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Systemic Delivery of NELL-1 through Intraperitoneal Route for the Reversal of Osteoporosis in OVX-Mice

A thesis submitted in partial satisfaction

of the requirements for the degree Master of Science

in Oral Biology

by

Abdulaziz A. H. Mohammad

2017

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2017

ABSTRACT OF THE THESIS

Systemic Delivery of NELL-1 through Intraperitoneal Route for the Reversal of Osteoporosis in OVX-Mice

by

Abdulaziz A. H. Mohammad

Master of Science in Oral Biology

University of California, Los Angeles, 2017

Professor Kang Ting, Chair

Bone health can be jeopardized by diseases such as osteoporosis, or by special environmental conditions like microgravity. Both conditions result in reduced bone mineral density (BMD) and an increased risk of bone fractures. NELL-1, a protein that binds integrin β1 and consequently induces Wnt/β-catenin, exhibits both bone-forming and anti-osteoclastic effects. Systemic delivery of NELL-1 via intravenous (IV) injections increases not only murine BMD and percent bone volume, but also new femoral bone formation. However, systemic delivery of NELL-1 under microgravity conditions in future spaceflight experimental studies would be more practical if it were accomplished through the intraperitoneal (IP) route. It is less technique sensitive, safer, and more patient friendly for eventual use by humans. The aim of this study was to experiment the

intraperitoneal delivery of NELL-1 to reverse osteoporotic bone loss in mice under normal gravity conditions. First, a post-menopausal osteoporotic model (OVX) was successfully reproduced in BALB/c mice and a significant reduction in BMD was evident by DXA and microCT analyses. Second, two IP dosages of NELL-1 were injected into the OVX mice, resulting in reversed bone loss and positive gains in BMD and BV/TV. In conclusion, NELL-1 can be effectively delivered by systemic intraperitoneal injections to reverse osteoporotic bone loss and regulate bone homeostasis.

The dissertation of Abdulaziz A. H. Mohammad is approved.

Tara Aghaloo

Xinli Zhang

Kang Ting, committee chair

University of California, Los Angeles

2017

Dedication

This thesis is dedicated to my family, especially my mother who is the main person contributing to my education

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Acknowledgements

I would like to thank my PI, Dr. Ting, for giving me this research opportunity at his lab and Dr. Zhang for his direct and thorough mentorship of my project.

I would like also to thank all members of Dr. Ting's lab for their support of and contribution to my project.

Introduction

Bone health is considered essential for daily physical activity, general body protection, and overall quality of life. Certain diseases and environmental conditions can affect the integrity of the skeletal structures. This commonly results in bone fracture, a skeletal manifestation that is costly to treat and quite debilitating to daily life.

Osteoporosis, a disease characterized by low bone mass and micro-architectural deterioration of bone tissue, can lead to enhanced bone fragility and a consequent increase in fracture risk [1]. Specifically, the World Health Organization defines osteoporosis as a bone mineral density (BMD) that lies 2.5 standard deviations or more below the average value for young healthy women [2]. It is estimated that 1.5 million individuals suffer an osteoporotic-related fracture every year in the United States [3]. Direct treatment of these fractures is estimated to cost between $12-18 billion annually in 2002 dollars, and indirect cost (e.g. productivity loss) would easily add to this figure [3]. Temporary treatment of osteoporosis consists of either anabolic or anti-resorptive agents. Parathyroid hormone is the only anabolic drug currently approved by the Food and Drug Administration (FDA) with a two-year limit of treatment due to risk of osteosarcoma. Ideal future therapeutic agents should have pro-osteoblastic and anti-osteoclastic elements with minimal side effects. Recently, the induction of the Wnt/ß-catenin signaling pathway or the inhibition of its antagonists have shown promise in treating bone metabolic disorders [4].

Microgravity-induced bone loss constitutes another critical medical condition where bone health is jeopardized. In fact, astronauts on long duration space flights suffer from an areal bone mineral density (aBMD) loss of 1-1.5% per month [5, 6]. Interestingly, microgravity-induced osteoporosis differs from osteoporosis on Earth in

three key aspects. Specifically, the microgravity environment alters cellular cytoskeleton, inhibits the proliferation and differentiation of mesenchymal stems cells into osteoblasts, and may directly induce osteoblast apoptosis [7]. The inflight exercise program planned to overcome microgravity-induced osteoporosis has been deemed insufficient, thus warranting future modalities to combat such bone loss [5].

NELL-1 (NEL-like molecule-1) is a pro-osteogenic protein that demonstrates both local bone-forming effects[8, 9] and anti-osteoclastic effects (**Fig. 1**) [10]. It has been associated with osteoporosis as a gene with specific polymorphisms in patients with reduced BMD [11]. Regarding its mechanism of action, NELL-1 has been shown to bind integrin β1 and consequently induce the Wnt/β-catenin signaling pathway [12]. Therapeutically, systemic delivery of NELL-1 via intravenous (IV) injections has been shown to increase not only murine femoral and lumbar BMD and percent bone volume, but also new femoral bone formation [13].

Cases with post-menopausal osteoporotic bone loss under a microgravity environment can be used as an extreme model of bone loss to study potential osteogenic therapeutics such as NELL-1. To date, systemic delivery of NELL-1 has been accomplished through IV routes and enhanced with PEGylation technology [13]. This technology almost tripled the elimination half-life time and distribution of the protein to bone tissues when compared to naked NELL-1 [13]. However, the investigation of NELL-1's effects under microgravity conditions would be more practical and feasible if protein is delivered through intra-peritoneal (IP) injection – a safer and more patient-friendly administration route. Astronauts prefer to use IP injections, delivered every two weeks in the space instead of weekly IV injections. In this study, we hypothesize that

NELL-1 is a promising systemic therapeutic agent for the treatment of osteoporosis with the ability to reverse osteoporotic bone loss and the ability to regulate bone homeostasis when administered via the intraperitoneal (IP) route. The IP delivery of chemically modified NELL-1 via PEGylation will be studied to reverse osteoporotic bone loss in mice under normal gravity conditions on Earth. The long-term objective of this study is to use IP-delivered NELL-1 for the reversal and perhaps the prevention of microgravity-induced osteoporosis in future spaceflights.

Materials and Methods

Animals

Three-month old, female BALB/c mice were obtained from Charles Rivers Laboratories (Wilmington, MA). All animals were handled under the supervision of the Division of Laboratory Animal Medicine (DLAM) at the University of California, Los Angeles in accordance with institutional guidelines of the Chancellor's Animal Research Committee (ARC) of the Office of Animal Research Oversite (OARO).

Preparation of NELL-PEG injections

NELL-PEG was prepared using linear PEG 5k (Sigma-Aldrich, USA) as previously described [14].

1. *Reproducing a post-menopausal osteoporotic model by use of ovariectomy (OVX)*

To reproduce a successful osteoporotic model that can be used to examine the effects of NELL-1, twenty female mice were divided into two groups: sham and ovariectomized (OVX) animals (**Fig. 2A**). After receiving OVX surgery or sham surgery, the mice underwent bi-weekly dual-energy X-ray absorptiometry (DXA) analysis for

thirteen weeks to observe bone loss in terms of changes in bone mineral density (BMD). *Ex vivo* micro-computed tomography (microCT) scans and histological analyses were carried out after sacrifice of animals on week 13.

1.1. In vivo assessment of BMD by dual-energy X-ray absorptiometry

To monitor changes in BMD, dual-energy X-ray absorptiometry (DXA; PIXImus2; GE Lunar Corp.) was used. Longitudinal assessment of the whole body (excluding the head), distal femur, proximal tibia, and lumbar vertebrae BMD (g/cm2) was performed every 2 weeks.

