated as the area under the curve to the proportional limit and failure point, respectively.

The L-4 vertebrae were tested in uniaxial compression (MTS Corp., Minneapolis, MN). Prior to testing, the endplates and spinous, transverse and articulate processes were removed using a diamond wafer saw (Buehler, Lake Bluff, IL) to obtain a sample 4.5 mm thick with plano-parallel surfaces. The samples were loaded to failure at a rate of 2 mm/min and the load-deflection curve was recorded. The ultimate load, stiffness and work were calculated as previously described. An automated computer method (Image Processing Language v. 4.29d, Scanco Medical, Switzerland) was used to determine the moment of inertia and cross-sectional area from the CT images of the femora and vertebrae, respectively. The force-displacement curves of the femora and vertebrae were then converted into stress-strain curves and the Young's modulus, ultimate stress, modulus of resilience and modulus of rigidity of each sample were determined using equations of mechanics of materials [15]. The apparent level stress (σ) in the femora subjected to three-point bending was determined using

$$\sigma = \frac{Mc}{I} \quad (1)$$

where M is the bending moment applied to the sample, c is the distance from the neutral axis to the periosteal surface in the direction of bending and I is the moment of inertia of the

cross-section at the midpoint of the sample. In this experiment the load was applied midway between the span; therefore, the bending moment was taken as

$$M = \frac{PL}{4} \quad (2)$$

where P is the applied load and L is the span length. To calculate the ultimate stress (σ_u), Eqs. (1) and (2) were used with P taken as the ultimate load, P_{max} . In the vertebral compression tests, stress was calculated as the applied force divided by the cross-sectional area. The Young's modulus (E) from the three-point bend tests was calculated using the flexure formula as

$$E = \frac{PL^3}{48I\delta_{max}} \quad (3)$$

where δ_{max} is the maximum displacement of the sample. In the compression tests, this was taken as the slope of the linear portion of the stress-strain curve.

Statistical analysis

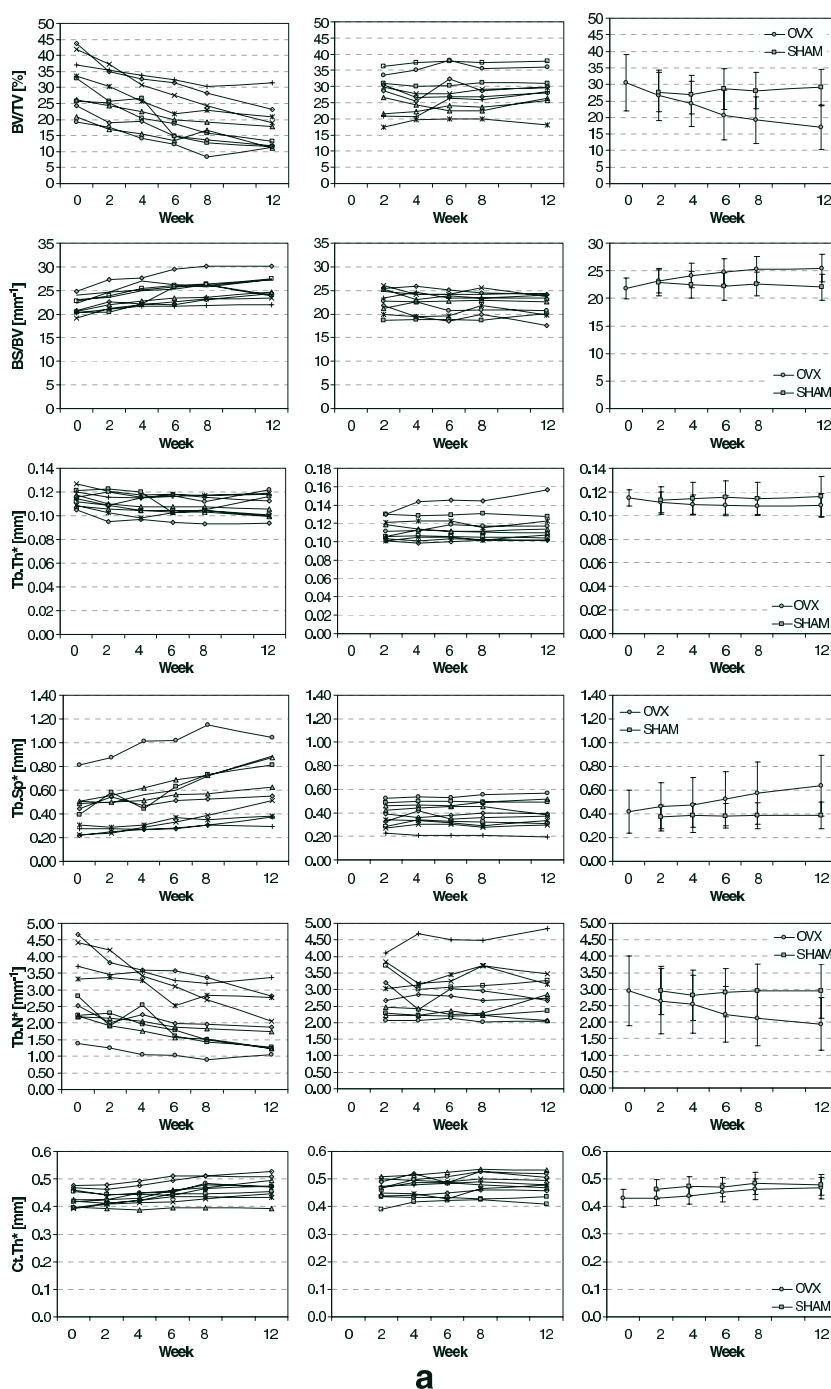
Descriptive statistics including mean and standard deviation of all variables were reported. A repeated measure two-way ANOVA was used to determine if there was a time by group interaction effect and/or a time effect for all continuous dependent variables for the architectural and biomarker data. There were two levels corresponding to the two groups (OVX and sham) and six and three levels corresponding to the time points of the architectural and biomarker data, respectively. Simple effects testing was performed to determine if a time or group effect existed when a time by group interaction was present. Paired t -tests were used to determine when the earliest detectable architectural changes occurred relative to the baseline data and if significant changes occurred compared to the previous time point. Unpaired t -tests determined if significant differences in BMD, BMC and mechanical properties between the OVX and sham groups existed. Results were considered to be statistically significant at $p < 0.05$ for all tests.

