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TITLE: Potential Therapeutic Use of Relaxin in Healing Cranial Bone Defects

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14. ABSTRACT

The overall objective is to provide proof-of-principle that recombinant human relaxin (rhRLX) administration will accelerate bone healing in a calvarial defect model in mice by promoting angiogenesis/vasculogenesis and osteogenesis, at least in part through incorporation of bone marrow-derived angio- and osteogenic progenitor cells into the lesion. Results from the second study conducted during this reporting period demonstrated: reproducible implementation of uniform cranial lesions of ~1.5 mm diameter and circulating concentrations of relaxin ranging from 0.35-3.41 ng/ml. However, after 10-12 days of healing, the lesion closure was comparable in the relaxin- and vehicle-treated mice (~50%). Consistent with this finding is that there were also no significant differences in bone/tissue volume (%) or bone and tissues mineralization densities (g/cm^3). Therefore, in the next study we will: (1) reach a circulating concentration of relaxin administered *systemically* by s.c. osmotic pump between the first and second studies i.e., ~10-20 ng/ml in one group of mice; (2) in another group, we will apply relaxin *locally* as collagen scaffolding; (3) make a larger lesion of 3 rather than 1.5 mm diameter, in order to reduce the overall %closure at 10-12 days—less closure may unmask differences between relaxin and vehicle treatments; and (4) utilize old mice of ~12 months of age, which relatively impaired bone healing due to age may be amenable to improvement by relaxin.

15. SUBJECT TERMS

NONE LISTED

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1. Introduction: Final Report Summary

This DOD Discovery Award addressed the general problem of failed or delayed healing of craniomaxillofacial fractures. The objective was to provide proof-of-principle that recombinant human relaxin (rhRLX) administration will accelerate bone healing in a calvarial defect model in mice by promoting angiogenesis/vasculogenesis and osteogenesis, at least in part through incorporation of bone marrow-derived angio- and osteogenic progenitor cells into the lesion. This hormone/growth factor has numerous biological attributes that are likely to benefit bone fracture healing, and it has an excellent safety profile in humans.

In year 01 of this award, we tested the hypothesis using a cranial defect model in chimeric mice transplanted with GFP^+ bone marrow. We followed defect closure by three-dimensional micro-computed tomography (3-D μCT). In addition, we quantitated blood vessel number and density by immunohistochemistry. As reported in the year 01 Annual Report, although we successfully established the animal model in all aspects, chronic administration of rhRLX at 1.0 μg/hr did not accelerate bone healing. Nor did it improve blood vessel number or density in the bone lesion. Because these results were negative, we did not further pursue the enumeration and location of GFP+ bone marrow-derived cells at the lesion site by immunofluorescence as originally proposed. However, the infusion rate of rhRLX, 1.0 μg/hr, produced higher plasma concentrations than expected—~53 ng/ml. Because we previously reported a biphasic effect of relaxin *in vivo*, we decided next to use a lower dose.

In year 02, we repeated the experiment using a lower dose of rhRLX of 0.05 ng/ml. In this second study, we demonstrated reproducible implementation of uniform cranial lesions of 1.5 mm diameter and circulating concentrations of relaxin ranging from 0.35-3.41 ng/ml. However, after 10-12 days of healing, the lesion closure was comparable in the relaxin- and vehicle-treated mice (~50%). Consistent with this finding was that there were also no significant differences in bone/tissue volume (%) or bone and tissues mineralization densities (g/cm^3).

In year 03, results from the third study conducted during this reporting period demonstrated: reproducible implementation of uniform cranial lesions of 3.0 mm diameter and circulating concentrations of relaxin of 4.9 $\pm$ 1.3 ng/ml ng/ml. However, after 10-12 days of healing, the lesion closure was comparable in the relaxin- and vehicle-treated mice (~70% each). Consistent with this finding was that there were also no significant differences in bone volume, bone/tissue volume (%) or bone and tissues mineralization densities (g/cm^3). In a parallel study, we applied relaxin *locally* in collagen scaffolding (1.0 μg/scaffold); however, again, the lesion closure was comparable in the relaxin- and vehicle-treated mice (~80% each). Consistent with this finding was that there were also no significant differences in bone volume, bone/tissue volume (%) or bone and tissues mineralization densities (g/cm^3). In these 2 protocols we also utilized older mice of ~13-14 months of age, the idea being that the relative impairment of bone healing due to age may be more amenable to improvement by relaxin.

For Final 2018-2019 reporting period, we completed analyzing all data, prepared figures and tables for publication, wrote the manuscript and submitted to *Physiological Reports* on April 10, 2019 (attached).