1.2. Ex vivo assessment of bone architecture by micro-CT

After euthanasia, mice skeletons were fixed in 4% paraformaldehyde (PFA) for 24 hours and stored in 70% ethanol at 4°C. Femurs, tibias, and vertebrae were scanned with SkyScan 1172 (Bruker microCT, Belgium). Regions of interests included the distal femur (2 mm from the tip of the growth plate), the proximal tibia (2 mm from the tip of the growth plate), and the lumbar vertebrae (coronal cross-sectional images with a 0.25-mm width and located 0.3mm from the growth plate, excluding the cortical bone) (**Fig. 3**). Assessments of bone volume fraction (BV/TV, %), trabecular thickness (Tb. Th, mm), trabecular number (Tb. N, mm-1), and trabecular separation (Tb. Sp, mm) were performed and analyzed using CTAn software (Bruker microCT, Belgium).

1.3. Histochemical and immunohistochemical analyses

The right femurs and tibias of all samples were fixed in 4% paraformaldehyde (PFA) for 24 hours, decalcified in 19% EDTA for 14

days, and then embedded in paraffin. Thin sections five-micron in diameter were prepared and stained with hematoxylin and eosin (H&E), Masson's Trichrome, and TRAP staining (Sigma-Aldrich, St. Louis, MO). Immunohistochemistry was carried out using anti-osteocalcin antibody (Santa Cruz Biotechnology, (FL-95): sc-30045) as a primary antibody and 3-amino-9-ethylcarbazole (AEC) as a chromogen. Olympus BX51 microscope (Olympus Corp., Waltham, MA) and cellSens software version 1.6 (Olympus Corp., Waltham, MA) were used to visualize and analyze results. Parameters of osteocalcin+bone-lining cells per bone perimeter (OCN+cells/Bpm, mm1) and TRAP+bone-lining cells per bone perimeter (TRAP+cells/Bpm, mm1) were used as previously reported [12].

2. *Effect of IP NELL-1 in reversing osteoporotic bone loss in OVX mice*

To test the effects of IP-injected NELL-1 in reversing bone loss, twelve mice were ovariectomized under settings identical to the first experiment. The mice were then followed up with bi-weekly DXA analysis and three *in vivo* microCT scans to record changes in BMD post-surgery and subsequent treatment (**Fig. 2A & 2B**). NELL-1 injections were delivered intraperitoneally at two dosages, 5mg/Kg and 10 mg/kg, on weeks five, seven, nine, and eleven. Mice were euthanized at week 13, and CFU and histological analyses were carried out post-mortem.

2.1. *In vivo assessment of BMD by dual-energy X-ray absorptiometry*

2.2. *In vivo assessment of bone architecture by microCT*

Animals were scanned with SkyScan 1176 (Bruker microCT, Belgium) three times: at week 0, 5, and 13. Regions of interests included

the distal femur (2 mm from the tip of the growth plate), the proximal tibia (2 mm from the tip of growth plate), and the lumbar vertebrae (coronal cross-sectional images with a 0.25-mm width and located 0.3mm from the growth plate, excluding the cortical bone). Assessment of bone volume fraction (BV/TV, %), and BMD were performed and analyzed using CTAn software (Bruker microCT, Belgium).

2.3. *CFU, histochemical, and immunohistochemical analyses*

To examine bone marrow mesenchymal stem cell (BMSC) content, the colony-forming unit-fibroblast (CFU-F) assay was carried out. Freshly harvested left humeri were immediately used to isolate BMSCs by marrow flushing. Isolated marrow cells were seeded on 6-well plates (1x106 cells/well) and cultured for 10 days in Complete MesenCult Medium (STEMCELL Technologies, Inc., Canada) at 37°C in 5% CO2. CFU-F derived colonies were stained using Giemsa Staining Solution (EMD Chemicals, Inc., NJ) and Alkaline phosphatase (Sigma Aldrich) for 5 minutes and counted microscopically.

Right femurs and tibias were used for histological purposes as described in 1.3..

Statistical analysis

Means and standard deviations were calculated for the results. The Student's t-test was employed for two group comparisons at 95% confidence intervals (*$P<0.05$ and **$P<0.01$).

Results

1. *A post-menopausal osteoporotic model was successfully reproduced by use of ovariectomy (OVX)*

1.1. Reduction of BMD in OVX group (measured by dual-energy X-ray absorptiometry)

Following ovariectomy surgery on week 0, OVX mice exhibited a gradual decrease in BMD while sham animals experienced a gradual increase in bone density (**Fig. 4**). This discrepancy was statistically significant after two weeks. On week 4, sham mice demonstrated 11% increase in BMD and the OVX mice lost 3% of the original BMD ($P<0.01$). Both groups maintained this difference following week four until euthanasia at week thirteen. Similar differences were found in the lumbar vertebrae BMD between the two groups (not reported).

1.2. Generalized deterioration of skeletal parameters in OVX mice (analyzed by micro-CT)

Post-mortem microCT confirmed the DXA findings of reduced bone density. BMD was statistically reduced in the femurs, tibias, and lumbar vertebrae of OVX animals when compared to the control. Femoral and tibial trabecular separation of OVX mice were increased while BV/TV, TB.Th, and Tb.N were reduced, indicating a general deterioration of trabecular parameters (**Fig. 5**). The lumbar vertebrae exhibited similar bone loss reflected in BV/TV, Tb.N, and Tb. Sp parameters (**Fig. 6**).

1.3. Histochemical and immunohistochemical analyses

Histological staining with H&E and Masson's Trichrome showed reduced trabecular formation in the tibia of ovariectomized animals compared to their sham counterparts. OVX group demonstrated significantly more TRAP positive and less osteocalcein positive cells on trabecular bone perimeter than sham group ($P<0.001$) (**Fig. 7**).

2. *IP NELL-1 reversed osteoporotic bone loss in OVX mice*

   2.1. *Enhancement of BMD (analyzed by dual-energy X-ray absorptiometry)*

The lumbar bone mineral density of OVX mice was reduced during the first four weeks following surgery (results not shown). After the onset of NELL-1 treatment beginning on week 5, however, the BMD started to rise again and the percent change in BMD almost equaled that of the sham animals (**Fig. 8**). Prior to harvest, both treatment groups, 5 and 10 mg/kg NELL-1, exhibited a positive percent BMD change greater than that of their sham counterparts.

   2.2. *Concomitant restoration of skeletal parameters with NELL-1 treatments (measured by In vivo microCT)*

In vivo microCT results revealed the same pattern seen in the DXA analysis. Lumbar absolute BMD values (**Fig. 9A**) and percent change BMD (**Fig. 9B**) decreased during the first four weeks and started to significantly increase after NELL-1 treatment began on week 5, reaching values higher than the initial pre-surgical levels. In addition, lumbar BV/TV followed the same pattern under different threshold analyses (**Fig. 9C**).

   2.3. *CFU, histochemical, and immunohistochemical analyses*

Compared to the 5 mg/kg treatment group, the number of BMSCs stained with Giemsa staining was increased in the 10 mg/Kg NELL-1 group, with and without osteoblast induction (**Fig. 10**). Even within the group itself, OB induction caused a significant increase in BMSC numbers. Similar results were found when the cells were stained with Alkaline Phosphatase (ALP) (**Fig. 11**). Taken together, these findings suggest that IP treatment with 10 mg/Kg NELL-1 resulted in greater proliferation of BMSCs than IP treatment with 5 mg/Kg NELL-1.

