Effects of daily restraint with and without injections on skeletal properties in C57BL/6NHsd mice

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Animal model experiments have an essential place in the infrastructure of biomedical science. The literature is replete with papers studying various physiological and organ systems in which manipulations to animals are made via administration of novel compounds. These studies typically involve groups of animals that are injected with placebo compounds. As there are studies that demonstrate that restraint and injection can affect behavior and corticosteroid levels in rodents[1,2] the basis of such placebo injections is to control for any potential effects caused by handling and injecting the experimental animals. But these stressors may not adversely affect all studies equally. While placebo injections make sense for studies that are focused on outcomes which may be directly or indirectly affected by stress hormones, for other studies the value of placebo injections is less clear. If placebo groups are not necessary for some studies, this would result in an overall reduction in both the number of animals used in research and the need to handle/inject a significant number of animals.

Bone is a dynamic organ that undergoes continual renewal throughout life[3]. The breakdown of this process leads to conditions such as osteoporosis, where bones lose mass and mechanical properties, ultimately leading to fracture. The mouse has become a highly utilized

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research model in skeletal biology due to the ease of genetic manipulation to answer mechanistic questions. Due to the relatively slow changes that occur in bone, most interventional studies involve treatment durations that last weeks or months. Studies of bone physiology often include control group (no manipulation) in addition to a placebo group (administration of vehicle), though changes of bone are relatively slow and likely not influenced by the stressors associated with restraint and injection. The goal of this study was to evaluate the effect of daily handling with and without placebo injections on skeletal properties of C57BL/6NHsd female mice. Our working hypothesis was that daily handling and injection would not significantly alter bone mass or mechanical properties compared to non-intervention controls.

Do bone studies need placebos?

Sixty female C57BL/6NHsd mice were purchased (Envigo, Indianapolis, IN) at approximately 8 weeks of age. All 60 mice were group housed (5 mice per cage) for the duration of the experiment. One week after arrival, cages of animals were randomly assigned to one of three experimental groups: animals that were only handled during weekly cage changes (CON, n=20); animals that were restrained but not injected 5 days per week (SHAM, n=20); and animals that were restrained and given an intraperitoneal (IP) injection (0.15 cc 0.9% saline solution) 5 days per week (INJ, n=20). SHAM and INJ mice were given their respective treatments between the hours of 9am and 12pm Monday–Friday throughout the experiment period. Restraint was done one handed using a standard dorsal neck scruff. SHAM mice had pressure put on their abdomen using a capped syringe to simulate the process of receiving an injection. INJ mice were restrained with the same approach and administered a saline IP injection. All mice were weighed weekly. The experiment lasted 8 weeks. All procedures were approved by the Indiana University School of Medicine Institutional Animal Care and Use Committee prior to initiating the study.

Behavioral assessment: After 8 weeks, all animals were scored behaviorally using a previously described technique[4]. The individual who scored all animals was blinded to the treatment groups through the assignment of arbitrary numbers to identify each cage for the scorer. Briefly, the cages were placed in a laminar flow work station, the top removed, and the scorer's hand placed in the front of the cage for 15 seconds. Mice were scored as fearful or inquisitive toward the hand. All mice were left in the cage during assessment, and the scorer assigned numbers to interactions. Animals exhibiting signs of barbering were also quantified at the end of the study.

Following behavioral scoring, mice were anaesthetized using isoflurane (5% inhaled in 0.5 L/min oxygen; Forane, Baxter Healthcare Corporation, Deerfield, IL) for a terminal blood collection, and then the animals were euthanized using cervical dislocation. Liver, heart, kidneys, thymus, and spleen weights were recorded. Tibiae and femora were dissected free, wrapped in saline-soaked gauze and stored at -20 °C until analysis.

Hematology: The blood sample collected at the end of the study was divided between a serum separator blood collection tube (serum) and an EDTA treated blood collection tube (whole blood). A complete blood count was run on the whole blood sample using an automated machine (Hemavet 950, Drew Scientific, Miami Lakes, FL). The whole blood count (thousands/mL), total number of neutrophils (thousands/mL), and total number of lymphocytes (thousands/mL) were measured for each sample and averaged per group. The neutrophil to lymphocyte ratio was calculated by dividing the total number of neutrophils by the total number of lymphocytes. This ratio has been demonstrated to be an accurate indicator of chronic stress in multiple species[5]. The serum sample was frozen at -80 °C until evaluation of the serum corticosterone levels. The serum corticosterone was evaluated using a mouse serum corticosterone ELISA kit (MP Biomedicals, Santa Ana, CA).