Dear Dr. Kirk P Conrad,

Thank you for your recent submission to *Physiological Reports*. *Physiological Reports* is an open access journal that levies article publication charges; therefore, I am writing to confirm the article publication charge based on the information you supplied during submission of the following manuscript:

Article Title: Potential Therapeutic Use of Relaxin in Accelerating Closure of Cranial Bone Defects in Mice

Manuscript ID: PHY2-2019-04-0128

2. Keywords

Mice, cranial defect closure, relaxin, osmotic pump, collagen scaffold, angiogenesis, vasculogenesis, 3-D microcomputed tomography, immunohistochemistry

3. Accomplishments

A. **Major Goals**

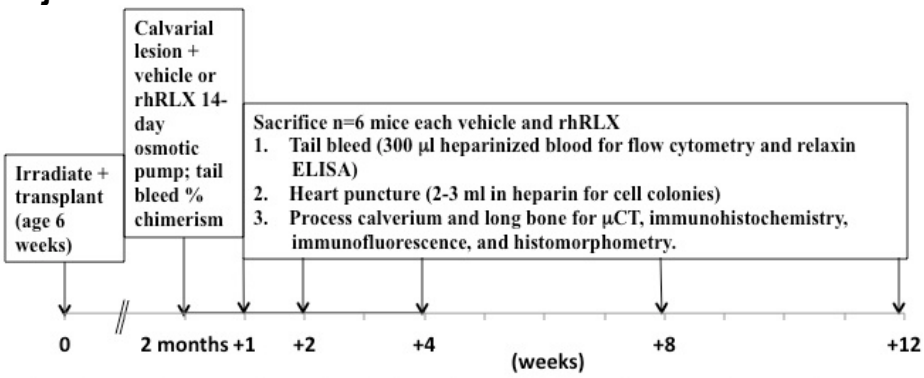


Figure 1. Potential therapeutic use of relaxin in healing cranial bone defects: Experimental Design

Major Tasks

a. Mouse manipulations
    i. Subtasks
        1. Completed
b. Necropsy
    i. Subtasks
        1. Completed
c. Bone analyses
    i. Subtasks
        1. Completed
d. Assays
    i. Subtasks
        1. Completed
e. Data analysis and statistics
    i. Subtasks

1. Completed

B. **What was accomplished under these goals?**

a. ***Major Activities***: For the abbreviated 2018-2019 final reporting period, we completed analyzing all data, prepared figures and tables for publication, wrote the manuscript and submitted to *Physiological Reports* on April 10, 2019 (manuscript attached).

b. ***Specific Objectives***: To prepare and submit our data for publication.

c. ***Significant Results***: See previous Reports, Report Summary above, and manuscript submission attached.

d. ***Other Achievements***: Nothing to Report

e. **What opportunities for training and professional development has the project provided?** Nothing to Report

f. **How were the results disseminated to communities of interest?** Manuscript submitted for publication.

g. **What do you plan to do for the next reporting period to accomplish the goals?** N/A

4. Impact

A. **What was the impact on the development of the principal discipline(s) of the project?** Unfortunately, the study results were negative.

B. **What was the impact on other disciplines?** Nothing to Report

C. **What was the impact on technology transfer?** Nothing to Report

D. **What was the impact on society beyond science and technology?** Nothing to Report

5. Changes/Problems

A. **Changes in approach and reasons for change:** N/A

B. **Actual or anticipated problems or delays and action or plans to resolve them:** N/A

C. **Changes that had a significant impact on expenditures:** Nothing to Report

D. **Significant changes in use or care of human subjects, vertebrate animals, biohazards, and/or select agents:** Nothing to Report

6. **Products:** Nothing to Report

7. **Participants & Other Collaborating Organizations**
   A. **What individuals have worked on the project?**
   PI, Co-Investigators and Staff (2018-2019)

   Kirk P. Conrad MD PI: 0.6 calendar month; Joshua F. Yarrow PhD Co-I (VA Medical Center, Gainesville, FL): 0.3 calendar month; Ignacio Aguirre PhD Co-I and Technician contributed 0.3 calendar month each.

   B. **Has there been a change in the active other support of the PD/PI(s) or senior key personnel since the last reporting period?** Nothing to Report

   C. **What other organizations were involved as partners?** Nothing to Report

8. **Special Reporting Requirements:** Nothing to Report

9. **Appendices:** Upload Submitted Manuscript to Physiological Reports.