Moreover, histological staining with H&E and Masson's Trichrome showed increased trabecular bone formation in the tibia of 10 mg/kg NELL-1 treatment animals compared to 5 mg/kg NELL-1 treatment animals. The mice treated with 10mg/kg NELL-1 demonstrated significantly less TRAP positive cells lining trabecular bone than the 5mg/Kg group. (**Fig. 12**).

Discussion

Bone health can be jeopardized by diseases such as osteoporosis, or by special environmental conditions like microgravity. Both conditions result in reduced BMD and an increased risk of bone fractures. Measures to counteract or possibly prevent this deterioration in bone density are currently being thoroughly investigated. Agents that induce Wnt/ß-catenin signaling or inhibit its antagonists demonstrate optimistic results in treating metabolic bone disorders [4]. NELL-1, a protein that binds integrin β1 and consequently induces the Wnt/β-catenin signaling pathway, exhibits both bone-forming and anti-osteoclastic effects. In fact, a recent genome-wide association study identified

NELL-1 polymorphisms in patients with reduced bone mineral density, suggesting that NELL-1 gene polymorphisms are associated with osteoporosis [11].

Regarding the potential route of administration of NELL-1, systemic delivery under microgravity conditions would be more practical if accomplished through the intraperitoneal route. This is due to the fact that systemic IP injections are less technique sensitive, safer, and more patient friendly for eventual use by human patients when compared to systemic IV injections. Furthermore, intravenous injections include risks of inflammation, thrombophlebitis of the vein, and necrosis of the surrounding tissues. Compared to the IV route, the intraperitoneal method also allows for a greater volume of injection that serves as a slow release and long-acting deposit of the drug [15]. Although the IV method offers the most rapid distribution of the drug, the IP route has been shown to result in longer retention of the drug with a longer mean residence time than that of the route IV[16]. Importantly, this aspect is critical in treating osteoporosis since bone is a peripheral tissue with limited blood supply, making it difficult to deliver medications to bone tissues [17]. Therefore, the goal would be to maintain a sustained release of the medication at targeted bone tissues for prolonged durations with minimal systemic side effects. Part of this goal was achieved with PEGylation technology which enhanced the half-life time of NELL-1 when administered intravenously [13]. In the present study, the objective was to evaluate the IP systemic delivery as a potential route for delivery of the PEGylated NELL-1 formulation.

The first aim was to reproduce a post-menopausal osteoporotic model in BALB/c mice. Several studies have shown that there is variation in the magnitude of responses of both the cancellous and cortical bones to ovariectomy in mice. This variation in

response is strain-dependent and varies with the skeletal site [18-20]. Our results confirmed that the BALB/c strain can be used as a post-menopausal osteoporotic model. After OVX, bone mineral density was significantly reduced at the distal femurs, proximal tibiae, and lumbar vertebrae. Other parameters reflective of gonadal hormone deficiency-related bone loss were also significantly affected (decreased BV/TV, TB.Th, & Tb.N, and increased Tb.Sp) and showed more consistency than those in similarly-designed studies of other strains like 129P3, C57BL/6, and B6129PF2 [20].

The second aim of this study was to experiment the IP delivery of NELL-1 to reverse osteoporotic bone loss. DXA analysis showed that IP treatment with NELL-1 in OVX animals successfully reversed bone loss with positive gains in BMD, exceeding that of the sham animals. Lumbar BMD, as measured by *in vivo* microCT, exhibited a pattern of severe reduction in the first four weeks after ovariectomy. Following NELL-1 treatment, BMD increased significantly and exceeded the pre-surgical levels. A similar pattern was observed in the analysis of lumbar BV/TV. In this study, IP NELL-1 was administered at two dosages: 5 and 10 mg/kg. These dosages were higher than the 2.5 mg/kg NELL-1 that was delivered intraperitoneally in another study [15]. However, Tanjaya et al. used a sham model and a weekly IP injection of the protein while in this study an OVX model and a bi-weekly administration were used instead. Thus, a higher dosage was required to overcome the osteoporotic bone loss and a less frequent injection schedule. The two-week interval was chosen because astronauts in spaceflights find weekly injections impractical and inconvenient. Interestingly, the IP delivery of NELL-1 resulted in BMD and BV/TV gains comparable to those obtained with IV delivery of the protein in another study [13]. Both NELL-1 IP dosages, 5 & 10 mg/kg,

resulted in bone loss reversal analyzed by DXA and *in vivo* microCT. Moreover, CFU-F analyses showed that IP NELL-1 treatment was capable of inducing BMSC proliferation, with IP treatment of 10 mg/Kg NELL-1 resulting in greater MBSC proliferation than 5 mg/Kg NELL-1 treatment. Histological staining with H&E and Masson's trichrome further demonstrated the enhancement in the number and distribution of trabecular bone in both treatment groups when compared to OVX and sham controls.

Conclusion

In the present study, we showed that NELL-1 can be effectively delivered by systemic intraperitoneal injections to reverse osteoporotic bone loss. The tested dosages of 5 mg/kg and 10 mg/kg NELL-1 were found to be sufficient in producing statistically significant improvements in various skeletal parameters. In fact, the mice treated with 10mg/kg NELL-1 demonstrated slightly increased trabecular bone formation than the 5mg/Kg group. The intraperitoneal route of administration can be unquestionably used to administer NELL-1 under microgravity conditions in future spaceflight. It should be noted that this study investigated the dosages of NELL-1 necessary to reverse ovariectomy-induced bone loss under a normal gravity environment. Therefore, future studies may examine higher dosages to reverse microgravity-induced bone loss, especially if the combined microgravity-OVX mouse model is to be used.

FIGURES AND TABLES

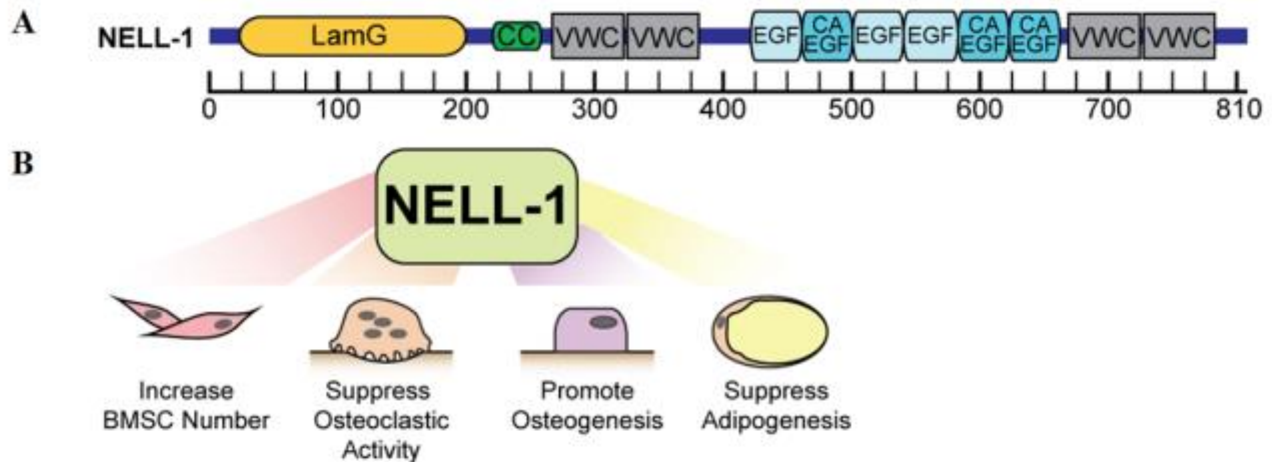


Figure 1. Structure and function of NELL-1. Distinct domains within the structure of NELL-1 include LamG: Laminin G domain, VWC: Von Willebrand type C domain, CC: Coiledcoil regions, and CA EGF: Calcium binding EGF-like domains (A). Known functions of NELL-1 to date (B). (James et al., 2013; Zhang et al., 2011b; James et al., 2012; James et al., 2011).