Table 1 Mean (SD) values for whole body BMD and BMC measured from DXA at the end of the twelve-week protocol. Significant differences were observed in whole body BMC but not BMD between the sham and OVX groups

	Sham	OVX
Mean BMD (g/cm^2)	0.175 (0.005)	0.174 (0.006)
Mean BMC (g)	12.17 (1.46)	13.65 (1.24)*

* Significant difference from sham ($p < 0.05$)

Fig. 1 Metric (a) and non-metric (b) morphological parameters of each individual rat in the OVX group (left column) and sham group (centre column). Group means and standard deviations are presented in the right column. The X axis indicates the time in weeks after OVX or sham operation. No data points are shown at week 0 in the sham group due to a change in scan region after the first measurement



a

Results

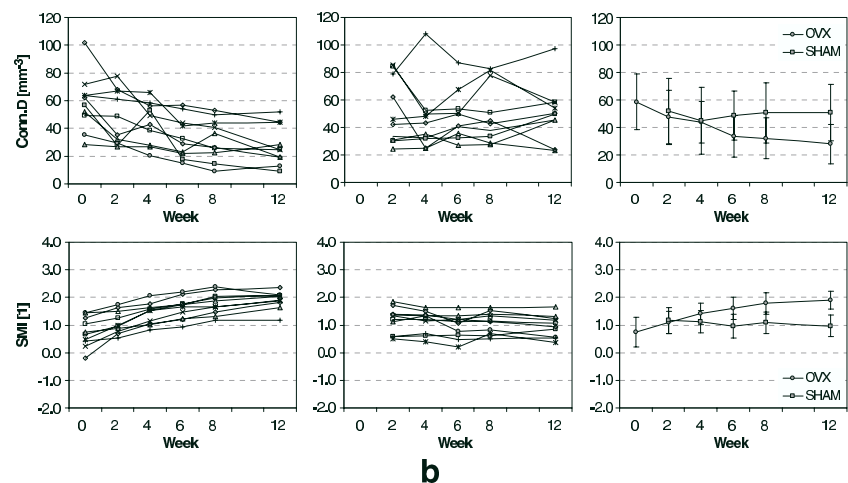
Animals

At the beginning of the protocol, the OVX and sham groups had mean masses of 362 ± 28 g and 362 ± 39 g respectively and were not significantly different ($p=0.984$). At week twelve, the mean mass of the OVX group increased from baseline by 31.5% (475 ± 37 g; $p<0.001$)

compared to an increase of 7.9% (390 ± 55 g; $p=0.008$) in the sham group, and was 21.9% higher than that of the sham ($p=0.001$).