Bone imaging: Micro-computed tomography (μCT) scans were taken of the right tibia and femur of each mouse using a Skyscan 1176 μCT system (Bruker microCT, Kontich, Belgium). Scans

were performed through a 0.5 mm Al filter with an isotropic voxel size of 9 μm. Projection scans were reconstructed and analyzed using manufacturer software. Standard cortical regions of interest (ROIs) for both tibia and femur were taken near the site of mechanical testing for assessment of geometry. Each standard site ROI was a set of 7 slices, perpendicular to the proximal-distal axis. As previously described[6], a custom MATLAB (MathWorks, Natick, MA) program was used to calculate the following parameters: total bone area (B.Ar), marrow area (Ma.Ar), cortical area (Ct.Ar), cortical area fraction (Ct.Ar/Tt.Ar), average cortical width (Ct.Wi), periosteal bone perimeter (Ps.Pm), endocortical bone perimeter (Ec.Pm), maximum and minimum second moment of inertia (I_{max} and I_{min}, respectively), width of the anteroposterior axis (AP.Wi), width of the mediolateral axis (ML.Wi), and AP.Wi to ML.Wi ratio (AP.Wi/ML.Wi). The proximal tibia and distal femur regions were assessed for trabecular bone properties. Trabecular ROIs were selected to encompass a 0.5 mm distance of the secondary spongiosa. Within this region the trabecular bone was manually segmented from the cortex and analyzed for bone volume per unit tissue volume (BV/TV), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp) and trabecular number (Tb.N).

Bone mechanics: Prior to testing, all samples were thawed to room temperature. Bones were tested to failure in 4 point bending (upper loading span of 3 mm, lower support span of 9 mm) in displacement control at a rate of 0.025 mm/s while hydrated with PBS. Using a custom MATLAB program[7], structural and apparent material properties were determined. Apparent material properties were derived using standard beam-bending equations for four-point bending and geometric data from microCT.

Results and observations

Considerable effort and cost can be consumed by the process of dosing animals in preclinical experiments. Studies routinely use saline dosing in control groups and although never

explicitly stated, the likely reason is to account for the effects that handling/injections have on the outcome of interest. The goal of the current study was to examine the effects of saline injections in mice, specifically on skeletal properties, and to determine if a reduction of animal use can be affected through the elimination of a placebo group that does not provide meaningful comparison data. Our data clearly show no effect of saline injections on skeletal morphology or mechanical properties following an 8-week study period. This suggests non-injected animals are a valid control when dosing studies focused on bone structure/mechanics are conducted in mice.

Comparisons among the three groups were made using a one-way ANOVA or Chi-square (behavioral data). When a significant main effect was present, post-hoc tests (Tukey HSD) were used to determine individual group differences. A p value of ≤ 0.05 was used for all determinations of significance. All data are presented as means +/- standard deviations.

Several studies have documented the effects of handling and/or injection on animal stress levels[1,2]. These studies and others have utilized a variety of outcome measures including assessing body/organ weight[8-11], animal activity/behavior [4,10], and biochemical assays[12]. We found no significant effect among the three groups in body or organ weight (**Table 1, Fig. 1a-b**). Blood levels of leukocytes were also similar across the three groups and were within normal limits[15]. The ratio of neutrophils to lymphocytes has been used as an index of animal stress in multiple species[5], and this study demonstrated no significant differences between the three groups (**Table 2, Fig. 1c**). Finally, qualitative evaluation of several aspects of behavior toward the end of the experiment showed no significant difference in barbering or fearfulness among animals in the three conditions (**Table 3**). Taken together, these data suggest there was no difference in animal stress between animals that remained untouched in their cages and those that were handled daily, with or without injection, over an 8-week time period.

Micro-CT based imaging of bone morphology represents a gold-standard and fairly

sensitive parameter of interventional effects on the skeleton. For example, removal of endogenous sex steroids, leads to a reduction in trabecular BV/TV[13]; mechanical loading leads to a robust increase in cortical bone area and cross-sectional moment of inertia[6]. Chronically high levels of corticosteroids, indicative of persistent stress, have well-established negative effects on trabecular BV/TV[14]. In our study, there were minimal effects of either handling or injecting animals on more than a dozen micro-CT based outcome measures. The tibia and femur were both assessed for trabecular and cortical bone parameters, the majority of which were not different among the groups. The lone parameter of the tibia that differed among the groups was trabecular thickness, which was lower in the SHAM animals compared to control. The femur had three properties that statistically differed among the groups: trabecular BV/TV and Tb.N (both significantly higher in INJ vs SHAM), and cortical thickness of the diaphysis (**Table 4, Figure 2**).There was significantly more trabecular bone (+35%) in the distal femur of animals injected daily compared to those that were handled but not injected. Neither of these groups was significantly different than cage controls which were intermediate to the two intervention groups. There was no difference in tibial BV/TV among the groups. Given that handled animals had femoral BV/TV values lower than cage controls, while injected animals were higher, it is unlikely that these effects were manifested due to treatments. A more plausible explanation for these group differences is simply the intrinsic variability in trabecular bone within mice.