A

		Number of Animals	Treatment
Aim 1.	Sham	10	none
	OVX (ovarectomized)	10	none
Aim 2.	OVX + 5 mg/Kg NELL-1 IP	6	4 injections
	OVX + 10 mg/Kg NELL-1 IP	6	4 injections

B

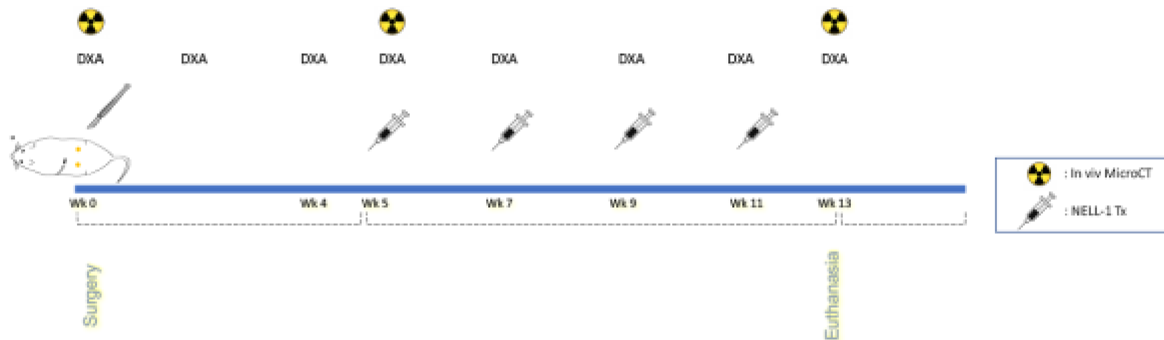


Figure 2. Experiment Aims and Design. Animal distribution among experimental Groups with NELL-1 dosages (A) and Aim 2 experimental design (B).

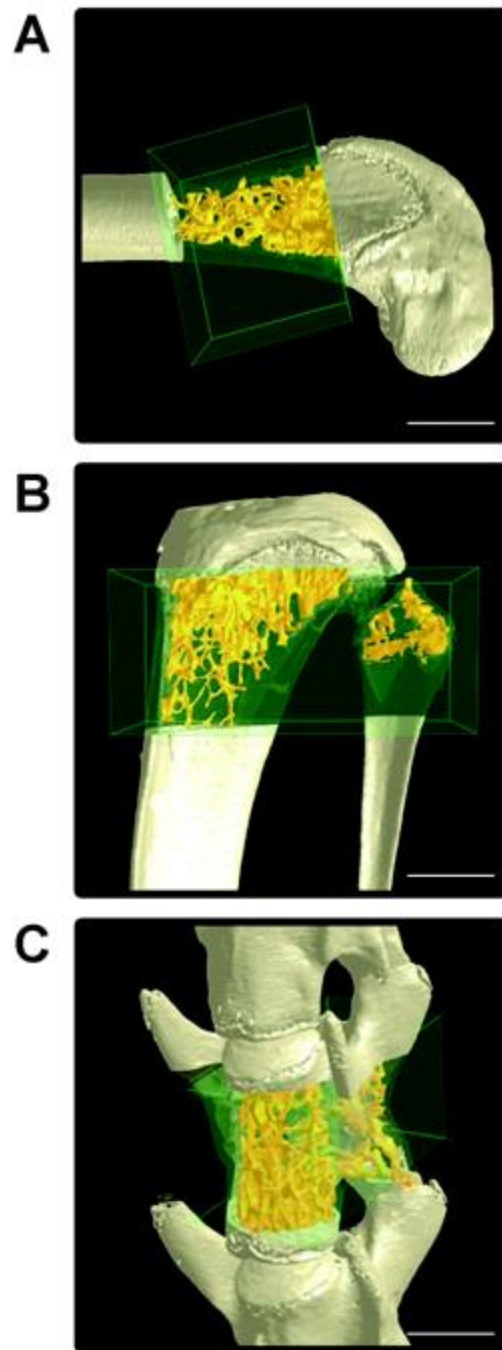


Figure 3. ROI for microCT analysis. ROI in micro-CT were the distal femur (A), proximal tibia (B), and lumbar vertebrae (C). (Shi et al., 2016)

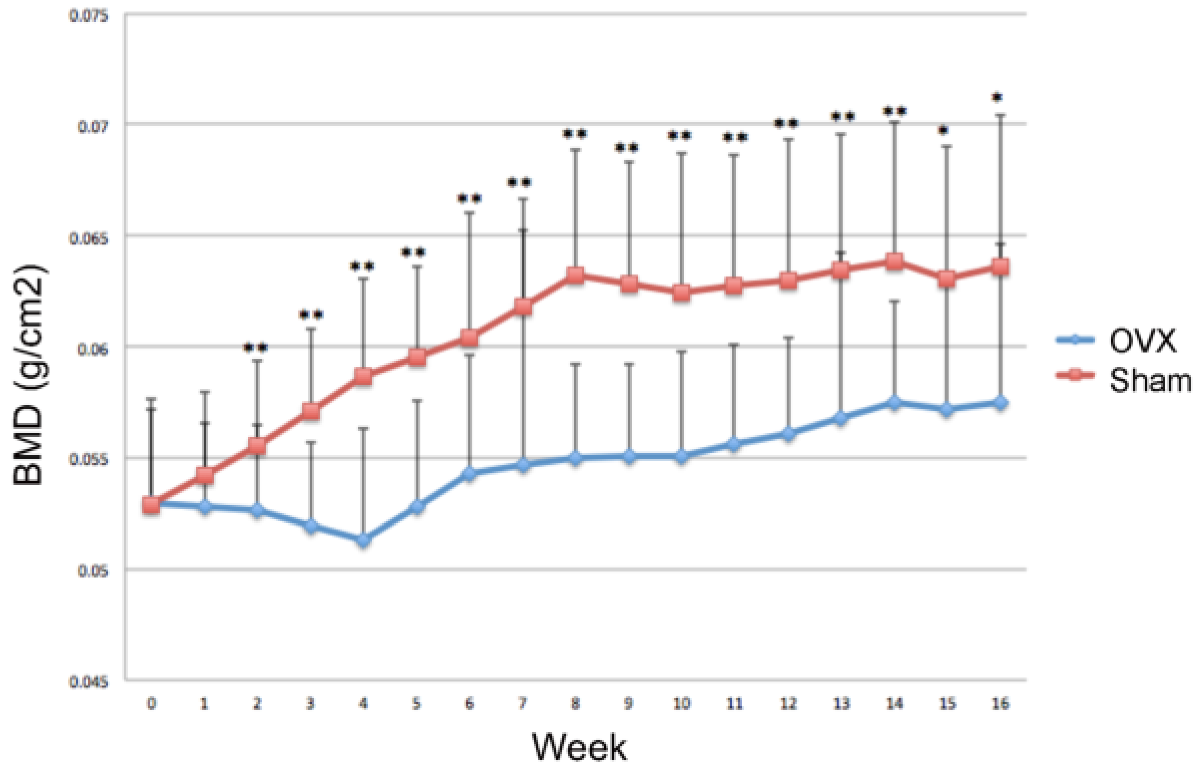


Figure 4. Femur BMD in sham and OVX groups. Distal Femur BMD measurements throughtout the whole experiment (ovariectomy surgery was done in week 0). *p<0.05, **p<0.01

