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Administration of low intensity vibration and a RANKL inhibitor, alone or in combination, reduces bone loss after spinal cord injury-induced immobilization in rats

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ABSTRACT

We previously reported an ability of low-intensity vibration (LIV) to improve selected biomarkers of bone turnover and gene expression and reduce osteoclastogenesis but lacking of evident bone accrual. In this study, we demonstrate that a prolonged course of LIV that initiated at 2 weeks post-injury and continued for 8 weeks can protect against bone loss after SCI in rats. LIV stimulates bone formation and improves osteoblast differentiation potential of bone marrow stromal stem cells while inhibiting osteoclast differentiation potential of marrow hematopoietic progenitors to reduce bone resorption. We further demonstrate that the combination of LIV and RANKL antibody reduces SCI-related bone loss more than each intervention alone. Our findings that LIV is efficacious in maintaining sublesional bone mass suggests that such physical-based intervention approach would be a noninvasive, simple, inexpensive and practical intervention to treat bone loss after SCI. Because the combined administration of LIV and RANKL inhibition better preserved sublesional bone after SCI than either intervention alone, this work provides the impetus for the development of future clinical protocols based on the potential greater therapeutic efficacy of combining non-pharmacological (e.g., LIV) and pharmacological (e.g., RANKL inhibitor or other agents) approaches to treat osteoporosis after SCI or other conditions associated with severe immobilization.

1. Introduction

Immobilization-induced bone loss occurs in many neurological conditions including stroke, spinal cord injury (SCI), multiple sclerosis, and amyotrophic lateral sclerosis. Approximately 305,000 individuals are living with SCI in the United States in 2023 (*National Spinal Cord Injury Statistical Center.*, 2024). The bone loss after SCI is rapid, progressive and severe, as well as unique in its localization to sublesional regions with the greatest declines observed at the distal femur and proximal tibia, anatomical regions where bone mineral density (BMD) may be reduced >50 % within the initial years after SCI (Bauman and Cardozo, 2015; Qin et al., 2010a; Qin et al., 2010b). The thinning bones of individuals with the neurological conditions place them at increased risk for fractures after falls or minor trauma. Such fractures result in

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hospitalization, increased cost, and decreased quality of life. About 46 % of individuals with SCI may suffer a fracture during their lifetimes, which represents a substantially elevated risk over that observed in the general population (Morse et al., 2009). Despite the pressing nature of this problem, to date, the most severe forms of immobilization-related bone loss (e.g., SCI) have been refractory to available interventions except the potent anti-resorptive agent, denosumab [a human monoclonal antibody to receptor activator of nuclear factor-kB ligand (RANKL)]. While promising, this agent suppresses bone formation that would be anticipated to be associated with less than desirable long-term effects on the skeleton (Peng and Qin, 2023), warranting the need for the development of better treatment options.

Whole body low intensity vibration (LIV) is receiving a great deal of attention as a potential means to slow or prevent osteoporosis. LIV increases bone formation and bone mass in healthy sheep (Rubin et al., 2001) and mice (Xie et al., 1985). LIV reduced bone loss in in postmenopausal women (Rubin et al., 2004) and children with cerebral palsy (Ward et al., 2004). Whether LIV administration in specific frequences, intensities, and durations improves bone mass in patients with SCI has not been appropriately investigated to date, although one case report suggests some beneficial effect (Davis et al., 2010). We had recently conducted an initial preclinical study to evaluate the effects of LIV on bone using a rat model of moderately severe SCI (Bramlett et al., 2014). LIV at 40 Hz/0.3 g was initiated at 28 days after SCI and continued for 35 days. LIV induced favorable changes in blood markers of bone formation and gene expression of cultured bone-forming cells (Bramlett et al., 2014). Specifically, LIV significantly increased serum osteocalcin, and markedly inhibited osteoclastogenesis of cultured marrow cells. In ex vivo cultured osteoblasts, LIV increased expression of Runx2 and reduced expression of SOST, both favorable changes (Bramlett et al., 2014). The SOST gene encodes sclerostin, a potent inhibitor of Wnt signaling that is almost exclusively expressed in osteocytes (Poole et al., 2005; Ke et al., 2012). We also observed a consistent trend to improve trabecular architecture although this change did not reach significance (Bramlett et al., 2014). Thus, our novel pilot findings established the potential benefits of LIV on the skeleton in an SCI model, and also in a model of severe neurologic disease or disorder. However, LIV did not significantly increase bone mass (Bramlett et al., 2014). In line with our findings, a recent study demonstrated that whole-body vibration (WBV) applied to rats 7 days after motor-complete SCI partially attenuated bone deterioration (Minematsu et al., 2016).

Our initial work and current knowledge have thus provided solid support for further study of the use of LIV as a convenient therapeutic option for SCI-related bone loss. Several questions may be raised from our early findings: i) Will a prolonged course of LIV that initiates at earlier time be more efficacious in preserving bone? ii) Do the beneficial cellular alterations that have been observed in our previous studies reflect changes in bone metabolism that would predict a net preservation in bone mass? iii) Will emerging pharmacological interventions, when applied in conjunction with LIV, be synergistic to the beneficial effects of LIV on sublesional bone? and iv) Will these benefits be observed in models of moderate or severe SCI? In this study, we sought to test the hypotheses that LIV will reduce bone loss if administrated for a longer period of time than that performed in our initial work (Bramlett et al., 2014) and that this mechanical approach when combined with pharmacological agents that reduce net bone loss (anti-RANKL antibody was tested herein) will more efficaciously preserve the sublesional skeleton after a moderate or a severe SCI than either intervention alone. We also tested the hypothesis that LIV can promote osteoblast differentiation potential of marrow stem cells and stimulate bone formation.

To test these hypotheses, we first evaluated whether an 8-week course of LIV will have a discernable effect to preserve bone in a rat model of moderate contusion SCI—that is, the identical model previously reported by our group but with LIV applied of longer duration (Bramlett et al., 2014). The mechanisms by which LIV might stimulate bone formation and reduce bone resorption was investigated. In addition, we tested whether an anti-RANKL antibody (IK22–5), when administered in conjunction with LIV, enhances the effects of LIV on sublesional bone loss in SCI. About 40–50 % of patients with SCI are motor-complete injuries in whom the bone loss is rapid, of greatest magnitude, and most refractory to current therapeutic approaches. Therefore, we next examined whether a prolonged course of LIV can protect against bone loss in non-weight bearing conditions in a rat model of severe SCI and whether such effects can be further augmented by the application of IK22–5 when combined with LIV.

Thus, in this study, we determined the efficacy of LIV alone or in combination with IK22–5 to restore bone integrity in rats if initiated 14 days after SCI. The temporal sequence of the study is somewhat analogous to the clinical and rehabilitation settings of SCI patients who have substantial bone loss prior to initiating an intervention to prevent further bone loss. The effects of the application of LIV or/and IK22–5 after an SCI were examined on bone mass, three-dimensional architecture of metaphysical trabecular bone, serum markers of bone metabolism, and differentiation potential of bone marrow progenitors, and on mass of paralyzed skeletal muscle.

2. Material and methods

2.1. Animals

Male Sprague Dawley rats 3 months of age (Harlan Laboratories) were maintained in temperature and humidity-controlled rooms and provided with a 12:12-h day to night cycle. Animals were fed standard rat chow ad libitum. All procedures with experimental animals were approved by the Institutional Animal Care and Use Committee of the University of Miami and were performed in accordance with applicable requirements of the National Institutes of Health Guide and the Department of Veteran Affairs. In rats, the rate of growth increases between 1 and 5 weeks, then declines until skeletal maturity, which is achieved by 11.5–13 weeks (Kember, 1973; Hunziker and Schenk, 1989). Therefore, 3 months (about 12 weeks) old adult rats (whose growth plates have been closed for a long time and bone mass has passed its peak amount) were used for this study to minimize the confounding factor of age-related longitudinal bone growth.

2.2. Experimental design, SCI surgery, treatments, and tissue collection

Because approximately 80 % of those with SCI in the general population are males (Center, 2018), male rats were used for these studies. Animals were randomly assigned to either Sham SCI (laminectomy only) or SCI (spinal cord contusion). A moderate severity or a severe SCI at the interspace between the ninth and tenth thoracic vertebra was produced using the Inifinite Horizon device with a moderate injury (150–175 kdyn) or severe (250-300kdyn) (Bramlett et al., 2014; Toro et al., 2021). Control animals underwent only a laminectomy. The SCI groups were randomly assigned to receive LIV, mouse IK22–5 or the combination. The SCI animals were assigned to groups based on BBB score at 4 weeks to prevent any confounding. Group sizes are as follows: Sham, $n = 8$; SCI, $n = 8$; SCI + LIV, $n = 7$; SCI + IK22–5, $n = 8$; and SCI + LIV + IK22–5, $n = 8$. In this study, LIV and IK22–5 were administered at 4 weeks after SCI at 40HZ/0.3 g twice daily for 15 min/session for 5 days each week and 2.5 mg three times each week for 2 months, respectively. The treatments for 8 weeks in this rat study is equivalent of approximately 5 years in humans (Ruth 1935), representing a sufficient treatment duration when compared to months of intervention in most clinical studies with electrical stimulation or LIV (Rubin et al., 2001).