Dual energy x-ray absorptiometry

The mean endpoint BMD and BMC from the DXA scans for the sham and OVX groups are provided (Table 1). There was no significant difference in mean whole body

Fig. 1 (continued)

BMD at the end of the twelve-week protocol ($p=0.57$), however, a significantly greater mean whole body BMC in the OVX group was detected ($p=0.026$).

Morphological analysis

The morphological parameters obtained from the in vivo micro-CT scans of all 20 rats are presented for both the individual and group average change (Fig. 1). Week 0 data for the sham group are not shown due to a change in scan region after the first scans. The relative change for all parameters with the exception of SMI in the OVX group with respect to the baseline data is also shown (Fig. 2). Averaged morphological data are provided (Table 2) for the study period with descriptive statistics to indicate significant changes between groups and relative to baseline values with a 95% confidence interval.

Representative changes in the 3D microstructure are illustrated in the micro-CT images of an OVX and sham rat at weeks 0, 2, 6 and 12 (Fig. 3). The repeated measures two-way ANOVA revealed a significant interaction between time and group for all of the morphological parameters. In the sham group, SMI changed significantly from the baseline measurements to become a more plate-like structure by week 6, and Ct.Th increased significantly by week 8. None of the remaining morphological parameters changed significantly in the sham group. In the OVX group, a change from the baseline value was detected after two weeks for all morphological parameters except for Tb.Sp, which showed a significant decrease after four weeks, and Ct.Th, which showed a significant increase after six weeks. The morphological data were studied for plateaus or regions where week-to-week changes were no longer detected. No plateau was reached in the OVX group for BV/TV and Tb.Sp as changes continued from weeks 8 to 12 in both of these parameters. Significant changes were not detected after week 8 for BS/

BV, SMI and Ct.Th, after week 6 for Tb.N and Conn.D, and after week 2 for Tb.Th in the OVX group.

Biomarkers of bone turnover

Results for osteocalcin and CTX-I levels are given (Table 3). The OVX group had significantly higher levels of osteocalcin (formation) than the sham group at week 0 ($p=0.023$) and week 6 ($p=0.007$). Additionally, osteocalcin levels in the OVX group were significantly higher at week 6 than at week 12 ($p=0.002$), indicating that the rate of bone formation decreased at the end of the protocol. At no point in the study was there a significant difference in CTX-I (resorption) levels between the OVX and sham groups; however, there was large variation in both the osteocalcin

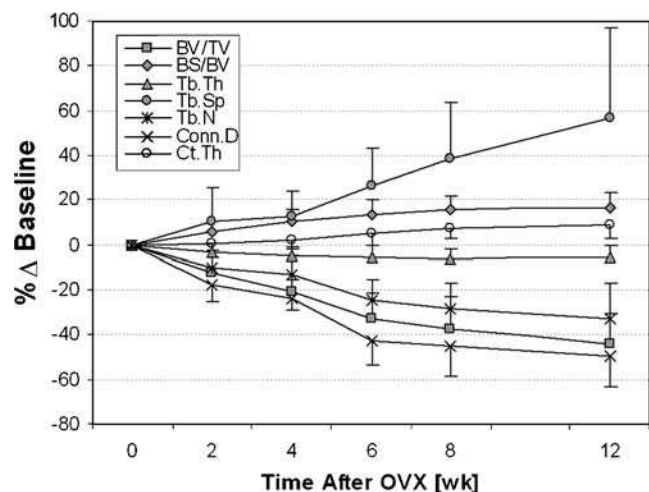


Fig. 2 Relative percent changes from baseline in the OVX group. The percent change was determined by dividing the value at each time point by the corresponding baseline value for each parameter. Positive numbers indicate an increase from baseline and negative numbers indicate a decrease from baseline

Table 2 Morphological outcomes by treatment group. Interaction and time effects are reported (F(df)), and p-values are reported for the repeated measures analysis (square brackets [])