While imaging outcomes are almost universally undertaken in preclinical work focused on skeletal properties, mechanical testing represents a holistic skeletal assay that integrates both bone mass and bone quality (the properties of the tissue independent of mass). Interventions that alter bone mass or bone quality can manifest as alterations in a variety of mechanical testing parameters. For example, chronic administration of corticosteroids results in significant reductions in bone strength (ultimate load and ultimate stress), along with several other properties[14]. Our study showed that a suite of mechanical properties were comparable among the three groups for

both the tibia and femur (**Table 5, Fig. 3**). These results show clear evidence that daily handling or long-term saline dosing have no significant negative effect on mouse mechanical properties and suggest there is no need to use daily injections in control animals.

Conclusions

The results of this study are limited in that only one mouse strain (C57BL/6), sex (female), and age (9–17 weeks) were studied. Whether the same lack of effect would occur universally in other situations is unknown and is not feasible to comprehensively study. The conclusions also do not apply to all possible bone outcomes. There may be parameters such as gene/protein expression that are more subtly affected by handling/injection, yet because our laboratory traditionally uses tissue-level assays, we chose to focus on imaging/mechanics as the main outcomes.

In conclusion, our study shows that over an 8-week study duration in mice, the effects of daily handling or daily injection with saline have modest effects on bone morphology and no effects on mechanical properties. These results suggest that for these tissue/organ level outcome measures there is no need to use saline-injections in control animals. This represents a refinement in experimental design that can result in a reduction of overall animal use in similar studies as well as in investigator time to do sham injections of large numbers of animals.

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Figure legends

Figure 1. Effect of daily animal handling and injection on body mass injection (A), thymus mass (B) and neutrophil/lymphocyte ratio (C).

Figure 2. Effect of daily animal handling and injection on trabecular bone volume (A) and cortical bone area (B). Upper right panel depicts 3D view of proximal tibia from the animal closest to the mean of each group, Lower right panel shows average cortical bone tracing across all animals in each group.

Figure 3. Effect of daily animal handling and injection on ultimate load of the tibia and femur.

Table 1: Body and organ masses

	Control	Sham	Injection	ANOVA P value
Baseline BW (g)	18.2 ± 1.2	18.2 ± 1.5	17.8 ± 1.5	0.604
Final BW (g)	21.9 ± 1.2	21.6 ± 1.9	21.2 ± 1.6	0.451
Liver/BW	0.049 ± 0.007	0.049 ± 0.009	0.051 ± 0.004	0.721
Spleen/BW	0.0034 ± 0.0006	0.0033 ± 0.0006	0.0033 ± 0.0004	0.687
Heart/BW	0.0058 ± 0.0007	0.0059 ± 0.0007	0.0058 ± 0.0006	0.701
Kidneys/BW	0.0111 ± 0.0029	0.0115 ± 0.001	0.0116 ± 0.0009	0.771
Thymus/BW	0.0023 ± 0.001	0.0025 ± 0.001	0.0027 ± 0.001	0.422

Data presented as means and standard deviations. BW, body weight.

Table 2: Hematology

	Control	Sham	Injection	ANOVA P value
White Blood Cells (thousands/mL)	3.19 ± 1.4	3.42 ± 1.36	3.54 ± 1.22	0.706
Neutrophils (thousands/mL)	0.60 ± 0.31	0.65 ± 0.32	0.68 ± 0.27	0.688
Lymphocytes (thousands/mL)	2.39 ± 1.0	2.58 ± 1.0	2.67 ± 0.9	0.668
Neutrophils/Lymphocytes	0.255 ± 0.76	0.244 ± 0.07	0.254 ± 0.05	0.864
Corticosterone (ng/mL)	163.74 ± 16.95	130.63 ± 16.95	140.08 ± 15.56	0.376

Data presented as means and standard deviations.

Table 3: Behavioral scoring

Group	Control	Sham	Injection	Chi square P value
Barbering (#)	7/20	6/20	3/20	0.330
Inquisitive (#)	13/20	18/20	2/20	0.154
Fearful (#)	7/20	4/20	5/20	0.551

Data presented as number of animals displaying feature as a ratio of total number of animals per group.