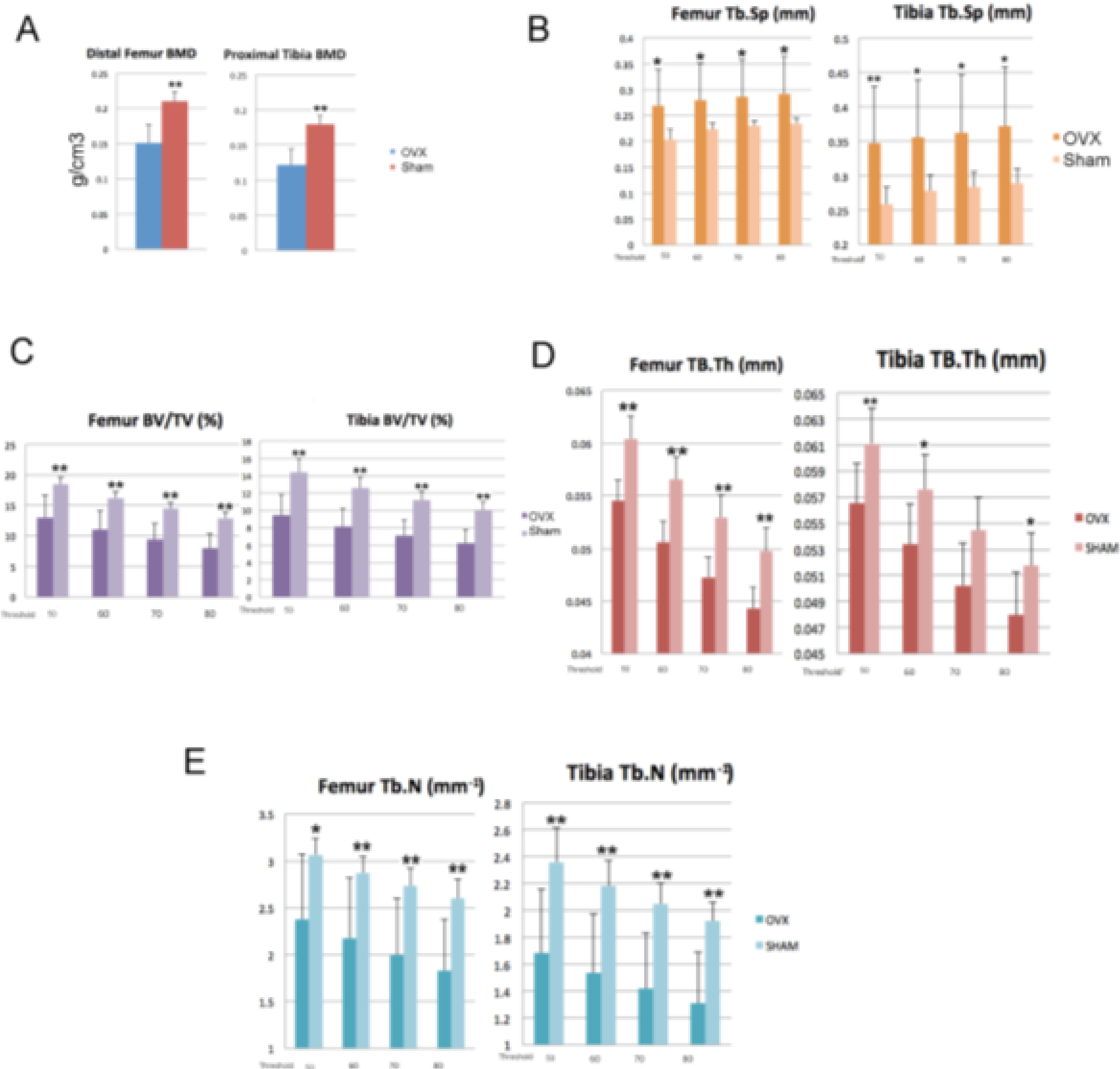


Figure 5. Femoral and Tibial Ex vivo microCT of sham and OVX animals.

Ovariectomized mice showed significant reduction in BMD (A), increase in Tb.Sp (B), and drop in other parameters: BV/TV, TB.Th, and Tb.N (C-E) compared to control. $^*p<0.05$, $^{**}p<0.01$

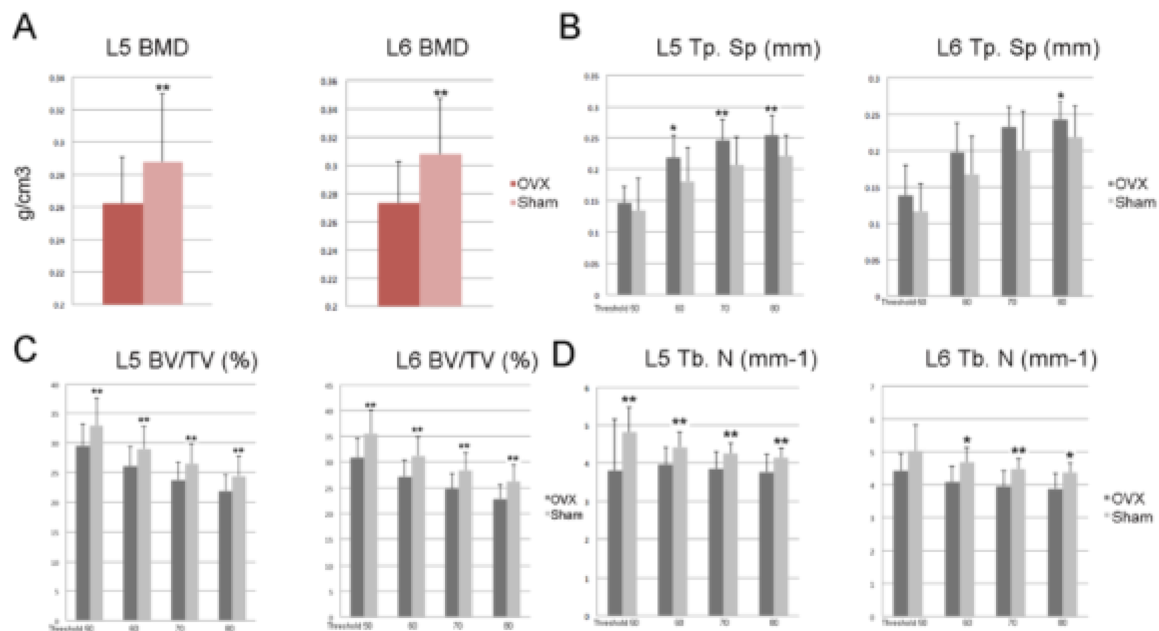
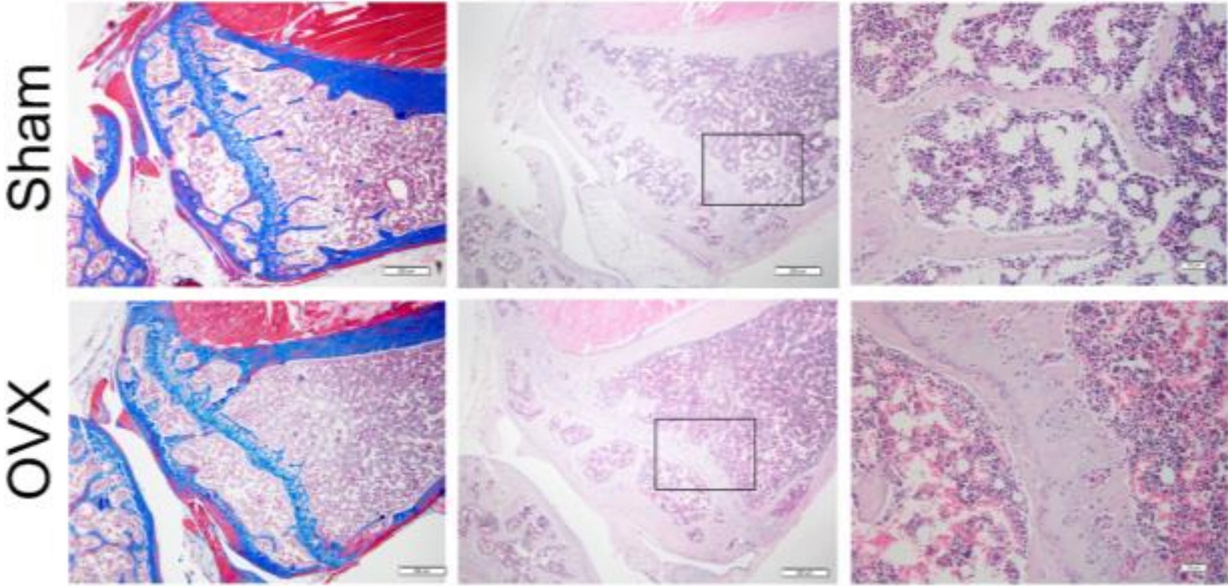