Interaction		Time effect	Age [wks]									
			0	2	4	6	8	12				
BV/TV [%]	OVX	14.5 (5,90) [<0.001†]	43.2 (5,90) [<0.001†]	30.5±8.6	26.6±7.6 -3.9 (-2.0, -5.8) [0.001†]	24.15±6.9 -6.4 (-4.4, -8.4) [<0.001†, 0.008†]	20.73±7.5 -9.8 (-6.8, -12.8) [<0.001†, 0.008†]	19.2±7.1 -11.3 (-7.8, -14.9) [<0.001†, 0.054]	17.1±6.7 -13.5 (-9.1, -17.8) [<0.001†, 0.038†]			
	SHAM		15.6 (5,90) [0.183]		27.6±6.0 -0.654 (0.859, -2.167) [0.354]	26.98±5.8 -0.654 (0.859, -2.167) [0.354]	28.6±6.1 0.989 (3.408, -1.431) [0.379, 0.087]	28.1±5.5 0.457 (2.450, -1.536) [0.616, 0.284]	29.1±5.5 1.445 (3.385, -0.495) [0.126, 0.084]			
BS/BV [1/mm]	OVX	19.6 (5,90) [<0.001†]	25.4 (5,90) [<0.001†]	21.8±1.8	23.1±2.1 1.3 (1.9, 0.65) [0.001†]	24.1±2.2 2.3 (3.1, 1.5) [<0.001†, 0.004†]	24.8±2.4 3.0 (3.9, 2.0) [<0.001†, 0.119]	25.2±2.4 3.4 (4.4, 2.4) [<0.001†, 0.006†]	25.4±2.5 3.6 (4.7, 2.6) [<0.001†, 0.660]			
	SHAM		1.9 (5,90) [0.169]		22.9±2.5 -0.42 (0.54, -1.4) [0.346]	22.5±2.4 -0.42 (0.54, -1.4) [0.346]	22.1±2.5 -0.78 (0.18, -1.8) [0.100, 0.188]	22.5±2.2 -0.39 (0.61, -1.4) [0.402, 0.215]	22.0±2.3 -0.88 (0.39, -2.1) [0.151, 0.234]			
Tb.Th [mm]	OVX	8.5 (5,90) [<0.001†]	4.3 (5,90) [0.001†]	0.115±0.007	0.111±0.009 -0.0040 (-0.0010, -0.0069) [0.014†]	0.109±0.008 -0.0056 (-0.0026, -0.0085) [0.002†, 0.158]	0.108±0.009 -0.0066 (-0.0019, -0.011) [0.011†, 0.611]	0.108±0.008 -0.007 (-0.0033, -0.011) [0.002†, 0.613]	0.109±0.010 -0.0063 (-0.0023, -0.010) [0.006†, 0.729]			
	SHAM		4.5 (5,90) [0.312]		0.113±0.011 0.00162 (0.0055, -0.0023) [0.372]	0.114±0.014 0.00162 (0.0055, -0.0023) [0.372]	0.115±0.014 0.00251 (0.0069, 0.0019) [0.225, 0.305]	0.114±0.014 0.00132 (0.0057, 0.0031) [0.513, 0.188]	0.116±0.017 0.00344 (0.0099, 0.0030) [0.260, 0.213]			
Tb.Sp [mm]	OVX	7.4 (5,90) [<0.001†]	23.55 (5,90) [<0.001†]	0.418±0.179	0.460±0.201 0.0416 (0.862, -0.0299) [0.064]	0.475±0.229 0.0573 (0.101, 0.137) [0.016†, 0.538]	0.526±0.228 0.109 (0.161, 0.563) [0.001†, 0.013†]	0.576±0.264 0.158 (0.239, 0.773) [0.002†, 0.013†]	0.636±0.256 0.218 (0.319, 0.117) [0.001†, 0.046†]			
	SHAM		12.7 (5,90) [0.952]		0.373±0.099 0.0159 (0.0414, -0.00957) [0.192]	0.389±0.100 0.0159 (0.0414, -0.00957) [0.192]	0.382±0.102 0.00907 (0.0264, -0.00828) [0.267, 0.392]	0.385±0.112 0.00112 (0.0316, -0.00911) [0.243, 0.726]	0.386±0.113 0.0129 (0.355, 0.00975) [0.230, 0.872]			
Tb.N [1/mm]	OVX	12.2 (5,90) [<0.001†]	12.1 (5,90) [<0.001†]	2.946±1.056	2.637±.986 -0.309 (-0.0642, -0.554) [0.019†]	2.546±0.875 -0.400 (-0.146, -0.654) [<0.006†, 0.459]	2.233±0.847 -0.713 (-0.450, -0.976) [<0.001†, 0.009†]	2.127±0.844 -0.819 (-0.486, -1.151) [<0.001†, 0.109]	1.944±0.803 -1.002 (-0.503, -1.502) [0.001†, 0.061]			
	SHAM		44.5 (5,90) [0.701]		2.959±0.731 -0.144 (-0.0642, -0.554) [0.019†]	2.816±0.771 -0.144 (-0.0642, -0.554) [0.019†]	2.904±0.734 -0.0555 (-0.450, -0.976) [<0.001†, 0.009†]	2.941±0.818 -0.0181 (-0.486, -1.151) [<0.001†, 0.109]	2.945±0.823 -0.0144 (-0.503, -1.502) [0.001†, 0.061]			