Table 4: Trabecular and cortical architecture of the tibia and femur

	Control	Sham	Injection	ANOVA P value
Tibia				
Trabecular BV/TV (%)	9.36 ± 2.12	8.75 ± 2.06	9.31 ± 1.51	0.540
Trabecular thickness (μm)	69.6 ± 0.6	65.3 ± 0.3 *	66.9 ± 0.3	**0.012**
Trabecular number (#/mm)	1.34 ± 0.26	1.34 ± 0.30	1.39 ± 0.20	0.793
Total cross sectional area (mm^2)	0.93 ± 0.08	0.92 ± 0.08	0.96 ± 0.08	0.365
Marrow area (mm^2)	0.33 ± 0.04	0.33 ± 0.04	0.33 ± 0.05	0.096
Cortical area (mm^2)	0.60 ± 0.04	0.59 ± 0.05	0.62 ± 0.05	0.077
Cortical thickness (mm)	0.22 ± 0.01	0.22 ± 0.01	0.23 ± 0.02	0.069
Cross-sectional moment of inertia (mm^4)	0.07 ± 0.02	0.07 ± 0.02	0.08 ± 004	0.430
Cortical tissue mineral density (g/cm^3 HA)	1.65 ± 0.18	1.72 ± 0.25	1.63 ± 0.19	0.372
Femur				
Trabecular BV/TV (%)	2.25 ± 0.73	1.97 ± 0.50	2.66 ± 0.80 #	**0.010**
Trabecular thickness (μm)	52.9 ± 0.8	48.3 ± 0.6	50.7 ± 0.9	0.196
Trabecular number (#/mm)	0.42 ± 0.12	0.41 ± 0.08	0.53 ± 0.14 *#	**0.005**
Total cross sectional area (mm^2)	1.57 ± 0.08	1.54 ± 0.12	1.57 ± 0.11	0.570
Marrow area (mm^2)	0.78 ± 0.06	0.78 ± 0.08	0.78 ± 0.07	0.943
Cortical area (mm^2)	0.79 ± 0.04	0.76 ± 0.04	0.79 ± 0.05	0.094
Cortical thickness (mm)	0.21 ± 0.01	0.20 ± 0.01*	0.21 ± 0.01	**0.045**
Cross-sectional moment of inertia (mm^4)	0.12 ± 0.01	0.12 ± 0.01	0.12 ± 0.01	0.764
Cortical tissue mineral density (g/cm^3 HA)	1.79 ± 0.04	1.78 ± 0.03	1.78 ± 0.03	0.441

Data presented as means and standard deviations. BV/TV, bone volume per total volume; HA, hydroxyapatite. $P < 0.05$ versus control (*) and sham (#).

Table 5: Mechanical properties of the tibia and femur

	Control	Sham	Injection	ANOVA P value
Tibia				
Yield Force (N)	17.3 ± 4.4	14.0 ± 2.8	15.7 ± 2.2	0.273
Ultimate Force (N)	15.1 ± 4.5	15.5 ± 2.8	17.1 ± 2.7	0.209
Displacement to Yield (μm)	278 ± 92	263 ± 50	286 ± 53	0.615
Post-yield Displacement (μm)	269 ± 210	311 ± 219	387 ± 202	0.250
Total Displacement (μm)	547 ± 234	574 ± 229	673 ± 206	0.222
Stiffness (N/mm)	63 ± 18	61 ± 13	64 ± 13	0.823
Total Work (mJ)	5.5 ± 3.0	5.8 ± 2.7	7.3 ± 2.8	0.126
Ultimate Stress (MPa)	206 ± 63	214 ± 45	218 ± 47	0.798
Modulus (GPa)	11.8 ± 3.9	12.0 ± 3.7	10.9 ± 3.0	0.644
Toughness (MPa)	5.5 ± 3.3	5.7 ± 2.6	7.1 ± 2.8	0.231
Femur				
Yield Force (N)	10.4 ± 1.1	9.9 ± 1.4	10.5 ± 1.1	0.169
Ultimate Force (N)	12.8 ± 1.0	12.5 ± 0.9	13.0 ± 1.2	0.287
Displacement to Yield (μm)	155 ± 18	153 ± 24	169 ± 29	0.113
Post-yield Displacement (μm)	990 ± 414	1031 ± 476	1129 ± 403	0.498
Total Displacement (μm)	1145 ± 411	1184 ± 472	1298 ± 399	0.430
Stiffness (N/mm)	78.8 ± 9.2	75.5 ± 9.7	73.3 ± 11.8	0.246
Total Work (mJ)	9.8 ± 2.5	9.6 ± 3.0	10.8 ± 2.7	0.318
Ultimate Stress (MPa)	142 ± 14	143 ± 15	145 ± 12	0.793
Modulus (GPa)	8.3 ± 0.9	8.2 ± 1.1	7.8 ± 1.4	0.402
Toughness (MPa)	11.4 ± 2.8	11.2 ± 3.0	12.6 ± 2.7	0.249

Data presented as means and standard deviations.