Starting with the LIV at 2 weeks post-injury was chosen based on several considerations. Briefly, animals have significantly regained the ability to weight bear at 14 days and gravitational loading might amplify any benefits of LIV. Voor et al. reported that rats with SCI of moderate severity lost ~25 % of their cancellous bone after the first 2 weeks of injury, while they were recovering plantar stepping; when the rats

recovered some weight-supported stepping, further bone loss was almost halted (Voor et al., 2011). Second, this delayed time prior to starting vibration may be somewhat comparable to that which occurs in a rehabilitation setting, where several weeks may pass before starting rehabilitation therapy after injury. Third, we recently demonstrated that when begun on day 29 after SCI and continued for 5 weeks, LIV increased serum OCN and Runx2 expression in cultured osteoblasts, accompanied by reduced SOST expression; in addition, LIV reduced osteoclastogenesis. However, this intervention regimen failed to promote evident bone accrual. We reason that LIV instituted earlier and continued for a longer duration would have resulted in increased bone mass.

Animals randomized to the LIV group underwent 8 weeks of treatment performed twice daily for 15 min each session for 5 days each week, as descried previously (Bramlett et al., 2014). Briefly, animals with SCI were divided among groups that received LIV and a group that was placed directly on the vibrating platform (Soloflex®) for the same period of time without activating it. The vibration device was programmed in order to achieve frequency of vibration within a range of about 40 Hz (0.3 g) as previously used in studies by us (Bramlett et al., 2014) and others (Herrero et al., 2011; Xie et al., 2006; Wysocki et al., 2011). An iPad and the Vibration app from Diffraction Limited Design, LLC were used to characterize the vibration (Bramlett et al., 2014).

Recent studies have demonstrated that the administration of IK22–5 (250 μg) 3 times/week for 8 weeks results in markedly increased bone mass in osteopenic FASL cKO mice (Wang et al., 2015) and prevents osteoporosis associated with arthritis (Kamijo et al., 2006), while injection of IK22–5 (25–50 μg) for 4 times within 10 days greatly promotes bone growth in young mice (Lezot et al., 2015). Because of the size and metabolic difference between mice and rats, IK22–5 at 2.5 mg for 3 times/week for 8 weeks was the dose chosen to test in this "proof-of-concept" study. The characterization of IK22–5 antibodies produced for this study is provided in Supplemental Fig. 1.

Animals were anesthetized with 3 % isoflurane at 12 weeks post-SCI for muscle, bone harvesting and blood collection. Blood was collected by intraventricular puncture and was centrifuged to separate serum from whole blood elements, and stored at −20 °C. Muscles were isolated by careful dissection, weighed, and flash frozen in liquid nitrogen. Hindlimbs were freed from the pelvis by cutting ligaments and connective tissues at the hip. Left hindlimbs were placed into sterile tubes containing ice-cold Minimum Essential Alpha Medium and kept at 4 °C until processing for isolation of bone marrow cells. Right hindlimbs were immersed in 4 % PFA overnight after which fixative was drained and replaced with 70 % ethanol in water for dual-energy X-ray absorptiometry (DXA) and μCT Analyses.

2.3. DAX analysis of BMD and μCT analysis of bone micro-architecture

Areal bone mineral density (BMD) measurements were conducted by using a small animal DXA (Lunar Piximus, Fitchburg, WI), as described in previous publications (Bramlett et al., 2014; Cardozo et al., 2010; Qin et al., 2015; Zhao et al., 2018; Sun et al., 2013; Qin et al., 2013; Qin et al., 2016). Hindlimbs were positioned on the DXA platform and the knee was flexed at an angle of 135°. Lunar Piximus software was used to acquire DXA images. The metaphysis of the distal femur and proximal tibia were chosen as regions of interest (ROI). The coefficient of variation for the repeated measurements for the ROI was about 1.5 %. See details in the Supplemental Materials.

Volumetric BMD and bone architecture of the distal femur were assessed using a μCT-40 (SCANCO Medical AG) at 16 μm isotropic voxel size, as previously described (Qin et al., 2015; Zhao et al., 2018; Qin et al., 2013; Qin et al., 2016; Peng et al., 2021; Zhao et al., 2021). Scanco software was used to perform image reconstruction and 3D quantitative analysis. Scans were initiated at the growth plate and moved proximally for a total of approximately 300 slices. A ROI consisting of 100 slices beginning 0.5 mm proximal to the growth plate and continuing in a proximal direction were included in the analysis. Standard nomenclature and methods for bone morphometric analysis were used (Parfitt et al., 1987). See details in the Supplemental Materials.

2.4. Immunohistochemistry

Immunohistochemistry was performed as we described previously (Peng and Qin, 2023; Qin et al., 2015; Zhao et al., 2018; Qin et al., 2016; Zhao et al., 2021; Qin et al., 2017; Qin et al., 2024; Zhao et al., 2024). Briefly, all femur or tibia tissues were fixed in buffered formalin solution (10 %) for 24–48 h. The specimens were soaked in 0.5 mol·L^{-1} ethylenediaminetetraacetic acid solution in a shaker at 4 °C for 3 weeks. The decalcification solution was changed 3 times a week. Samples were processed for paraffin embedment (4-μm thick sections). The paraffin-embedded tissue sections were stained with hematoxylin (Thermo Fisher Scientific) and eosin (Thermo Fisher Scientific), for histological observation. TRAP staining was used to detected osteoclasts according to the manufacturer's instructions (387 A, Sigma-Aldrich). Immunostaining was conducted using the standard protocol. The sections were washed 3 times in Tris-buffered saline Tween-20 for 5 min and permeabilized with 0.3 % Triton-X (Sigma-Aldrich) for 15 min. The tissue slides were then blocked with 3 % bovine serum albumin in PBS for 1 h and incubated in primary antibodies against osteocalcin (OCN; 1:20, M188, TaKaRa Bio, Shiga, Japan) overnight at 4 °C in a humidified chamber. On the second day, the sections were incubated in the secondary antibody conjugated with horseradish peroxidase (Jackson ImmunoResearch Laboratories, West Grove, PA, USA) to develop with DAB. Hematoxylin counterstaining was performed to label the nuclei. An Olympus BX52 microscope (Olympus Scientific Solutions Americas, Inc.) with the OsteoMeasure™ system was used for sample image capture. NIH ImageJ software was used for quantitative analysis.

2.5. ELISA assays for serum levels of C-terminal telopeptide (CTX) and osteocalcin

Serum CTX and osteocalcin were determined by ELISA using commercially available kits according to the manufacturers recommended procedures. Serum levels of CTX was assayed using a RatLaps kit from Immunodiagnostic Systems (Fountain Hills, AZ). Serum concentration of osteocalcin was measured using an Osteocalcin EIA kit from Biomedical Technologies Inc. (Stoughton, MA). All samples were assayed in duplicate.

2.6. Ex vivo osteoclastogenesis and osteoblastogenesis assays

Culture and differentiation of bone marrow progenitors was assessed, as previously described (Bramlett et al., 2014; Cardozo et al., 2010; Qin et al., 2015; Sun et al., 2013; Qin et al., 2013; Qin et al., 2016) and are described in greater detail in the Supplemental Materials. Briefly, cells were flushed from the marrow cavity with α-MEM and seeded into tissue culture wells in this medium. For osteoclastogenesis assay, cells were cultured for 2 days in α-MEM supplemented with macrophage colony-stimulating factor (M-CSF, 5 ng/ml). The non-adherent cells were collected, purified by centrifugation in Ficoll-Plus (GE Life Sciences), then seeded into wells and cultured in α-MEM supplemented with M-CSF (30 ng/ml) and RANKL (60 ng/ml) for 5 days. Osteoclasts were identified by staining for tartrate-resistant acid phosphatase (TRAP) using a kit (Sigma-Aldrich, St. Louis, MO).

For osteoblastogenesis assay, the harvested bone marrow cells were cultured in α-MEM supplemented with 15 % preselected FCS (Hyclone, Logan, UT, USA) and ascorbic acid-2-phosphate (1 mM). Recruitment of marrow stromal cells to the osteoblast lineage was assessed at 10 days for counting colony-forming unit-fibroblasts (CFU-F) with alkaline phosphatase (AP) staining kit (Sigma- Aldrich), or at 28 days for counting mineralized nodules [CFU-osteoblastic (ob)] by Von Kossa staining.