Conn.D [1/mm ³]	OVX	3.1 (5,90) [0.013†]	7.49 (5,90) [<0.001†]	58.49±20.21	47.47±19.46 -11.01 (-0.47, -21.56) [0.042†]	51.72±23.93	(0.174, -0.461) [<0.333] 43.90±15.21 -14.58 (-4.51, -24.67) [<0.010†, <0.433] 45.08±24.29 -6.64 (8.61, -21.89) [0.350] 1.410±0.390 0.66 (0.908, 0.413) [<0.001†, <0.001†] 1.121±0.413 -0.0452 (0.0608, -0.151) [0.360] 0.438±0.0308 0.007 (-0.0142, -0.0045) [0.153, 0.011†] 0.473±0.0353 0.010 (-0.0163, -0.0052) [0.085]	(0.206, -0.317) [0.643, 0.266] 33.54±14.94 -24.95 (-16.06, -33.84) [<0.001†, 0.016†] 48.53±19.96 -3.12 (11.74, -13.69) [0.614, 0.380] 1.608±0.404 0.859 (1.119, 0.599) [<0.001†, <0.001†] 0.966±0.431 -0.200 (-0.00890, -0.391) [0.042†, 0.043†] 0.451±0.0335 0.02 (-0.0206, -0.0065) [<0.013†, <0.006†] 0.472±0.0374 0.009 (-0.0191, -0.0061) [0.171, 0.832]	0.235, -0.271) [0.875, 0.561] 32.13±14.79 -26.36 (-16.80, -35.93) [<0.001†, 0.461] 50.74±21.86 -0.97 (14.13, -16.20) [0.866, 0.533] 1.783±0.398 1.033 (1.311, 0.756) [<0.001†, <0.001†] 1.081±0.396 -0.0850 (0.0851, -0.255) [0.288, 0.103] 0.464±0.0347 0.033 (-0.0183, -0.0058) [<0.001†, 0.006†] 0.484±0.0389 0.021 (-0.0208, -0.0066) [0.011†, 0.030†]	(0.264, -0.292) [0.909, 0.973] 27.96±14.45 -30.53 (-17.59, -43.48) [<0.001†, 0.130] 50.68±20.61 -1.04 (14.13, -16.20) [0.881, 0.991] 1.900±0.320 1.150 (1.484, 0.817) [<0.001†, 0.086] 0.972±0.398 -0.195 (0.0336, -0.423) [0.086, 0.073] 0.467±0.0395 0.036 (-0.0276, -0.0087) [0.003†, 0.496] 0.479±0.0367 -0.016 (-0.0171, -0.0054) [0.016†, 0.244]		
	SHAM		13.3 (5,90) [0.574]									
	SMI	OVX	31.4 (5,90) [<0.001†]	59.2 (5,90) [<0.001†]	0.750±0.543	1.093±0.415 0.344 (0.521, 0.166) [0.002†] 1.166±0.475						
	SHAM		2.7 (5,90) [0.007†]									
Ct.Th [mm]	OVX	39.7 (5,90) [<0.001†]	7.74 (5,90) [<0.001†]	0.431±0.0318	0.430±0.0267 -0.001 (0.0082, -0.0102) [0.811] 0.463±0.0350							
	SHAM		103.7 (5,90) [<0.001†]									

Measurements for each week include group averages (mean±SD), and changes with respect to baseline values (week 0 in OVX and week 2 in sham) and a 95% confidence interval. The first p -value represents change with respect to baseline, and the second p -value represents change with respect to previous measure
† $p<0.05$