Figure 6. Lumbar Ex vivo microCT of sham and OVX animals. Ovariectomized mice showed significant reduction in BMD (A), increase in Tb.Sp (B), and drop in other parameters: BV/TV and Tb.N (C & D) compared to control. *p<0.05, **p<0.01


Trichrome (40X)
H & E (40X)
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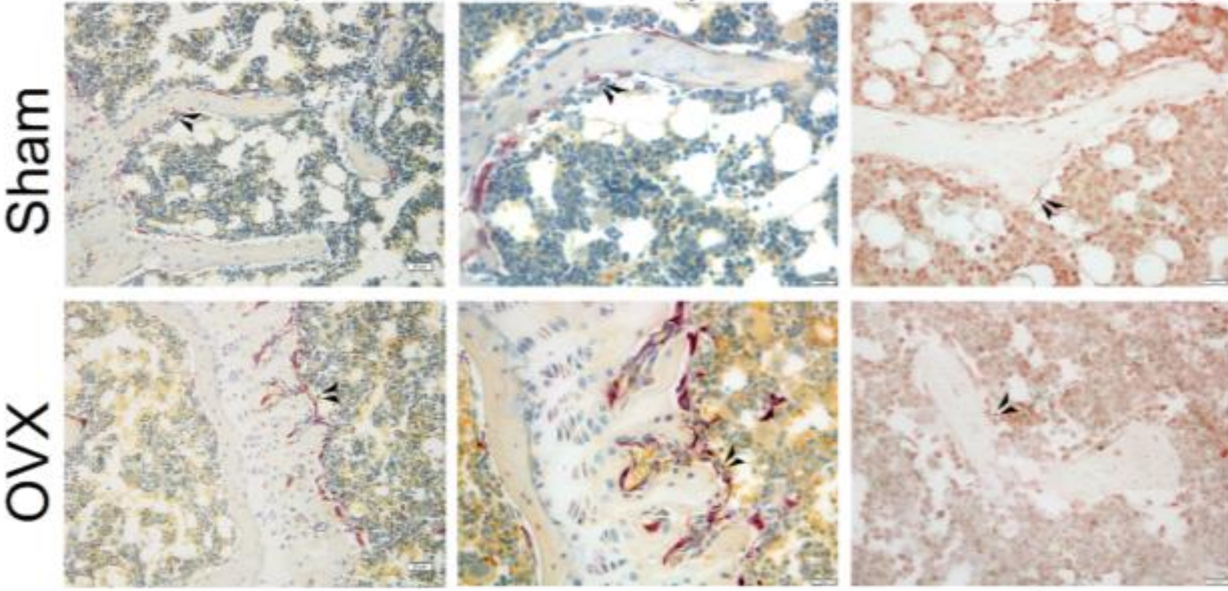
Sham
OVX
A
B
C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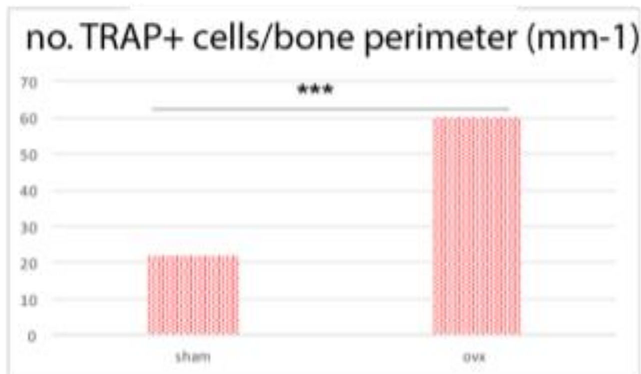
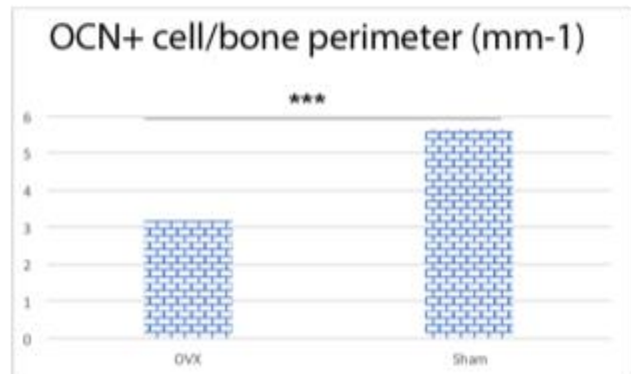
TRAP (400x)
OCN (400x)
Sham
OVX
D
E
F





G H

Figure 7. Histological staining of Sham and OVX groups. Trichrome staining (A) and H&E staining (B&C) exhibited less trabecular bone formation at the proximal tibial metaphysis in the OVX group than in the sham group. TRAP staining exhibited an increase in TRAP positive cells in the OVX mice compared to the sham control (D-G). Immunostaining exhibited a less number of osteocalcin (OCN) positive cells with a weaker staining in the OVX group compared to the sham group (F&H). *p<0.05, **p<0.01, *** p<0.001