2.7. Locomotor function assessment

The Basso, Beattie and Bresnahan (BBB) locomotor rating scale was employed to assess the use of the hindlimbs in rats. A score of 0 indicates no hind-limb movement whereas a score of 21 indicates unimpaired locomotion of that observed in normal uninjured rats. The test was performed pre-operatively, one day post injury, and then once weekly postoperatively, as described previously (Basso et al., 1995) and in Supplemental Materials. Briefly, animals were placed in an open field environment for 3–5 min and observed by two blinded raters to assess BBB. In addition, BBB subscores were used to quantify subtle changes in motor functions in all rats, including toe clearance, paw position, trunk stability and tail use that are independent of forelimb–hindlimb coordination (Popovich et al., 2012), as described in greater details in Supplemental Materials.

2.8. Statistics

Standard power analysis was used to determine the requisite minimum number of animals to ensure sufficient statistical power, as described previously (Lenth, 2006). Data are expressed as mean ± SEM. The number of independent samples (n) is noted in the legend of each figure. The statistical significance of differences among means was tested using one-way analysis of variance and Bonferroni's post hoc test to examine the significance of differences between individual pairs of means. Repeated measures analysis was used for the BBB task followed by appropriate post hoc. Differences were considered significant at $P < 0.05$. Statistical calculations were conducted using Prism 4.0c (GraphPad Software, CA).

3. Results

3.1. Effects of LIV or/and IK22–5 on body weight and muscle mass in rats with moderate contusion SCI

Animals with moderate contusion SCI lost 12.3 % percent of their preoperative body weight. LIV or/and IK22–5 administration did not result in significant body weight change (Supplemental Fig. 2A). Muscle weight was expressed as relative mass corrected for pre-surgery body mass. Gastrocnemius muscle mass after animals received a moderate contusion injury was significantly reduced (−22.5 %, $p < 0.05$) compared to sham-operated animals (Fig. 1A). Although not reaching statistical significance, gastrocnemius muscle mass (+13.8 % and + 13.5 %) after LIV or its combination with IK22–5 trended to increase in weight (Fig. 1A). Triceps muscle weights were not significantly different among the five groups (Fig. 1B).

3.2. Effects of LIV or/and IK22–5 on BMD in rats with moderate contusion SCI

A small animal DXA was used to examine whether LIV, IK22–5 or the combination of these interventions could alter bone loss in rats after moderate contusion SCI. At the distal femur (Fig. 1A), BMD was decreased by −10.6 % ($p < 0.01$) after SCI, and significantly increased

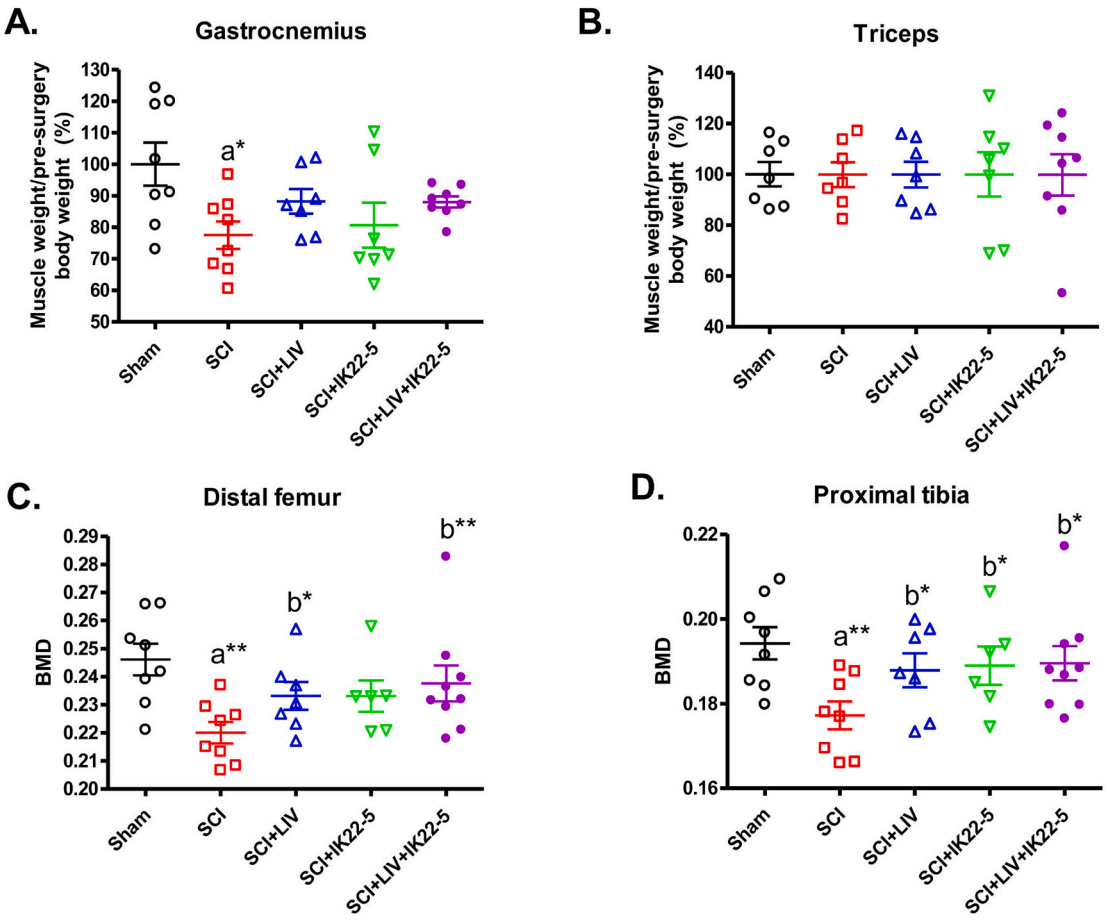


Fig. 1. Effects of LIV, IK22–5 and their combinations on muscle and bone in rats with moderate contusion SCI. Mass of gastrocnemius (A) and triceps (B) muscles in each group are shown. Bone mineral density (BMD) was measured by a small animal dual-energy X-ray absorptiometer (DXA) at distal femur (C) and proximal tibia (D). Data are expressed as mean ± SEM. $N = 7–8$ per group. $^{*}p < 0.05$ and $^{**}p < 0.01$ by one way ANOVA with a Newman–Keuls post hoc test. "a" indicates a comparison with Sham animals; "b" indicates a comparison with SCI animals.

by LIV (+7.1 %, $p < 0.05$) and LIV + IK22–5 (+7.6 %, $p < 0.01$), respectively. An almost identical pattern of BMD change was also detected at the proximal tibia (Fig. 1B) where BMD was reduced by −8.8 % ($p < 0.01$) after SCI. Compared to SCI, BMD at the proximal tibia increased +6.0 % ($p < 0.05$), +6.6 % ($p < 0.05$), +7.0 % ($p < 0.05$) following LIV, IK22–5, LIV + IK22–5 treatment, respectively. The larger magnitude of changes on BMD at the distal femur and proximal tibia in animals with the dual combination treatment, as compared to each treatment alone, suggests a possible synergistic effects of LIV and IK22–5 on bone mass.

3.3. Effects of LIV or/and IK22–5 on trabecular bone microstructure in rats with moderate contusion SCI

A high-resolution μCT was used to assess changes in trabecular bone architecture at the distal femoral metaphysis (Fig. 2). After SCI, trabecular bone volume (BV/TV) at this site was significantly reduced (−16.1 %, $p < 0.01$; Fig. 2B-a), with decreased trabecular number (Tb. N) (−17.4 %, $p < 0.05$; Fig. 2B-b) and trabecular thickness (Tb. Th) (−13.9 %, $p < 0.05$; Fig. 2B-c) as well as increased trabecular space (Tb. Sp) (+15.8 %, $p < 0.05$; Fig. 2B-d). Trabecular connectivity (Conn. D) was greatly reduced (−35.6 %, $p < 0.05$; Fig. 2B-e), associated with transformation from plate-like to rod-like structures [as reflected by the structure model index (SMI); Fig. 2B-f]. LIV or IK22–5 significantly restored trabecular BV/TV by increasing connectivity and by decreasing the value of the SMI (Fig. 2B-f). LIV + IK22–5 administration almost completely restored each of these bone microstructure parameters to that of control animals, suggesting a synergistic effect of LIV + IK22–5 on bone integrity.

Consistent with the findings by Rubin et al. (Rubin et al., 2001), we found that there was no significant change in cortical bone volume at femoral midshaft (Supplemental Fig. 3). This data indicates that these anabolic effects were highly specific to cancellous (porous or trabecular) bone.