Article Title: Potential Therapeutic Use of Relaxin in Accelerating Closure of Cranial Bone Defects in Mice

Manuscript ID: PHY2-2019-04-0128

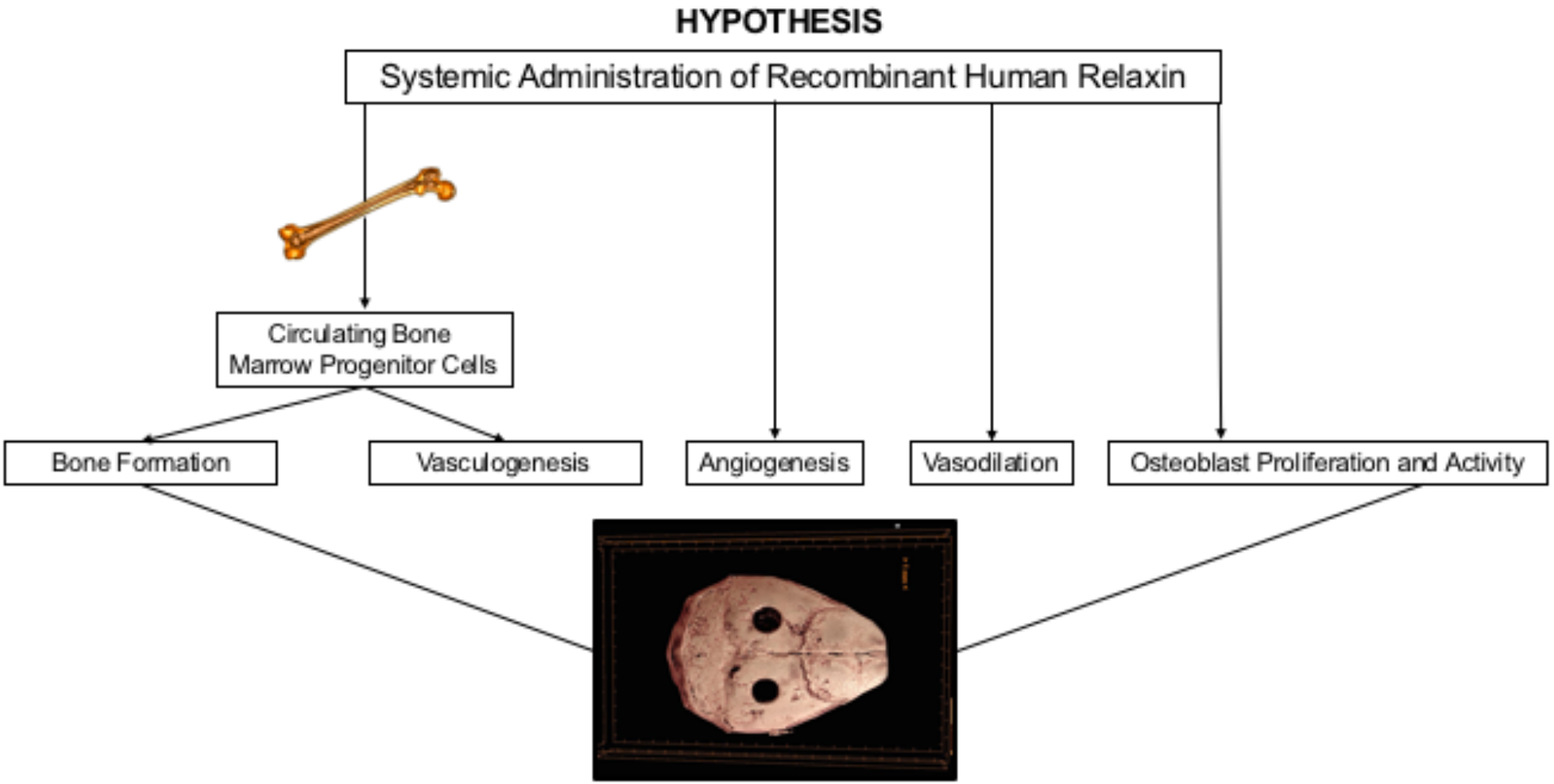


Figure 1. Bone perfusion is a rate-limiting step in bone formation, and hence, healing. We hypothesized that, in a murine calvarial lesion model, administration of recombinant human relaxin would increase circulating bone marrow derived angio-/osteogenic progenitor cells and enhance their uptake into the lesion site promoting vasculogenesis and bone formation, while concurrently stimulating angiogenesis, and bone formation by direct effects on osteoblasts. The current study was not supportive of a beneficial role for recombinant human relaxin delivered either systemically or locally via collagen scaffolds applied to the lesions.