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Figure 1

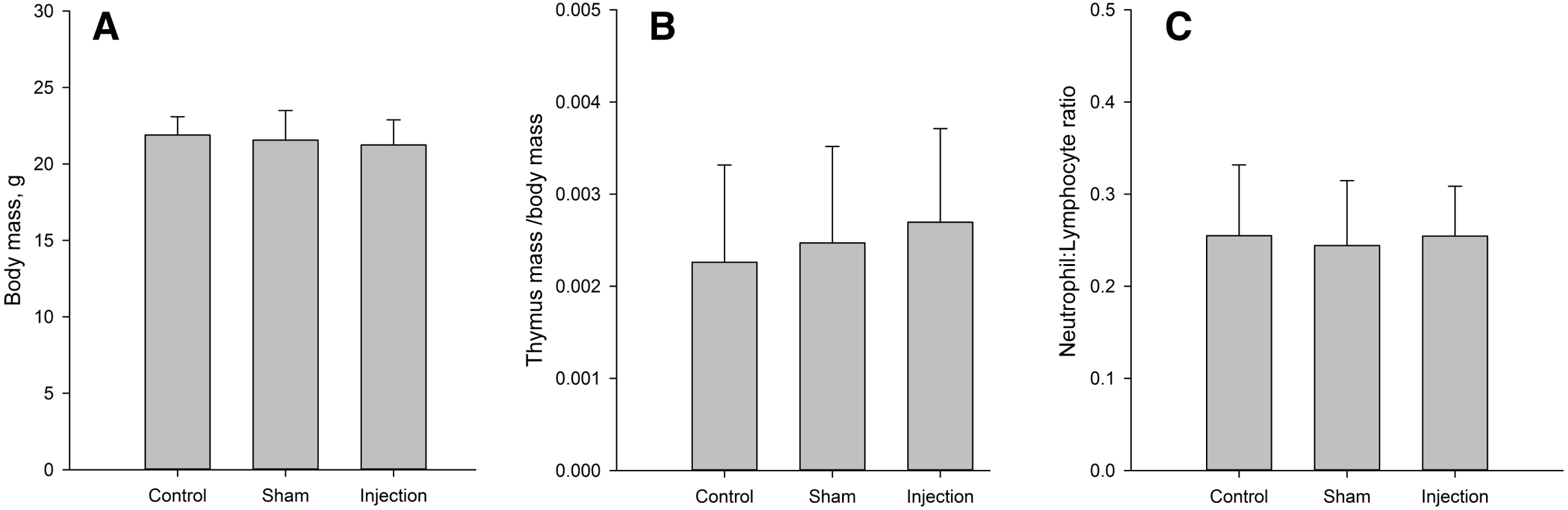
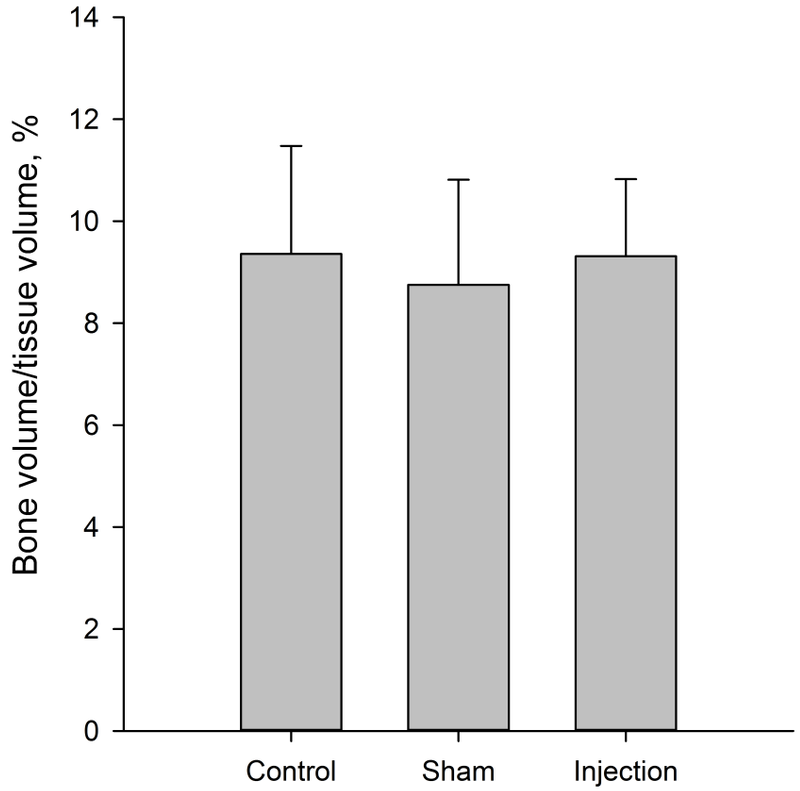
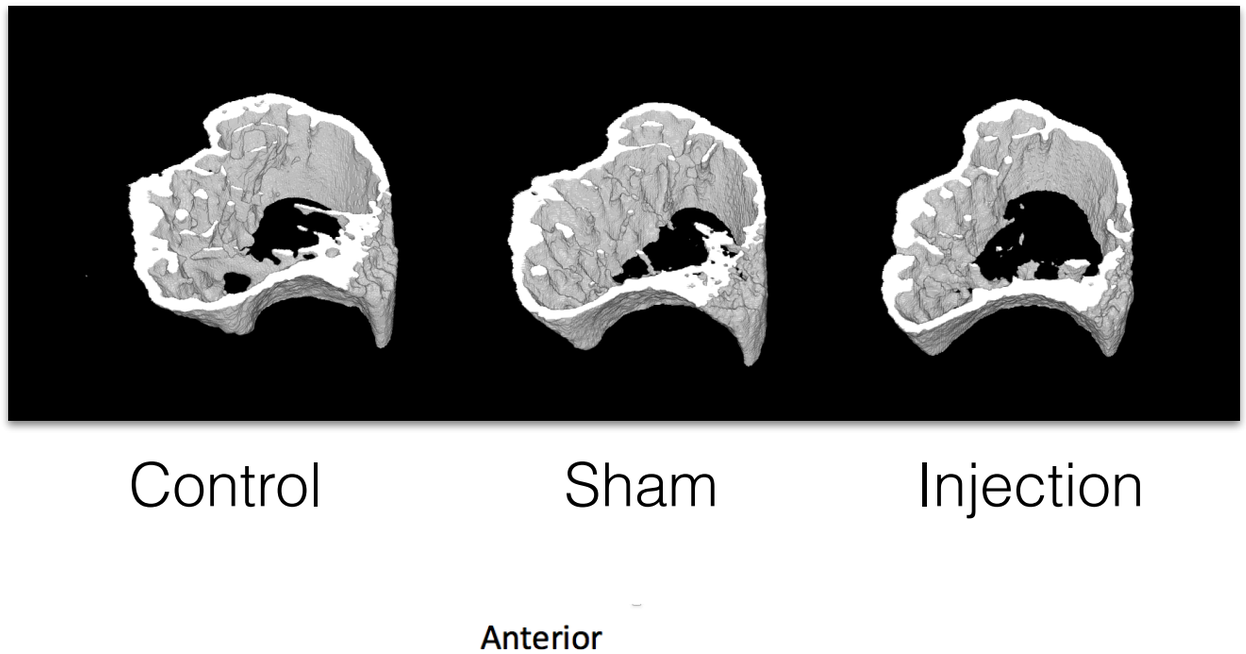