3.4. Effects of LIV or/and IK22–5 on bone formation and bone resorption in rats with moderate contusion SCI

ELISA assays were performed to examine the serum levels of bone biomarkers for formation, osteocalcin, and resorption, CTX. Consistent with previous findings (Peng and Qin, 2023; Bramlett et al., 2014; Qin et al., 2013; Zhao et al., 2021), osteocalcin levels were decreased after SCI (−37.1 %, $p < 0.05$, Fig. 3A). Importantly, we found LIV or LIV + IK22–5 significantly increased serum osteocalcin levels (+64.2 %, $p < 0.05$; +59.3 %, $p < 0.05$, respectively; Fig. 3A), suggesting promotion of bone formation. Following SCI, serum CTX level was increased by +56.6 % ($p < 0.01$; Fig. 3B). LIV or IK22–5 and their combination significantly decreased the concentration of CTX in blood by −25.9 % ($p < 0.05$), −31.1 % ($p < 0.05$) and − 34.3 %, ($p < 0.05$), respectively, suggesting inhibition of bone resorption (Fig. 3B).

In addition, we examined osteoblasts activity using immunohistological staining for osteocalcin and osteoclasts activity using TRAP

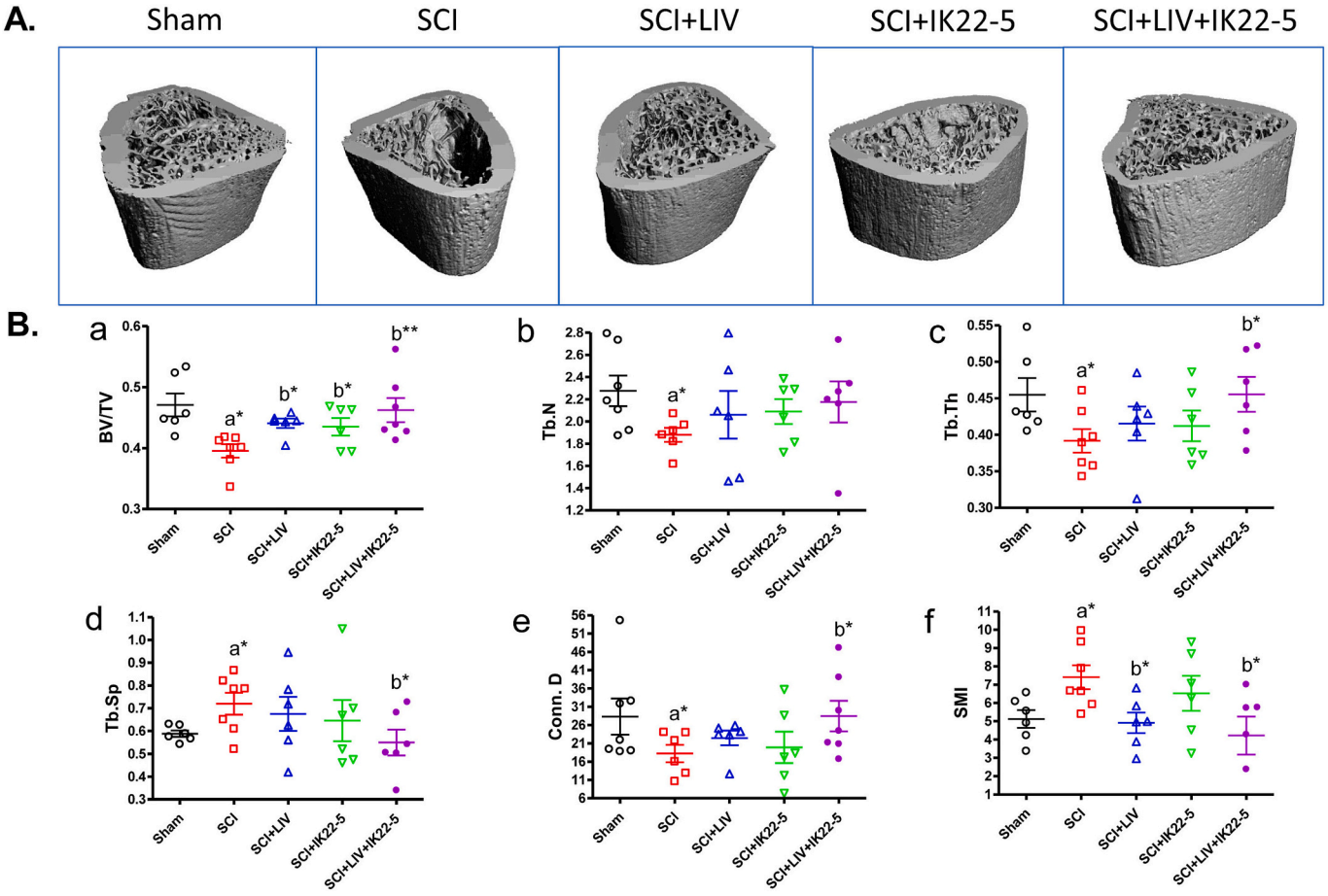
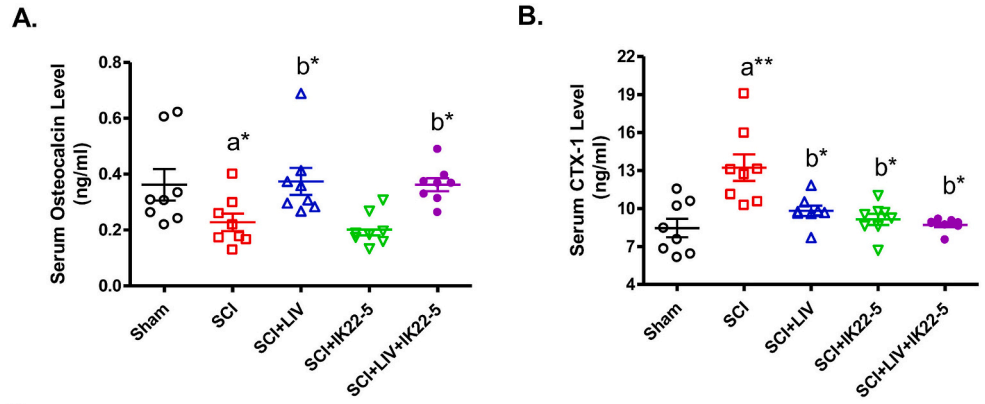


Fig. 2. Effects of LIV, IK22–5 and their combinations on trabecular architecture of the distal femur bone in rats with moderate contusion SCI. (A) Representative 3D images of bone microarchitecture. Measurements are shown for (B) (a) trabecular bone volume per total volume (BV/TV), (b) trabecular number (Tb.N), (c) trabecular thickness (Tb.Th), (d) trabecular space (Tb.Sp), (e) connectivity density (Conn.D), and (f) structure model index (SMI). Data are expressed as mean ± SEM. $N = 6–8$ per group. Significance of differences was determined by using one-way ANOVA with a Newman–Keuls post hoc test. $^*P < 0.05$ and $^{**}P < 0.01$ versus the indicated group. "a" indicates a comparison with Sham animals; "b" indicates a comparison with SCI animals.



C.

	Sham	SCI	SCI+LIV	SCI+IK22-5	SCI+LIV+IK22-5
OCN^+/mm^2	31 ± 3	20 ± 2 ^{a*}	33 ± 4 ^{b*}	21 ± 3 ^{b*}	36 ± 5 ^{b*}
$TRAP^+/mm^2$	75 ± 7	132 ± 12 ^{a**}	102 ± 11 ^{b*}	92 ± 9 ^{b*}	86 ± 8 ^{b*}

Fig. 3. Effects of LIV, IK22–5 and their combinations on levels of serum biomarkers of bone resorption and formation in rats with moderate contusion SCI. ELISA tests for (A) Osteocalcin and (B) CTX. (C) Quantitative analysis of the osteocalcin (OCN) staining and TRAP staining of the distal femurs. Data are expressed as mean ± SEM, N = 7–8 per group. Significance of differences was determined using one-way ANOVA with a Newman–Keuls post hoc test. *p < 0.05 and **P < 0.01 versus the indicated group. "a" indicates a comparison with Sham animals; "b" indicates a comparison with SCI animals.

staining in distal femur. Consistent with our ELISA findings, the number of osteocalcin positive cells was decreased after SCI (−36 %, p < 0.05, Fig. 3C); LIV or LIV + IK22–5 significantly increased serum osteocalcin levels (+65 %, $p < 0.05$; +80 %, $p < 0.05$, respectively; Fig. 3C), suggesting promotion of bone formation. Following SCI, the number of $TRAP^+$ staining cells was increased by +76 % (p < 0.01; Fig. 3C). LIV or