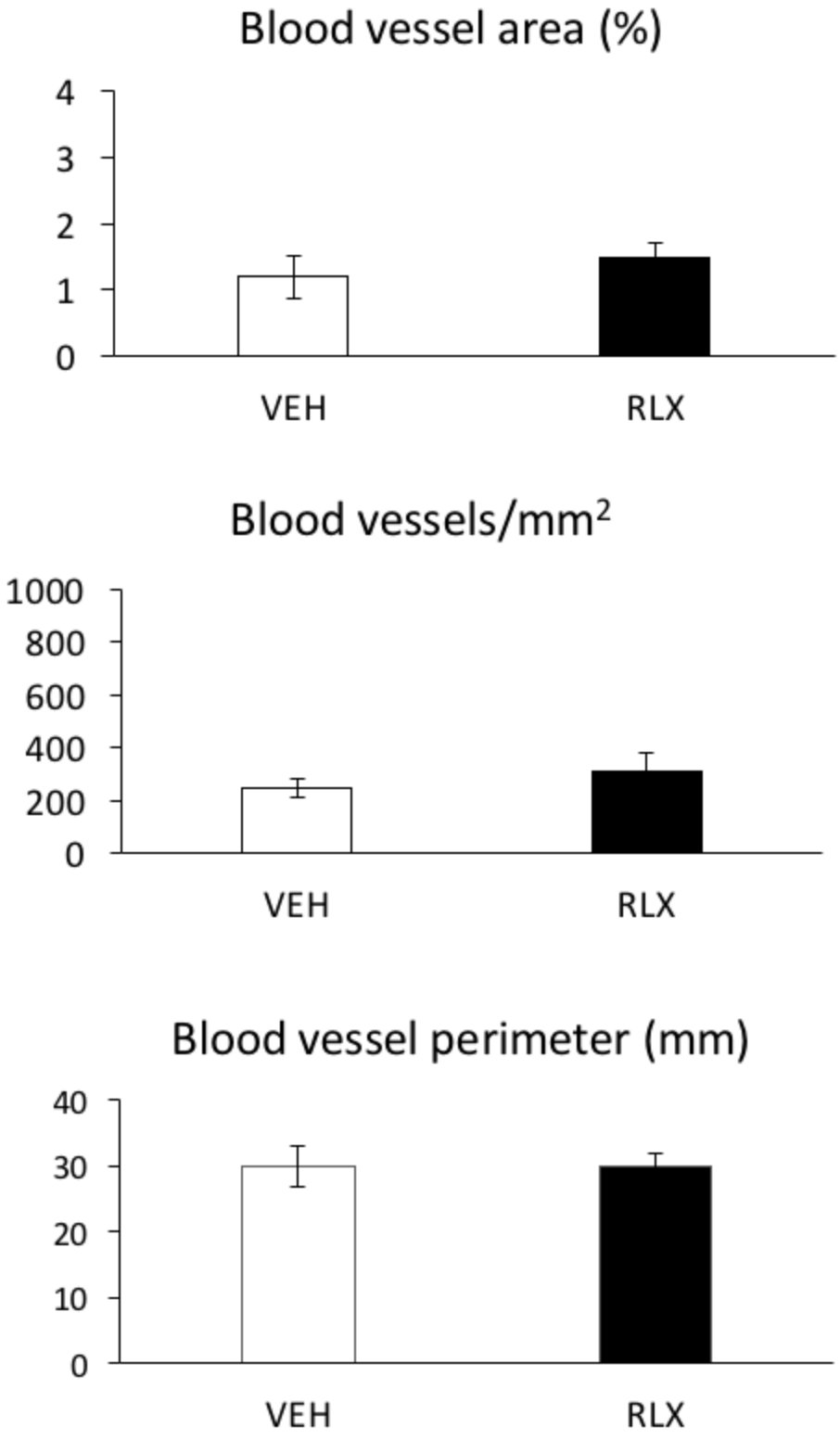


Figure 2. Protocol 1. Quantification of blood vessels in the calvarial defects.