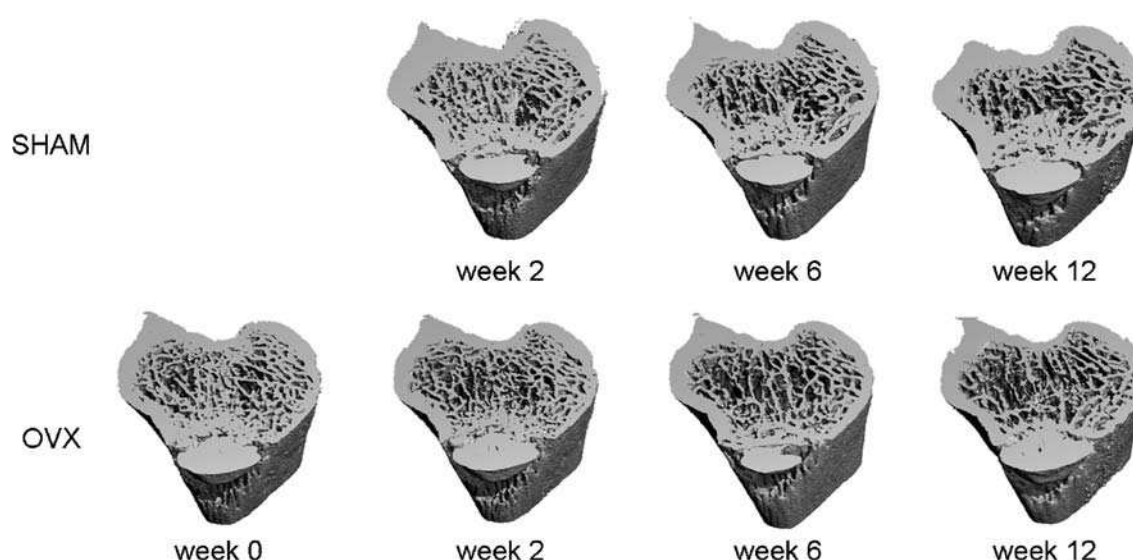


Fig. 3 Visualization of bone loss in a transaction of the tibial metaphysis of an OVX and sham rat (median BV/TV for each group) at the time of surgery, and two, six and twelve weeks following surgery

and CTX-I measures. Simple effects testing showed a significantly higher CTX-I concentration at week 0 compared to week 12 for the OVX group ($p=0.001$) and week 6 to week 12 for both groups (OVX: $p=0.041$, sham: $p=0.001$).

Mechanical testing

The results from the three-point bend tests of the femora and compression tests of the L-4 vertebrae are provided (Table 4). For the three-point bend tests, there was a significant reduction in ultimate stress (14.1%, $p<0.001$) but not ultimate load (7.1%, $p=0.072$) in the femora of the OVX group compared to the sham. The extrinsic stiffness and Young's modulus, on the other hand, were not significantly different between groups. Significant reductions in work to yield (22.0%, $p=0.008$) and modulus of resilience (32.2%, $p=0.002$) in the OVX group compared to the sham were observed; however, there was no significant difference in work to failure or modulus of toughness between the groups. Trends toward increases in the moment of inertia and decreases in the distance from the periosteal surface to the neutral axis were observed in the OVX group. For the compression tests of the L-4 vertebrae, there

was a significant decrease in ultimate load in the L-4 vertebrae of the OVX group compared to sham (16.3%, $p=0.048$) but no significant change in ultimate stress ($p=0.2142$). There were no significant differences in any of the other mechanical parameters measured for the L-4 vertebral compression tests. Trends toward decreases in the cross-sectional area were observed for the OVX group.