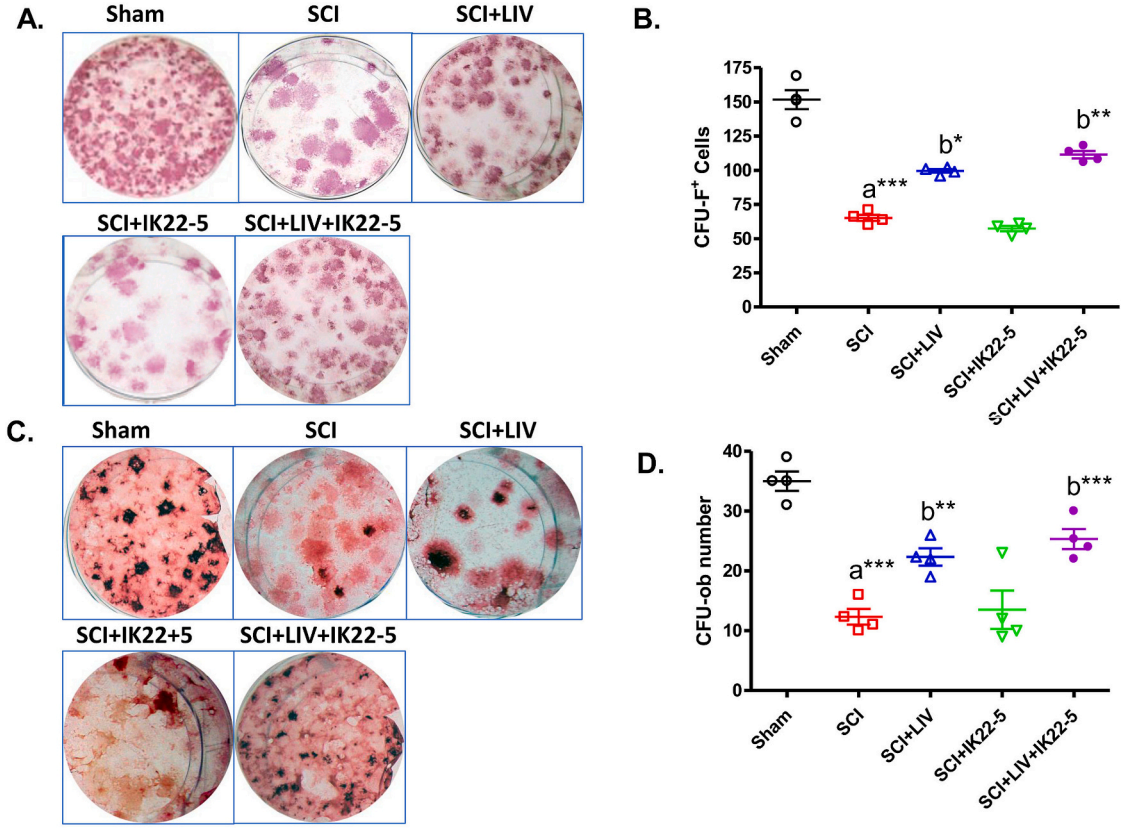


Fig. 4. Effects of LIV, IK22–5 and their combinations on osteoblastogenesis. Bone marrow stem cells were collected from the femur and tibia. Procedures for osteoblast formation from bone marrow stem cells were performed as previously described (Bramlett et al., 2014; Cardozo et al., 2010; Qin et al., 2015; Sun et al., 2013; Qin et al., 2013; Qin et al., 2016). (A) Representative images of bone formation by CFU-F staining of cultured bone marrow stromal cells, and (B) quantification of $CFU\text{-}F^+$ cells. (C) Representative images of bone formation by CFU-osteoblastic (ob)/Von Kossa staining of cultured bone marrow stromal cells, and (D) quantification of CFU-ob cell numbers. $N = 3–4$ for each group. Data are expressed as mean ± SEM. *p < 0.05, **p < 0.01, ***$p < 0.001$ by one way ANOVA with a Newman–Keuls post hoc test. "a" indicates a comparison with Sham animals; "b" indicates a comparison with SCI animals.

IK22–5 and their combination significantly decreased the number of TRAP $^{+}$ staining cells by −23 % ($p < 0.05$), −31.1 % ($p < 0.05$) and − 30 %, ($p < 0.05$) in distal femur, respectively, suggesting inhibition of bone resorption (Fig. 3C).

3.5. Effects of LIV or/and IK22–5 on osteoblastogenesis

LIV or IK22–5 or their combination was tested to determine the effect of these interventions on osteoblastogenesis and osteoclastogenesis in cultures of bone marrow stem cells. We compared the effects on bone cells of LIV combined with IK22–5 with the effects observed for LIV or IK22–5 alone. Osteoblastogenesis was first evaluated by CFU-F staining using bone marrow mesenchymal stem cells derived from the femurs (Fig. 4A, B). Consistent with our previous findings (Peng and Qin, 2023; Bramlett et al., 2014; Sun et al., 2013; Qin et al., 2013; Zhao et al., 2021; Qin et al., 2015), SCI decreased the CFU-F^{+} cells by −56.7 % ($p < 0.001$) compared to sham animals. Interestingly, LIV or LIV + IK22–5 treatment after SCI increased the osteoblastic colony number by +50.1 % ($p < 0.05$) and + 68.5 % ($p < 0.05$), respectively. Similarly, the values for mineralized nodules by CFU-ob/Von Kossa staining were significantly reduced in the vehicle-treated SCI group (−64.8 %, $p < 0.001$). Importantly, LIV or LIV + IK22–5 treatment after SCI increased the osteoblastogenesis by +81.1 % ($p < 0.01$) and + 105.4 % ($p < 0.001$), respectively (Fig. 4C, D).

3.6. Effects of LIV or/and IK22–5 on osteoclastogenesis

Consistent with our previous findings (Peng and Qin, 2023; Bramlett et al., 2014; Sun et al., 2013; Qin et al., 2013; Zhao et al., 2021; Qin et al., 2015), an osteoclastogenesis assay using bone marrow