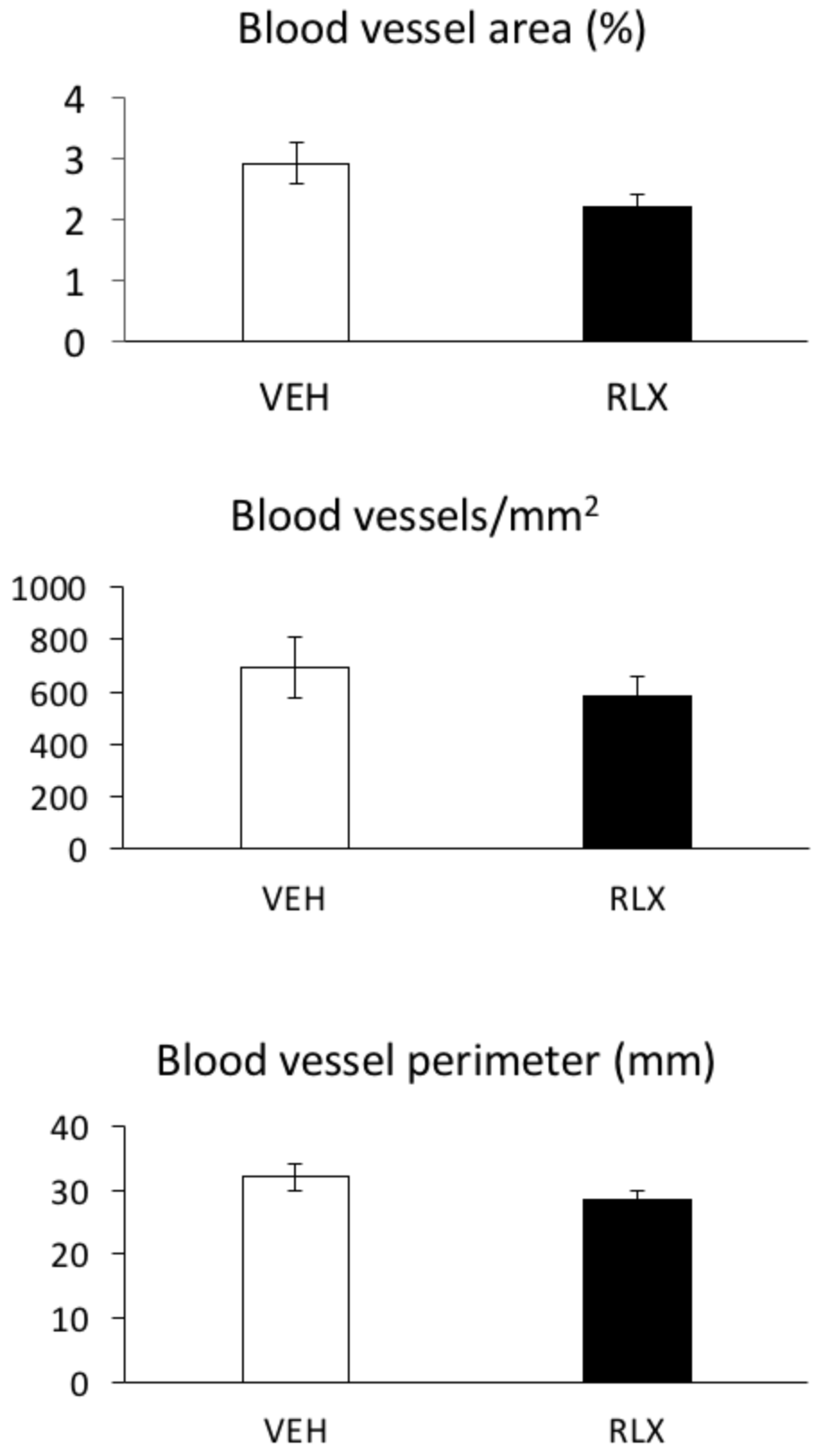
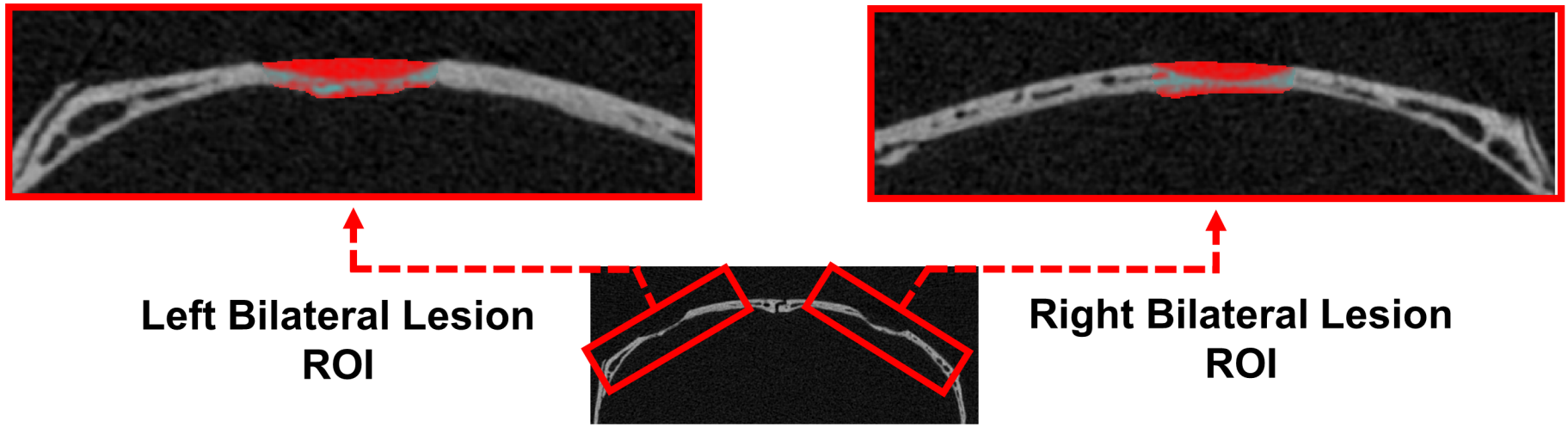