A
Body mass, g
30
25
20
15
10
5
0
Control
Sham
Injection
B
Thymus mass /body mass
0.005
0.004
0.003
0.002
0.001
0.000
Control
Sham
Injection
C
Neutrophil:Lymphocyte ratio
0.5
0.4
0.3
0.2
0.1
0.0
Control
Sham
Injection

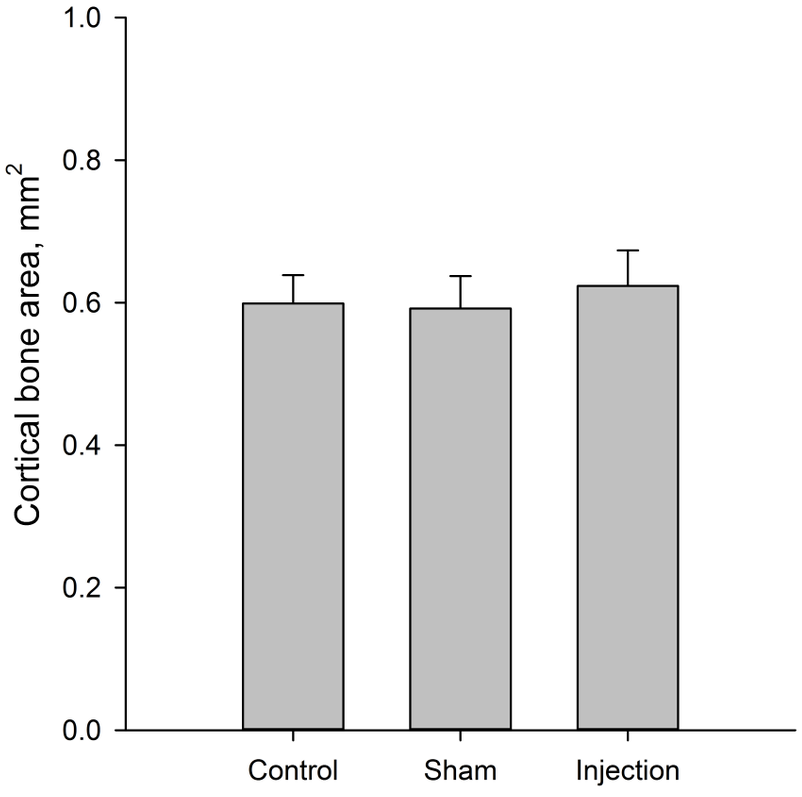
Figure 2

A


Bone volume/tissue volume, %
14
12
10
8
6
4
2
0
Control
Sham
Injection


Control
Sham
Injection
Anterior

B


Cortical bone area, mm2
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0.8
0.6
0.4
0.2
0.0
Control
Sham
Injection