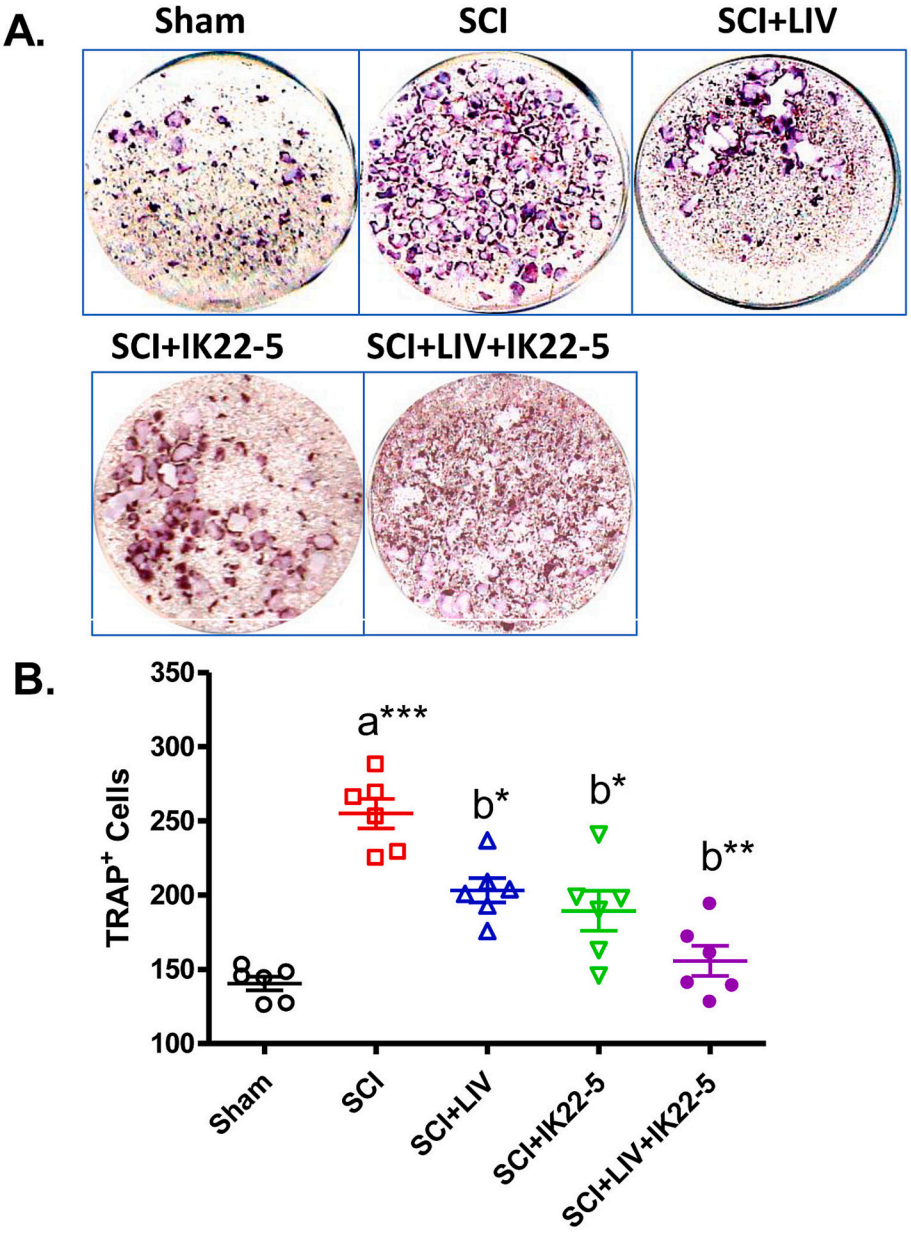


Fig. 5. Effects of LIV, IK22–5 and their combinations on osteoclastogenesis. Bone marrow stem cells were collected from the femur and tibia. Procedures for osteoclast formation from bone marrow stem cells were performed, as previously described (Bramlett et al., 2014; Cardozo et al., 2010; Qin et al., 2015; Sun et al., 2013; Qin et al., 2013; Qin et al., 2016). (A) Representative images of bone formation by TRAP staining of cultured bone marrow cells, and (B) quantification of TRAP^{+} cells. Data are expressed as mean ± SEM. N = 3–4 for each group. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ by one way ANOVA with a Newman–Keuls post hoc test. "a" indicates a comparison with Sham animals; "b" indicates a comparison with SCI animals.

hematopoietic stem cells derived from the femurs of SCI animals revealed significantly increased $TRAP^+$ multinucleated cells by +81.5 % ($p < 0.001$) when compared to sham animals (Fig. 5 and Supplemental Fig. 4). Importantly, LIV, IK22–5, or LIV + IK22–5 treatment following SCI markedly decreased $TRAP^+$ cells by −20.3 % ($p < 0.05$), −25.7 % ($p < 0.05$), and − 38.9 % ($p < 0.01$), respectively.

3.7. *Effects of LIV on functional recovery in rats with moderate contusion SCI*

To evaluate the potential impact of the LIV on functional recovery after SCI, hindlimb locomotor function was first determined prior to SCI, one day post-SCI, then weekly thereafter for 8 weeks using the BBB locomotor scale method (Basso et al., 1995; Basso et al., 1996; Pinzon et al., 2008). At one day after SCI, mean BBB scores in the 3 experimental groups (sham, SCI, SCI + LIV) were similar (ranging from 1.1 to 2.4), indicating that the groups were equivalent in terms of lesion severity. There was no difference for the BBB score between SCI group and SCI group with LIV interventions at 1–8 weeks (ranging from 9 to 10, indicating weight support in stance only without consistent stepping), suggesting that LIV failed to improve overall function recovery in rats with moderate severity contusion (Fig. 6A).

BBB subscore, which measures toe clearance, paw position, trunk stability, and tail usage, independent of forelimb–hindlimb coordination, was analyzed and demonstrated similar degrees of impairment in the 3 groups one day after SCI. However, the analysis revealed a significant interaction between group and time ($p < 0.001$). Post hoc analysis showed that one week after SCI, the BBB subscore in SCI + LIV group improved over time, and there was significant difference between SCI group (ranging from 0 to 3) and SCI + LIV group (ranging from 2 to

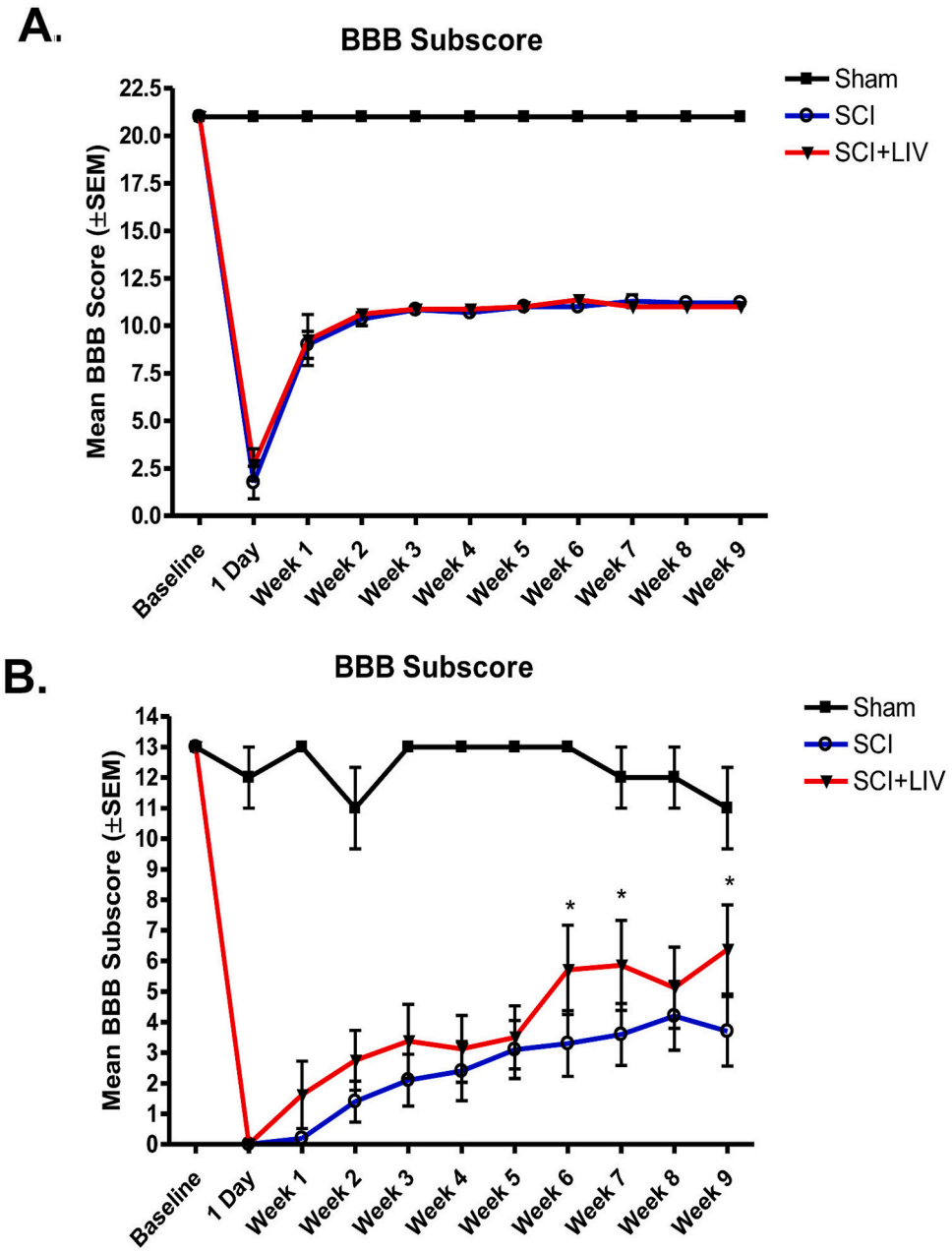


Fig. 6. Effects of LIV intervention on functional recovery after moderate contusion SCI. Hindlimb locomotor function was determined prior to SCI, Day 1, and then weekly thereafter for 8 weeks using the BBB locomotor scale method (A) and the BBB sub-score analysis (B). Data are expressed as mean ± SEM. N = 7–8 per group. *$p < 0.05$ by two-way repeated ANOVA and Bonferroni posttests.

6) at Week 6, Week 7 and Week 9 at which time points the LIV-treated rats stepped frequently or consistently, along with improved trunk stability, and tail usage (Fig. 6B).

3.8. Effects of LIV or/and IK22–5 on trabecular bone mass in rats with severe contusion SCI

Severe Contusion SCI led to more robust loses of body weight and bone mass than moderate contusion SCI did. Animals with severe contusion SCI lost 16.7 % percent of their preoperative body weight (Supplemental Fig. 2B). BMD values by DXA were decreased by −15.1 % ($p < 0.01$) after SCI, and significantly increased by LIV (+8.1 %, $p < 0.05$), IK22–5 (+8.3 %, $p < 0.05$) and LIV + IK22–5 (+11.9 %, $p < 0.01$), respectively (Fig. 7A). An almost identical pattern of BMD change was also detected at the proximal tibia (Fig. 7B) where BMD was reduced by −6.4 % ($p < 0.01$) after SCI. Following LIV, IK22–5, or LIV + IK22–5, BMD at the proximal tibia increased +4.5 % ($p < 0.05$), +4.2 % ($p = 0.06$), +5.6 % and ($p < 0.01$), respectively, compared to SCI animals without benefit of receiving any treatment intervention. The larger magnitude of change in BMD at the distal femur and proximal tibia in animals that received the combination treatment suggests a potential synergistic effects of LIV and IK22–5 on bone mass compared to each treatment alone.