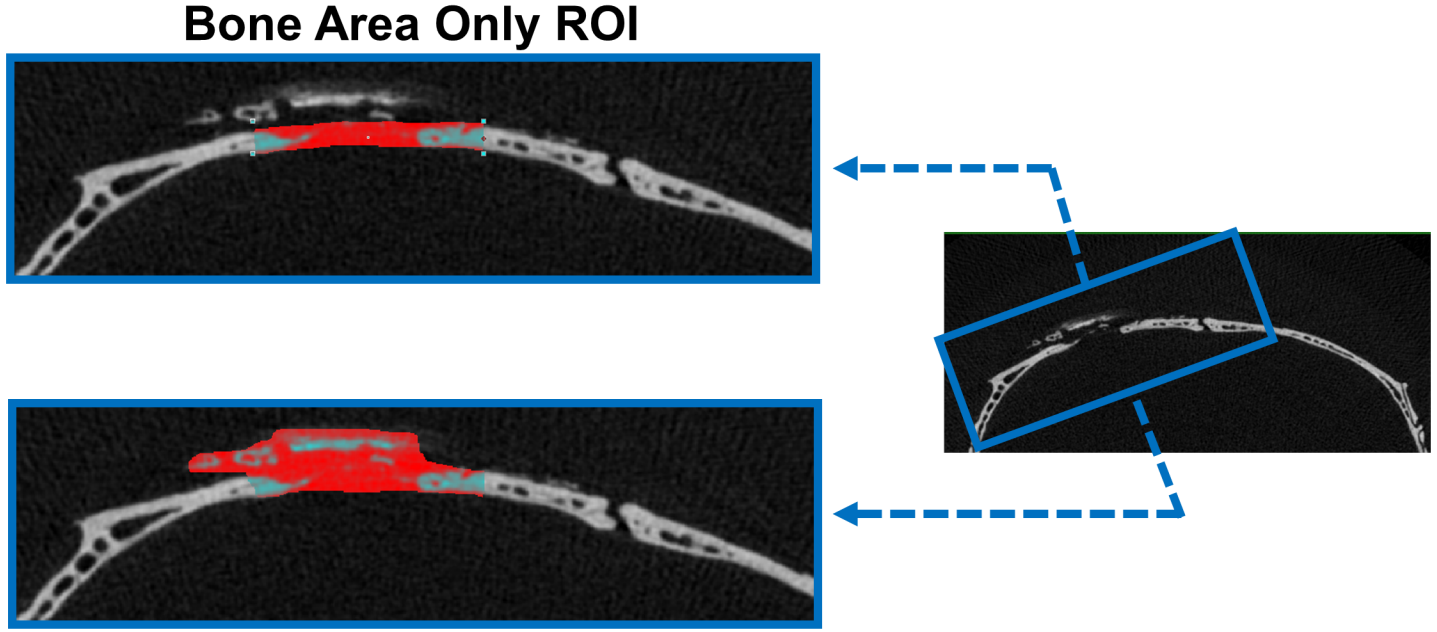