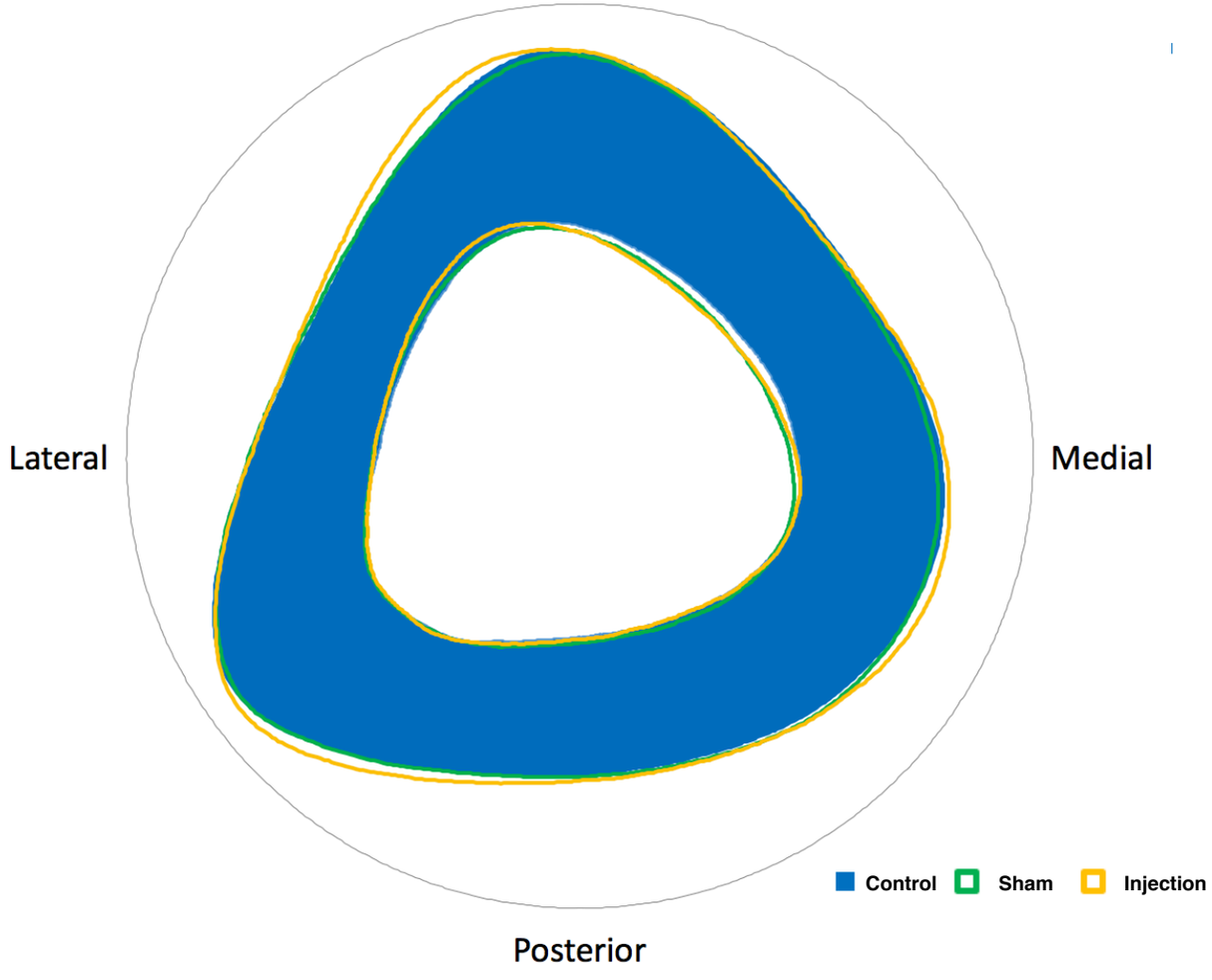
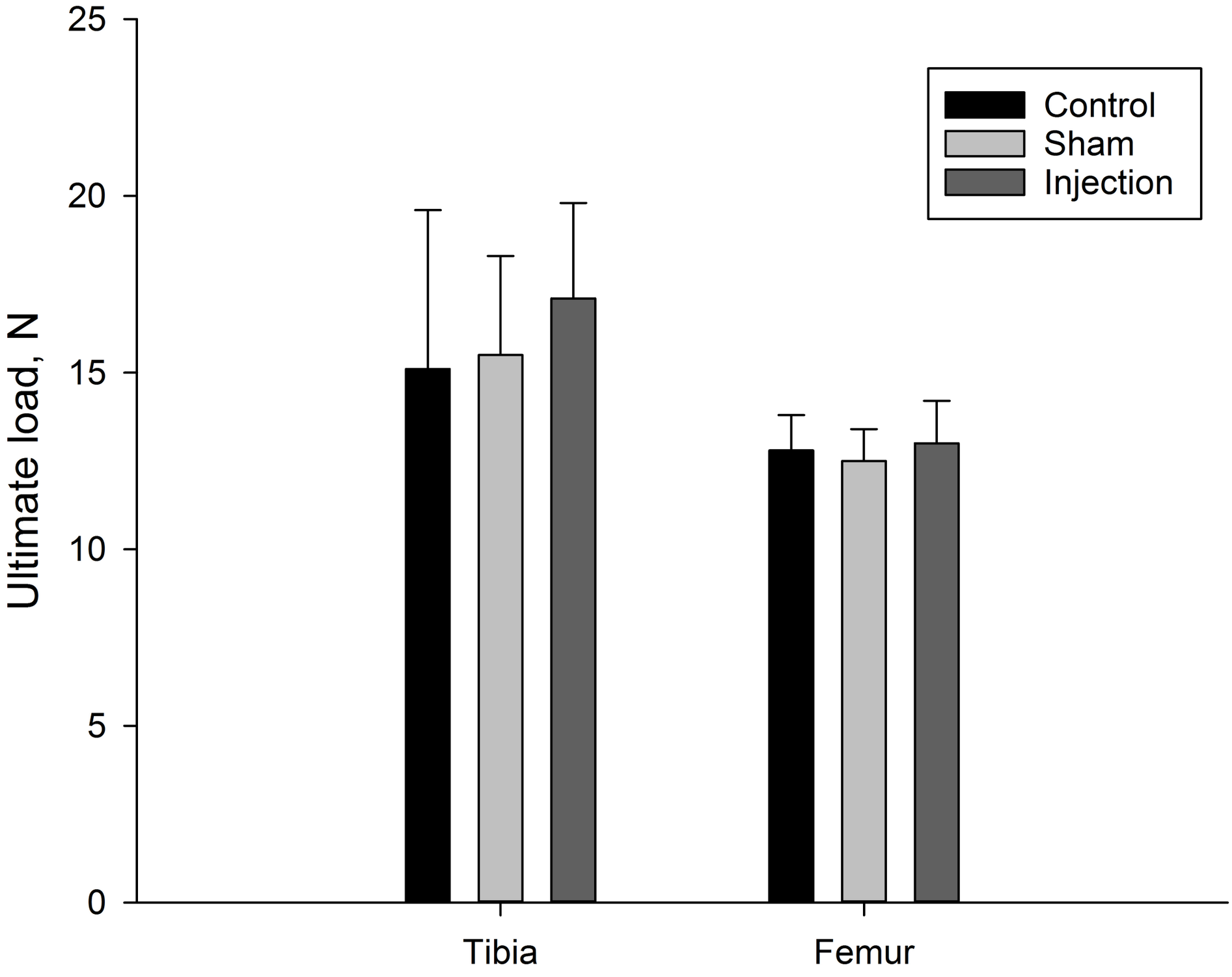

Lateral
Medial
Posterior
Control
Sham
Injection

Figure 3


Ultimate load, N
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20
15
10
5
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Control
Sham
Injection
Tibia
Femur