High-resolution μCT analysis revealed that after severe SCI, trabecular BV/TV at the distal femoral metaphysis was significantly reduced (−24.2 %, $p < 0.01$). LIV or IK22–5 alone significantly restored bone volume of the trabecular region. Furthermore, the combination of LIV + IK22–5 administration almost completely restored trabecular BV/TV, further suggesting a synergistic effect of LIV and IK22–5 on bone mass (+17.1 %, $p < 0.01$; Fig. 7C, D).

4. Discussion

Our study determined the efficacy of a mechanical intervention (LIV) alone or in combination with a RANKL inhibitor (IK22–5) to restore bone integrity after a moderate or a severe SCI and to improve our understanding of the molecular mechanisms by which these changes occur. An 8-week course of LIV that was begun 14 days after moderate or severe contusion injury offered significant protection against bone loss in rats. LIV stimulated bone formation (reflected by the increased serum osteocalcin levels) and improves osteoblastic differentiation potential of bone marrow stromal stem cells while inhibiting osteoclastic differentiation potential of a pool of hematopoietic cells to reduce bone resorption (reflected by the reduced serum CTX levels). Our findings suggest that a combination of LIV and IK22–5 reduced SCI-related bone loss more than each intervention alone. LIV is efficacious in maintaining sublesional bone mass, suggesting that such noninvasive physical-based intervention approach would be an inexpensive and practical approach to implement in the treatment of bone loss after SCI. Our suggestive finding that the combination of LIV with IK22–5 is better able to preserve sublesional bone integrity after SCI is a novel finding, providing groundwork for the development of future clinical protocols based on physical activity (e.g., LIV) and pharmacological (e.g., RANKL inhibitor or other bone-sparing agents) approaches to prevent or reverse bone after SCI or other conditions associated with severe immobilization, with the promise of identifying more effective and safer therapeutic options.

In our prior study, LIV at 40 Hz/0.3 g that was initiated at 14 days after SCI and continued for 35 days led to some positive changes in parameters related to bone remolding (e.g., significantly increased serum osteocalcin and markedly reduced osteoclastogenesis of cultured marrow cells), although this work was unable to demonstrate a

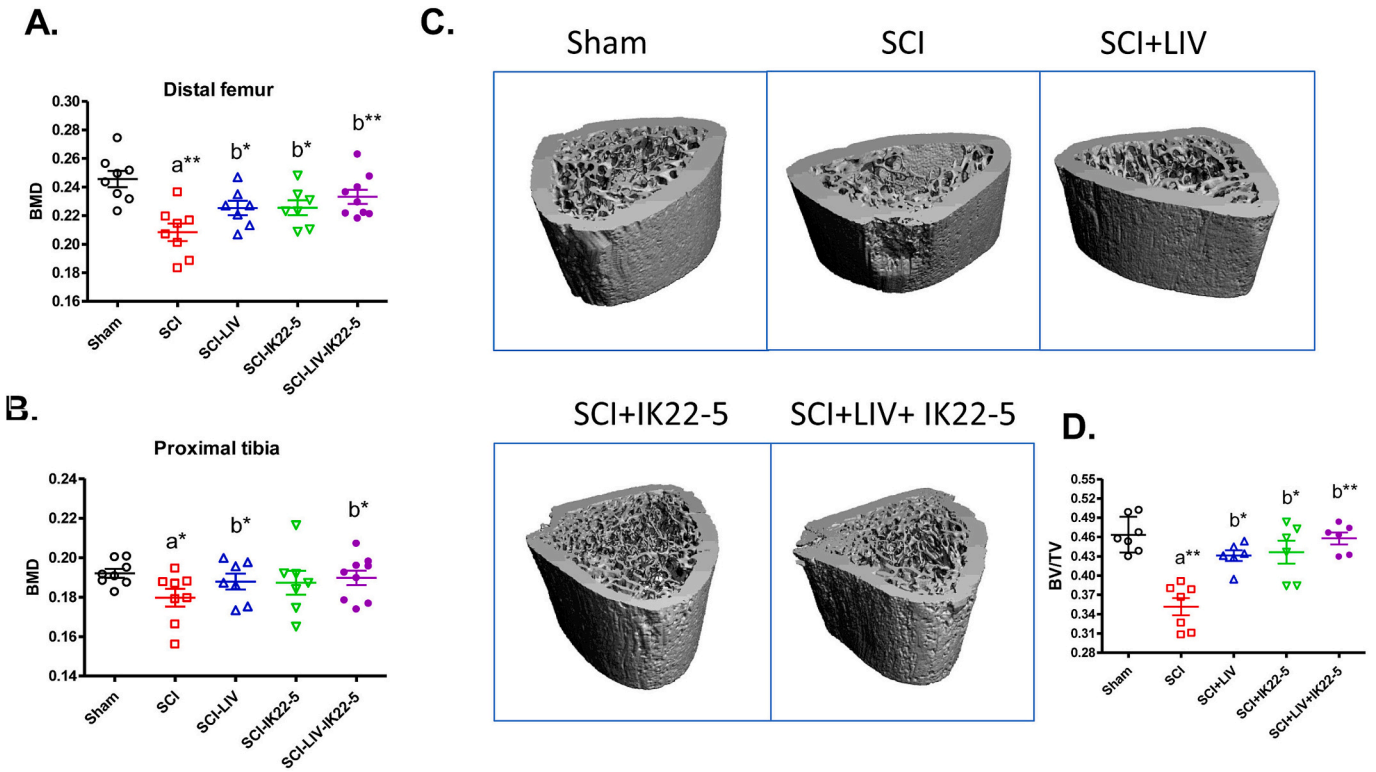


Fig. 7. Effects of LIV, IK22–5 and their combinations on bone in rats with severe contusion SCI. Bone mineral density (BMD) was measured by a small animal dual-energy X-ray absorptiometer (DXA) at distal femur (A) and proximal tibia (B). (C) Representative 3D images of bone microarchitecture. Measurements are shown for (D) trabecular bone volume per total volume (BV/TV) at the distal femur. Data are expressed as mean ± SEM. N = 7–8 per group. Significance of differences was determined by using one-way ANOVA with a Newman–Keuls post hoc test. *P < 0.05 and **P < 0.01 versus the indicated group. "a" indicates a comparison with Sham animals; "b" indicates a comparison with SCI animals.

significant increase in bone mass (Bramlett et al., 2014). In the present study, a set of innovative approaches were implemented to improve the efficacy of the LIV intervention on bone and include: i) instituting the LIV administration earlier to increase the effectiveness of the intervention; ii) prolonging the duration of LIV from 5 to 8 weeks with the prior protocols and the one herein initiating the intervention 14 days after SCI (Bramlett et al., 2014)), and iii) adding a pharmacological agent to the current study (Bramlett et al., 2014). Bone loss after SCI is largely due to markedly increased bone resorption. Osteocyte release of RANKL is a key factor driving the increased osteoclastogenesis in unloading-related bone loss (Kearns et al., 2008). In rodent SCI models, we observed that there is an 8-fold increase in RANKL mRNA expression within 56 days after injury (Sun et al., 2013). Inhibitors of RANKL reduced unloading-related bone loss in several animal models (Kearns et al., 2008). Denosamub, a commercial human anti-RANKL antibody, has recently been shown to preserve sublesional bone mass after acute SCI (Gifre et al., 2016; Cirnigliaro et al., 2020). Of note, RANKL inhibitors in animal models were reported to be more effective than bisphosphonates in reducing immobilization-related bone loss (Kearns et al., 2008). Among other advantages of LIV-based combination therapy, LIV has the potential to reduce the effective dosage of pharmacological agents, which then would minimize adverse dose-related side effects and cost. In addition to the osseous effects, LIV is believed to be associated with other accompanying benefits that may include improvements in functional recovery (Wirth et al., 2013), muscle blood flow (Herrero et al., 2011), spasticity (Murillo et al., 2011), and bladder function (Wirth et al., 2013).