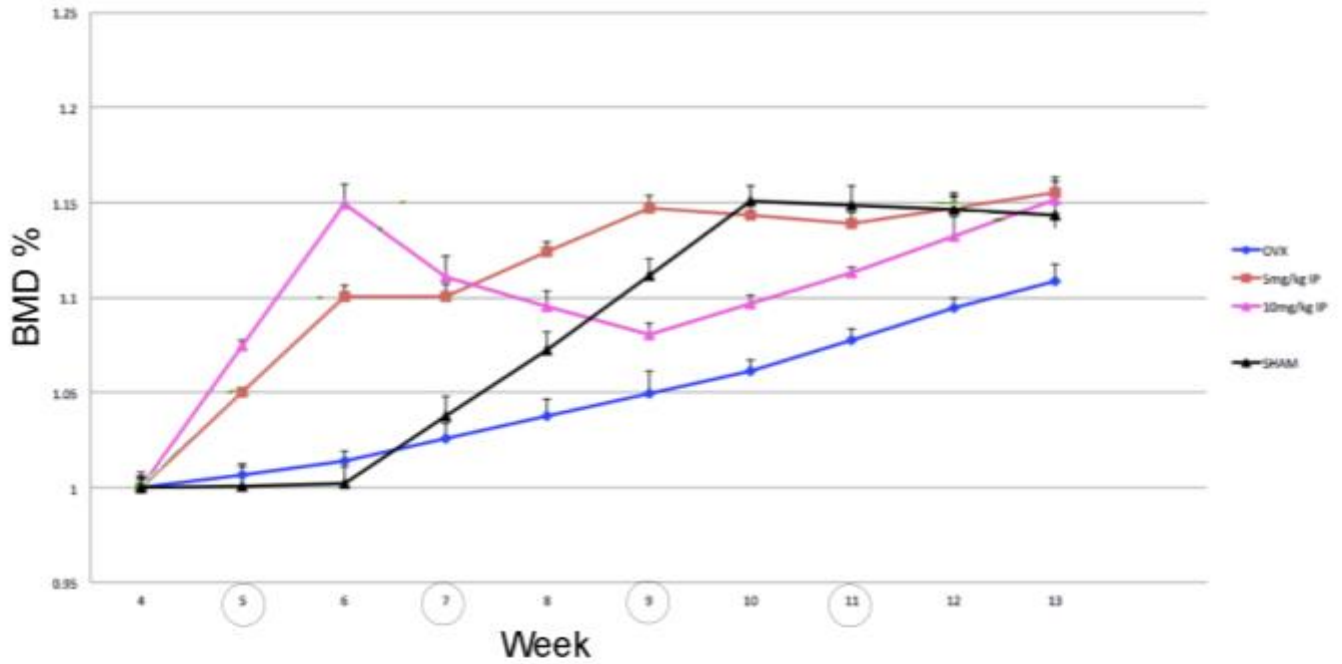


Figure 8. Lumbar BMD in sham,OVX, and OVX-Treatment groups. Comparison of lumbar BMD percent change among groups: sham, OVX, OVX-5 mg/Kg NELL-1, and OVX-10 mg/Kg NELL-1.

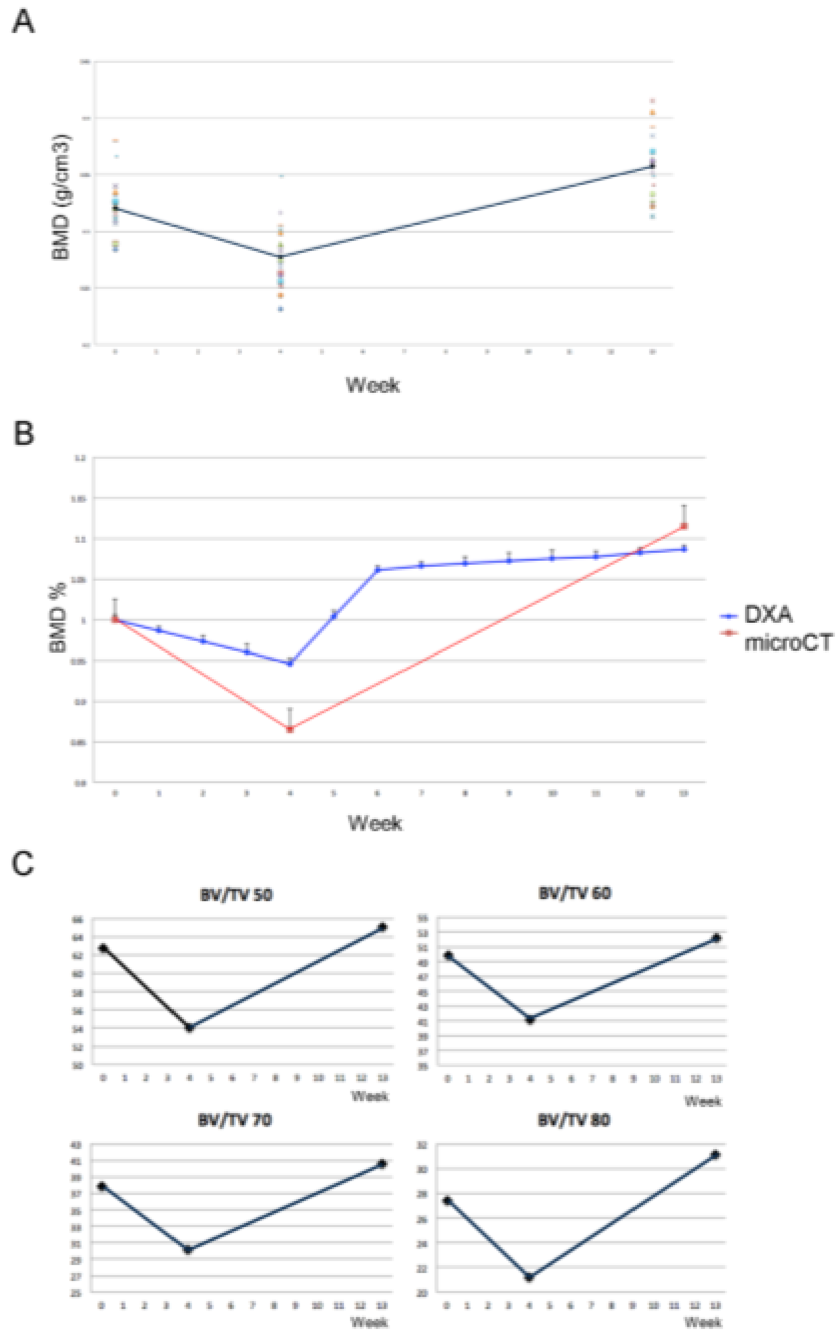


Figure 9. Longitudinal In vivo microCT of Lumbar Vertebrae (L5/6) of Mice treated with 5 & 10 mg/Kg Nell-1. Absoulte measurements of lumbar BMD of L5-L6 of all animals (A), lumbar BMD percent change measured by DXA and In vivo microCT (B), and lumbar BV/TV records under different thresholds (C).