Discussion

This study investigated the short-term effects of OVX on changes in bone architecture, turnover and biomechanics. Estrogen deficiency had a detrimental effect on the trabecular architecture of the tibial metaphysis of the rat, and significant changes occurred within two weeks of OVX in most parameters, including a decrease in Conn.D, which indicates that irreversible changes had taken place. Biomarker data indicated that bone formation was highest during the first six weeks after OVX, suggesting that bone turnover (and loss) may also be highest during this time. Despite the structural changes at the proximal tibia, there were only moderate differences in mechanical properties of

Table 3 Mean (SD) concentrations of osteocalcin and CTX-I

	Week 0		Week 6		Week 12	
	Sham	OVX	Sham	OVX	Sham	OVX
Osteocalcin (ng/mL)	4.77 (2.84)	8.95* (3.61)	4.56 (5.18)	11.56* (2.98)	4.25 (4.03)	6.34 ^{o,†} (2.84)
CTX-I (ng/mL)	63.20 (19.56)	49.69 (6.49)	50.97 (14.17)	58.10 (18.44)	90.45 [†] (15.72)	84.46 ^{o,†} (16.13)

* Significant difference from sham ($p<0.05$)

^{o,†} Significant difference from week 0 and week 6, respectively ($p<0.05$)

Table 4 Mechanical properties at different skeletal sites. Mean (SD) values of stiffness (S), ultimate load (F_u), elastic modulus (E), ultimate stress (σ_u), work to failure (W_f), modulus of toughness (u_t), work to yield (W_y) and modulus of resilience (u_r) 12 months after surgery. Standard deviations are in parentheses

	Three-point bending of femur		Compression of L-4 vertebrae	
	Sham	OVX	Sham	OVX
S (N/mm)	653.6 (83.3)	649.2 (87.5)	3111 (899.0)	3151 (981.5)
F_u (N)	191.3 (20.1)	177.7 (25.2)	352.0 (60.3)	294.8* (42.6)
E (GPa)	10.1 (1.47)	9.66 (1.37)	2.36 (0.768)	2.36 (0.978)
σ_u (MPa)	209.7 (18.7)	180.1* (23.4)	60.7 (8.18)	55.9 (8.70)
W_f (Nmm)	69.5 (12.5)	68.9 (19.1)	73.9 (26.8)	63.1 (21.3)
u_t (MPa)	5.37 (1.03)	4.81 (1.17)	2.91 (0.869)	3.03 (0.831)
W_y (Nmm)	19.3 (4.72)	15.1* (4.11)	22.9 (5.34)	22.8 (10.6)
u_r (MPa)	1.52 (0.520)	1.03* (0.288)	0.912 (0.204)	0.875 (0.235)

* Significant difference from sham ($p < 0.05$)

the OVX and sham groups at the femur midshaft and L4 vertebra. The results of this study are consistent with the hypothesis that both micro-architectural and mechanical changes occur within the first twelve weeks of OVX in the rat model.

Significant changes in the trabecular architecture occurred as early as two weeks after OVX. BV/TV significantly decreased from baseline in the OVX group after two weeks, and after eight weeks had decreased by an average of 37%. This is a smaller reduction than found in previous studies that report up to a 57% decrease at the same eight-week time point after OVX [5, 16, 17], and is potentially due to the use of retired breeders, which have less bone than virgin rats [18]. Previous studies have conflicting results on the effect of OVX on trabecular thickness, with some showing an increase in thickness [4, 9], and others showing a decrease [5]. In this study, trabecular thickness was significantly lower than the baseline value at every time point after OVX; however no relative changes occurred after week 2, indicating that a plateau had been reached. It is possible that trabecular thickness continued to decrease throughout the study and the trend is masked as the thinner trabeculae become disconnected, resulting in a structure with fewer, thicker trabeculae. This is supported by the observed decrease in trabecular number and connectivity density, as well as increased trabecular separation. There was no delay in the reduction of connectivity density following OVX as has been previously reported [5], indicating that thinner trabecular structures may separate immediately after OVX. This might be reflected by the smaller observed increase in SMI in this study compared to the previous, as small increases in Conn.D can occur as the trabecular structure becomes more rod-like. Although thinning trabeculae will lead to a weaker bone structure, this may not be as critical to bone health as the loss of entire trabecular elements or connections [19] which are difficult to repair. Anabolic treatments for osteoporosis, such as parathyroid hormone are

able to restore bone mass; however it is unclear whether any connectivity can be restored [20–23], suggesting that Conn.D may be an indicator of irreversible bone loss.

A plateau was reached for BS/BV, SMI, Tb.N, Conn.D, Tb.Th, and Ct.Th, highlighting the importance of early treatment for bone loss due to the short time frame in which these parameters experience the largest magnitude of change. Inter-group variation was high for all parameters, which may result from a different number of breeding cycles undergone previously by each rat. Although there was no significant difference in endpoint BMD detected from the whole body DXA, the OVX group had a higher mean BMC than the sham group, potentially related to the observed increase in Ct.Th. Increased Ct.Th in both groups is consistent with previous findings [24] and suggests that this parameter may be affected by age in addition to estrogen deficiency.