Figure 3. Protocol 2. Quantification of blood vessels in the calvarial defects.

Supplemental Fig 1A. Bilateral Lesion ROI



Supplemental Fig 1B. Bone Area Only and Bone + Scaffold ROI



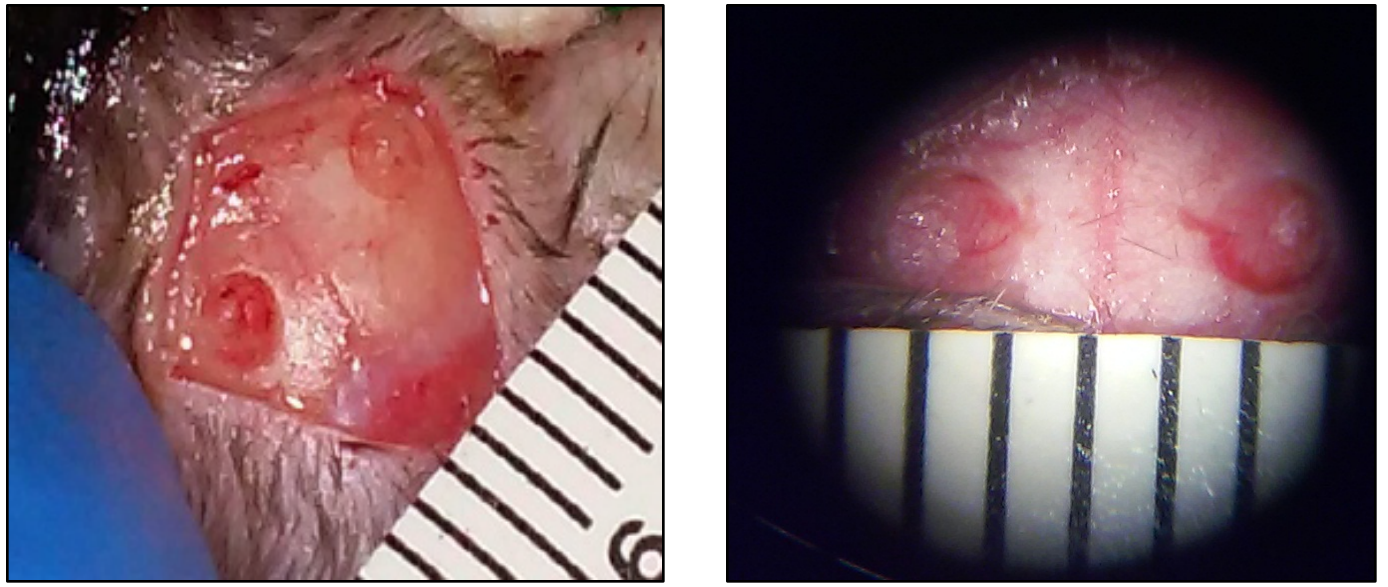


Figure S2. Representative Photomicrographs of Bilateral Cranial Lesions

Potential Therapeutic Use of Relaxin in Accelerating Closure of Cranial Bone Defects in Mice

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Abstract

Bone fractures are associated with considerable morbidity and increased mortality. A major limitation to healing is lack of bone blood flow, which is impaired by physical disruption of intra-skeletal and/or periosteal vasculature by the fracture. Thus, pharmacological interventions that improve osseous blood flow, thereby accelerating bone fracture closure are needed. Relaxin is secreted by the ovary and circulates in rodents and humans during pregnancy. Because relaxin possesses attributes that could benefit bone fracture healing including stimulation of angiogenesis, vasculogenesis (and potentially osteogenesis) by mobilizing and activating bone marrow progenitor cells, and blood flow via vasodilation, we tested whether relaxin administration would accelerate closure of a calvarial defect in mice. Whether administered systemically by osmotic pump or locally by collagen scaffolds for ~2 week period after lesioning, relaxin did not accelerate bone healing. Despite implementing relaxin doses that reached plasma concentrations spanning the physiological to supra-physiological range, testing the closure of 2 different sizes of calvarial lesions, allowing for different intervals of time from instigation of cranial lesion to euthanasia, and investigating mice of different ages, we did not observe significant benefit of relaxin in bone lesion healing. Nor did we observe stimulation of blood vessel formation in the bone lesion by the hormone. An incidental finding was that relaxin appeared to enhance trabecular bone growth in an uninjured control bone (femur). Although the results of this study were not supportive of a beneficial effect of relaxin on calvarial defect closure, future investigation is needed employing different animal species and experimental models of bone fracture.

Key Words: bone fracture, blood flow, angiogenesis, calvaria, femur, bone scaffold

Running Title: Relaxin and Bone Healing

Introduction

Bone fractures significantly increase the risk of mortality, and medical expenditures range from approximately 7000 to nearly $19,000 per fracture (depending on site), such that annual hospitalization costs exceed that of myocardial infarction, stroke, or breast cancer (26). These direct costs are compounded by indirect costs associated with absenteeism and short-term disability. Moreover, the prevalence of osteoporosis is much higher in women than men as is fracture incidence (26). In the US Military, return-to-duty criteria necessitate full recovery from bone fracture for front-line active duty service members, which can take upwards of several months. Unfortunately, US Military involvement in Operation Enduring Freedom/Operation Iraqi Freedom and other recent conflicts resulted in a high incidence of traumatic fractures for front-line active duty service members (17, 27). The incidence of traumatic fractures is expected to increase in future conflicts, in part due to the increasing usage of improvised explosive devices by adversaries. Consequently, failed or delayed bone healing is a major clinical problem in both the general population and the military, which necessitates the development of novel pharmacologic agents to accelerate bone healing.

A major rate-limiting step in bone healing is the degree of osseous blood flow, which is impeded by concomitant physical disruption of the intra-skeletal and/or periosteal vasculature following fracture. In addition, blood flow to the bone may be further compromised due to pre-existing vascular disease in individuals who smoke, or have metabolic syndrome, elevated cholesterol or diabetes mellitus (8). Angiogenesis (branching of existing blood vessels) is essential for bone fracture healing, and recent studies also reveal important roles for vasculogenesis (*de novo* formation of blood vessels) and osteogenesis both mediated by bone marrow derived progenitor cells (BMPC) (19, 22, 28). Therefore, therapeutic agents are needed that will improve bone perfusion, and hence, healing of fracture lesions.