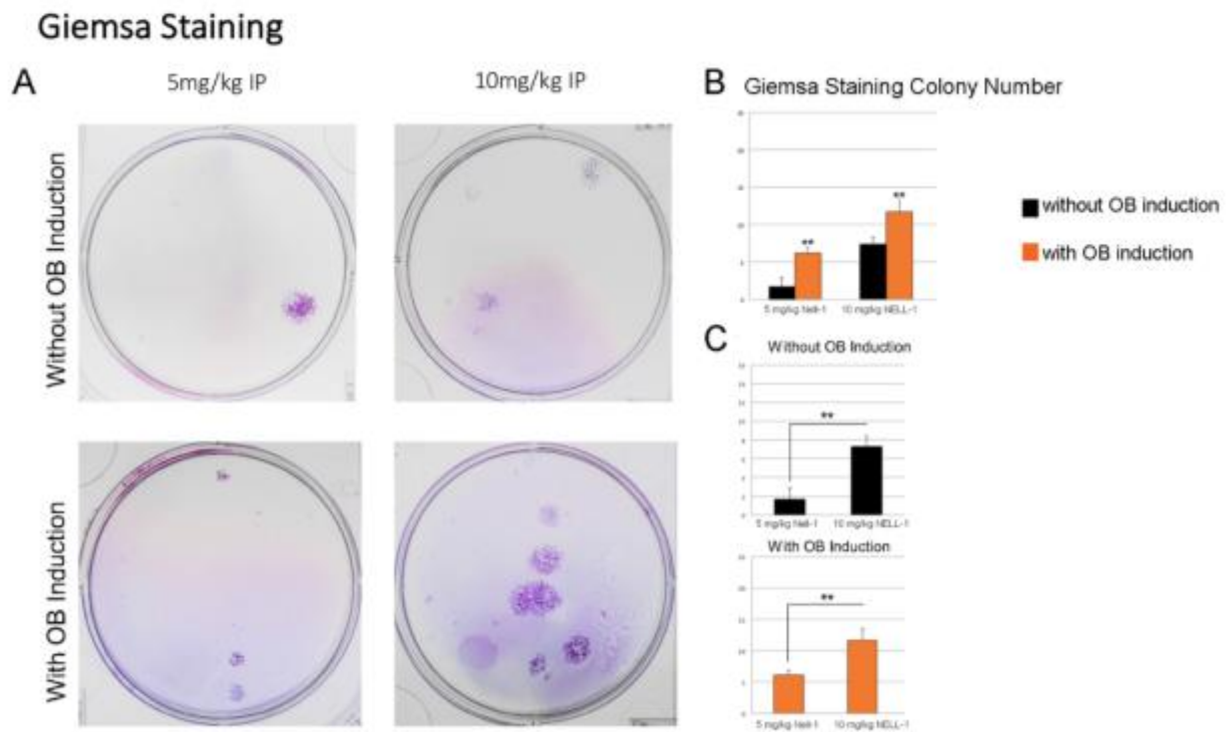


Figure 10. CFU-F assay Of Treatment Groups 5 and 10 mg/Kg NELL-1. CFU-F-derived colonies were stained using Giemsa staining (A) and quantification of colony number microscopically (B&C). *p<0.05, **p<0.01

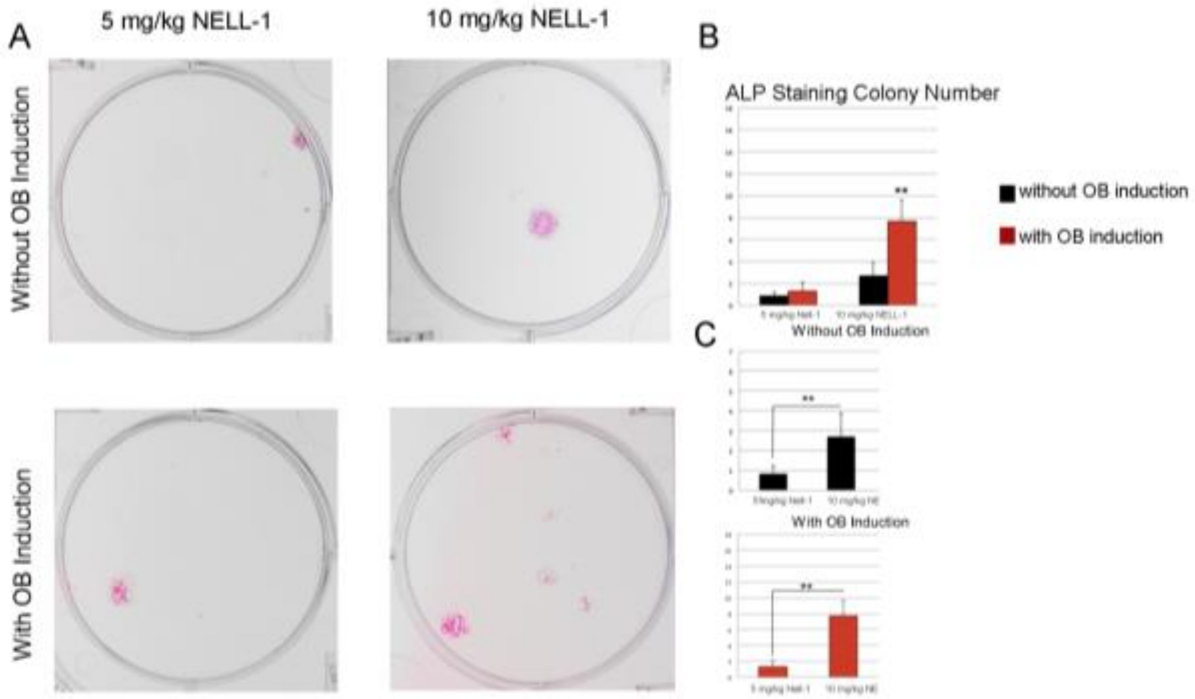
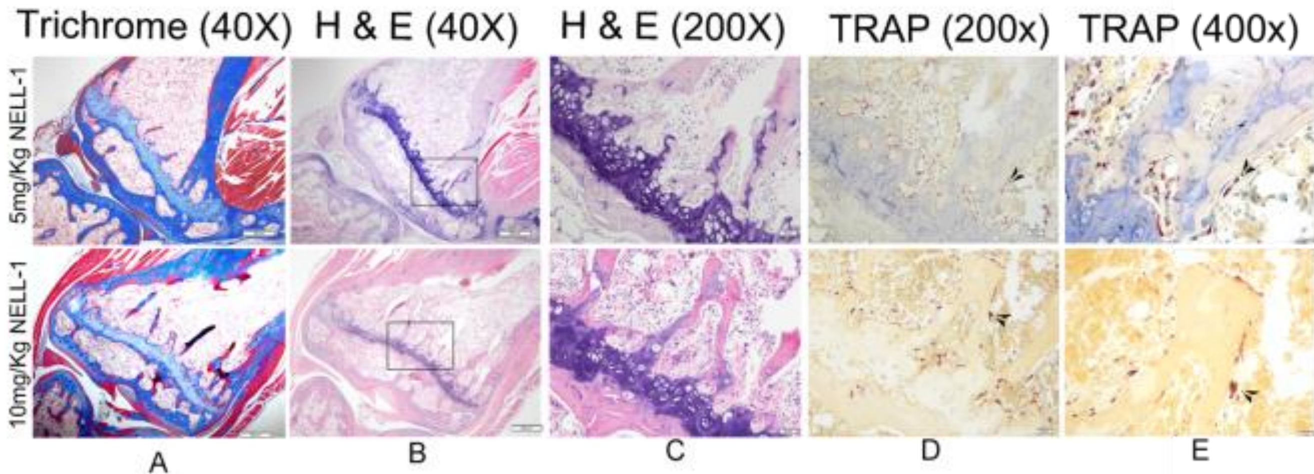


Figure 11. CFU-F assay Of Treatment Groups 5 and 10 mg/Kg NELL-1. CFU-F-derived colonies were stained using Alkaline Phosphatase (A) and quantification of colony number microscopically (B&C). *p<0.05, **p<0.01



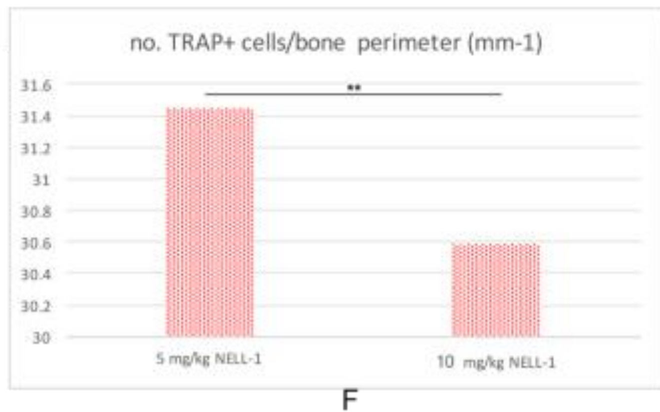


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Fig 12. Histological staining of 5mg/kg NELL-1 and 10 mg/kg NELL-1 groups. Trichrome staining (A) and H&E staining (B&C) exhibited greater trabecular bone formation at the proximal tibial metaphysis of the 10 mg/kg NELL-1 group compared to the other group. TRAP staining exhibited a reduction in TRAP positive cells in the 10 mg/kg NELL-1 group compared to the 5 mg/kg NELL-1 group (D-F). *p<0.05, **p<0.01, *** p<0.001

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