In the present study, we found that LIV is capable of reducing bone loss in both moderate and severe SCI rats, indicating that anabolic effects of LIV do not appear to require gravitational loading, a finding consistent with the previous observations by Rubin et al. (Garman et al., 2007a; Judex et al., 2007). One study from Rubin's group used an apparatus to accelerate specific segments of the murine skeleton without loading–that is, bone was subjected to oscillatory motions without application of gravity- or function-related deformations to the tissue (Garman et al., 2007a). In that study, the left tibia of eight adult mice was exposed to small (0.3 g or 0.6 g) 45 Hz sinusoidal accelerations for 10 min/day, while the right tibia served as an internal control. The study demonstrated that tiny acceleratory motions, independent of direct loading of the matrix, can greatly improve bone formation rate (BFR). The findings were confirmed in a model in which weight bearing and locomotion were removed from the tibiae via hindlimb unloading. In the latter study, LIV-elicited acceleratory motions resulted in 70 % greater BFR in trabeculae of the metaphysis (Garman et al., 2007b). These findings indicate that "the physical acceleration of a cell may represent a generic signal that transmits information via the cytoskeletal or cell/matrix interrelationships, rather than requiring substrate deviation (e.g., ground reaction forces)" (Sen et al., 2011). This notion can be further supported by the molecular and cellular findings related to bone remodeling from literature and the present study, as discussed below.

In the majority of cases of acute SCI, implementation of a therapy to protect bone integrity is often instituted after medical and/or surgical treatment has been rendered when substantial bone loss has already occurred. Because no clinical guideline currently exist for anti-osteoporosis therapy in patients with acute SCI, the majority of 18,000 new SCI cases each year (*National Spinal Cord Injury Statistical Center.*, 2024) do not receive any intervention to prevent bone loss and, as such, have unabated progressive bone deterioration over the duration of their paralysis and associated severe immobilization (Jiang et al., 2007; Cirnigliaro et al., 2019). We have demonstrated that LIV alone or in combination with other pharmacological approaches (e.g., RANKL antibody) was able to substantially reverse bone loss when administered beginning at 14 days after moderate or severe SCI, when significant bone loss has already occurred in the rodent model (Jiang et al., 2007). In the latter studies of SCI rats, loss of trabecular bone in the proximal tibia approaches 70 % at 3 weeks after a complete spinal cord transection (Jiang et al., 2007). As stated earlier, this lag time prior to initiating LIV after acute SCI in our rodent protocol may be comparable to that which occurs in rehabilitation settings, where several weeks may pass before starting rehabilitation therapy after traumatic injury. Our findings are in line with previous literature regarding the effects of mechanical reloading on bone (e.g., functional electrical stimulation), suggesting that gains in bone mass may occur even years after SCI (Qin et al., 2010a; Chang et al., 2013). Microgravity in spaceflight results in significant bone loss, but one year after returning to earth, most astronauts have significant reversal of their adverse skeletal changes (Lang et al., 2006). In addition, we demonstrated that when begun on day 29 after complete spinal cord transection, the anabolic steroid nandrolone was able to reduce bone loss, indicating that anabolic influences remained effective even after an extensive interval after SCI. Thus, our work suggests that it may be possible to reverse loss of bone mass and strength even after extended periods of immobilization.

The integrity and mass of bone are maintained through a continual cycle of bone resorption by osteoclasts and deposition of new bone by osteoblasts, the net rate of which determines whether bone mass increases or declines (Raggatt and Partridge, 2010). In addition to the previous data showing an increase in bone formation in mice tibia (Garman et al., 2007b), a more recent study in rats suggested that the WBV-induced improvement on bone after SCI would have resulted from renewed bone formation (the increased serum osteocalcin) and/or net bone formation (Minematsu et al., 2016). Although the reduced levels of serum osteocalcin following SCI were completely reversed by 5 weeks of LIV in rats in our pilot study, the LIV regimen failed to protect against SCI-related reduction of osteoblast differentiation in ex vivo bone marrow stem cell cultures (Bramlett et al., 2014). However, in our present study we found that the more prolonged LIV (for 8 weeks) was able to largely reverse SCI-induced reduction in osteoblast differentiation (examined by CFU-F staining; Fig. 4A) and mineralization (examined by CFU-ob staining; Fig. 4B) in the similar ex vivo cultures. Consistent with the previous findings (Bramlett et al., 2014), more prolonged administration of LIV protected against SCI-induced osteoclastogenesis in ex vivo cultures (Fig. 5). Collectively, together with the favorable changes for the serum markers of bone resorption and bone formation, our findings strongly suggest that LIV-mediated enhancement of bone formation and inhibition of bone resorption are biologically universal mechanisms for bone adaptation after SCI in various species of animals. It thus follows that LIV, if administered in the correct frequency, intensity, and duration, may also be anticipated to be protective against bone loss in patients with SCI.

In line with earlier studies (Bramlett et al., 2014), we failed to detect significant increase in skeletal muscle weights. However, a trend of improved gastrocnemius muscle weights was observed. It remains to determine if these numeric changes might actually contribute to improvements in neuromuscular function, but increased muscle strength can occur without any change in muscle volume or cross-sectional area in mice (Bramlett et al., 2014; McKeehen et al., 2013). LIV did not improve BBB score in our current study, which indicates lack of improvement in over-ground locomotion. However, signs of a salutary functional treatment effect became evident when the data was analyzed by the BBB subscore. Importantly, the latter approach revealed that LIV improved forelimb–hindlimb coordination-independent activities for toe clearance, paw position, trunk stability, and tail usage, suggesting a subtle functional recovery after contusion SCI. Our findings are consistent with the previous observation that compared to sham-treated rats, WBV did not improve motor function, but improved body support when applied at 14 days after SCI and continued for 12 weeks (Schwarz et al., 2015). Our study design did not allow for the examination of the morphological substrates (e.g., lesion size, spared whiter matter) and other cellular changes on the site of the spinal cord lesion that can influence those functional measurements by the BBB subscore analysis. Nevertheless, it can be appreciated from the prior work where WBV initiated at 14 days after SCI partially restored synaptic input to the

ventral horn (Wirth et al., 2013), suggesting that synaptic plasticity and adaptations in the distal spinal cord could contribute to enhance body weight support. The increased local blood flow in vibrated skeletal muscles might also play a role for better motor performance (Yarar-Fisher et al., 2014). Additional work employing LIV intervention with higher frequency/intensity and in combination with other pharmacological interventions may lead to more pronounced musculoskeletal and functional outcomes after SCI.

There are a few limitations of our study. RANKL is produced by many cell types outside of bone micro-environment, such as activated T cells. Thus, one of the potential side effects for RANKL inhibition may be immuno-suppression (Ferrari-Lacraz and Ferrari, 2011). Although current studies have shown no significant alterations of inflammatory processes by RANKL inhibitors (Ferrari-Lacraz and Ferrari, 2011), nor any increase in the infection rate in patients with postmenopausal osteoporosis (Kendler et al., 2022), a future study may address if there is increased rates of infection of animals with SCI, especially at the surgical site. Another limitation of our study was that only male rats were studied. Thus, it should be shown that the beneficial effects of LIV and/or RANKL inhibition can be extended to females with SCI, which would be strongly anticipated.

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CRediT authorship contribution statement

Yuanzhen Peng: Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation. **Helen M. Bramlett:** Writing – review & editing, Validation, Supervision, Investigation, Funding acquisition, Formal analysis, Data curation. **W. Dalton Dietrich:** Writing – review & editing, Investigation, Data curation. **Alex Marcillo:** Validation, Methodology, Investigation, Formal analysis, Data curation. **Juliana Sanchez-Molano:** Methodology, Investigation, Formal analysis, Data curation. **Ofelia Furones-Alonso:** Validation, Methodology, Formal analysis, Data curation. **Jay J. Cao:** Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation. **Jenney Huang:** Formal analysis, Data curation. **Andrew A. Li:** Formal analysis, Data curation. **Jian Q. Feng:** Visualization, Validation, Methodology, Investigation, Data curation. **William A. Bauman:** Writing – review & editing, Validation, Investigation, Funding acquisition. **Weiping Qin:** Writing – review & editing, Writing – original draft, Supervision, Resources, Project administration, Investigation, Funding acquisition, Formal analysis, Data curation, Conceptualization.

Declaration of competing interest

YP, AM, OFA, JSM, JC, JH, AL, JQF, WAB, WQ have nothing to disclose. HMB and WDD are co-founders and managing members of InflamaCORE, LLC and have licensed patents on inflammasome proteins as biomarkers of injury and disease as well as on targeting inflammasome proteins for therapeutic purposes. HMB and WDD are Scientific Advisory Board Members for ZyVersa Therapeutics.

Data availability statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Author contributions

WQ was responsible for the overall study design and data analysis. YP, HMB, WDD, AM, OFA, JC, XEG and WAB provided consultation on and performed the experimental procedures to generate the data, data analysis as well as data interpretation for the study. The manuscript was drafted by WQ and was revised and approved by all authors. WQ takes responsibility for the integrity of the data analysis.

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