It should be pointed out, however, that the higher soft tissue mass in the OVX rats may have contributed to “soft tissue contamination” [25] resulting in an artificial overestimation of BMC.

OVX resulted in a significant increase in bone formation markers but not resorption markers. The elevated osteocalcin levels in the OVX group at week 0 are likely a result of the blood samples being taken at the time of the first scan, which was performed several days after the surgery to allow the animals time to recover. The osteocalcin results imply that bone turnover slows between weeks 6 and 12; therefore, the rate of bone loss may also decrease during this period. The osteocalcin data correspond with the longitudinal data for BS/BV, SMI, Tb.N and Conn.D where plateaus were reached after week 6. It is possible then, that there is a small window after the initiation of estrogen deficiency in which interventions can be applied to prevent the resulting bone loss.

Moderate changes in mechanical properties of the femora and vertebrae were observed following OVX. The mechanical properties of the femora were assessed with three-point bend tests, and the values obtained are similar to

those of previous studies of skeletally mature rats for ultimate load ultimate stress, stiffness and work to failure [26–28]. Some studies, however, found lower values for elastic modulus, stiffness, ultimate stress and ultimate load [26, 29]. These differences may be attributed to dehydration of the sample when removed from the saline soaked gauze prior to testing [30]. A significantly lower ultimate stress was observed in the femora of the OVX rats compared to those of the sham, indicating that the intrinsic mechanical properties of the bone may possibly have been affected by OVX at this site. This would need to be verified by direct measures (i.e., nanoindentation, synchrotron micro-CT). Significant reductions in modulus of resilience and work to yield suggest that the behavior of the bone tissue in the elastic region may also be affected 12 weeks after OVX. The results from the compression tests of the L-4 vertebrae were similar to previous for ultimate load [22] and Young's modulus [31]; however higher values for ultimate load [28], and lower values for stiffness, Young's modulus and ultimate stress [22] have been observed. As with the femora, this may be due to dehydration of the sample when tested without the saline-soaked gauze. A reduction in ultimate load was observed in the L-4 vertebrae indicating that OVX was detrimental to the ability of the vertebrae to withstand load.

Some limitations need to be considered with regards to the structural, biomechanical and biomarker data. As mentioned previously, the use of retired breeders added variability and reduced trabecular bone content in the baseline architecture and led to smaller changes due to OVX than previously reported [5]. Nevertheless, the repeated measures study made possible by in vivo micro-CT provided sufficient statistical power to detect changes as early as two weeks. Regarding the biochemical data, it is possible that the six-week intervals were too large to detect changes in resorption. This may, however, be specific to the use of CTX-I as elevated resorption assessed by hydroxyproline has been observed 42 days after OVX in six-month-old Sprague-Dawley rats [32]. The time of day at which the serum was collected was not controlled and the rats did not fast the night before collection. For biomechanics, the flexure formula was used to determine the Young's modulus of bone samples in three-point bending, and is known to have limitations due to implicit assumptions about bone geometry [33]. Nonetheless, it has been able to detect significant changes in the mechanical properties of OVX rats previously [22]. Mechanical testing cannot be performed in vivo; therefore, temporal changes in the mechanical properties could not be determined in this study. In the future, finite element (FE) models that incorporate density measurements to determine tissue level properties of the samples may provide a sufficiently accurate alternative to assess mechanical properties of bone

over time in vivo [34, 35], and this would help to identify key milestones of change in mechanical properties in a similar manner as the time points of architectural changes were assessed in this study. Those methods, however, remain to be sufficiently developed for that purpose.

Quantitative data demonstrating architectural, biochemical and biomechanical changes in an OVX rat model provided important baseline information of the effects of OVX on bone loss. The comprehensive 12-week dataset will provide a basis for the design of future in vivo studies to examine the effect of interventions on bone architecture and strength, particularly with respect to when irreversible architectural changes occur. OVX caused irreversible changes to the trabecular architecture after two-weeks and these changes were most dramatic within the first six weeks. Biomechanical properties were altered twelve weeks after OVX. This study highlights that early identification and treatment of osteoporosis may be critical to optimize treatment outcomes, due to the short time frame in which significant changes in bone quality can occur.

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