A logical approach to improve bone fracture healing would be to harness a therapeutic agent, which possesses the following attributes: (i) abets local angiogenesis, (ii) stimulates

vasculogenesis and osteogenesis by mobilizing and activating BMPC, (iii) enhances blood flow via vasodilation, (iv) directly stimulates bone formation, (v) counteracts inflammation, and (vi) is clinically safe. To-date, we are unaware of a single FDA-approved agent that possesses all of these beneficial characteristics. However, as detailed next, the hormone relaxin fulfills these criteria, and thus may provide a novel approach to hasten bone fracture healing (**Figure 1**).

Relaxin is a natural hormone in women secreted by the corpus luteum, which circulates in the luteal phase as well as during pregnancy contributing to maternal vasodilation (6, 25). Although it is unlikely to circulate in men, relaxin administration is nevertheless bioactive, leading to vasodilation, because males also have relaxin receptors in arteries (10, 11, 14, 15, 21). Several investigators reported that relaxin promotes angiogenesis in cycling and pregnant endometrium, skin wounds, muscle injury and ischemic myocardium (reviewed in (16)). Recombinant human relaxin (rhRLX) increases human BMPC migration and nitric oxide production *in vitro* (24). Moreover, administration of rhRLX increases circulating BMPC, and enhances their incorporation and differentiation into endothelial cells in a matrigel plug assay in mice, i.e., vasculogenesis (24). Circulating relaxin contributes to maternal vasodilation in pregnancy, and administration of relaxin to female and male, rodents and humans elicits renal and systemic vasodilation (6). Relaxin stimulates osteoblast differentiation *in vitro* suggesting that this novel agent may stimulate bone formation *in vivo* (20). A potential advantage of relaxin over other agents like granulocyte colony-stimulating factor (G-CSF) routinely used to mobilize BMPC is that relaxin may exert an anti-inflammatory rather than inflammatory response (4, 18). In the case of G-CSF, its inflammatory response leads to undesirable side-effects (13). Finally, based on several clinical trials in heart failure, rhRLX (Serelaxin) has a good safety profile (14, 23). In light of the aforementioned attributes, relaxin may be a novel therapeutic for improving bone fracture healing. The purpose of this work was to determine whether rhRLX administration accelerates bone healing of a calvarial defect model in mice.

Materials and Methods

Mice

In this proof-of-principle study, we investigated male rats, because males are currently the majority of front-line soldiers. Power analysis was based on Wang and coworkers (28), in which they tested the role of AMD3100, a C-X-C chemokine receptor type 4-antagonist to mobilize BMPC and accelerate closure of the same calvarial defect model as employed herein. These investigators observed significant differences compared to vehicle-infused mice using 6 treated and control animals each. We purchased C57Bl/6J male mice from Jackson Lab, and all animal procedures were approved by the University of Florida Institutional Animal Care and Use Committee, and the Animal Care and Use Review Office of the USAMRMC Office of Research Protection.

Mouse Experimental Procedures

In **Protocols 1-3**, we used different rates of rhRLX infusion by subcutaneous osmotic pump (Alzet Osmotic Pumps, Durect, Co.), in order to span low, medium and high concentrations in the blood. In **Protocol 4**, we employed a collagen scaffolding to deliver relaxin locally to the bone lesion. Protocol procedures are detailed below.

Protocol 1—Recombinant human relaxin subcutaneous infusion: 1.0 µg/h (24)

The general protocol was adapted from the study by Wang et al. (28). Male mice of 13-15 weeks of age were anesthetized with isoflurane using a portable anesthesia machine (Summit Medical, Bend, OR). After shaving the head and aseptically preparing the surgical area, a mid-line skin incision was made with a scalpel blade starting between the ears and ending over the occipital bone. The incision window was first positioned over the left and then right parietal bones, in order to make 1.5 mm bilateral calvarial lesions with a dental burr being careful not to injure the underlying dura mater. Photomicrographs, including scale, of the lesion site and micrometer-

based measurements of lesion dimension were obtained to establish the original lesion area. The scalp wound was then closed with interrupted 6-0 nylon suture.

During the same surgical setting, after shaving the left or right flank of the animal anterior to the hip and then aseptically preparing the surgical area, a small skin incision was made with scissors, and a subcutaneous tunnel formed for insertion of an Alzet osmotic pump (Model 1002 14-day infusion) containing either recombinant human relaxin (rhRLX) or vehicle (20 mM sodium carbonate, pH 5.2). The skin was subsequently closed with 1 or 2 miniature auto-clips.

After surgical recovery, the mice were placed into their home cages for ~11 weeks to allow for healing of cranial lesions prior to euthanasia and quantitation of lesion closure, and blood vessels parameters in the lesion area (see below). Approximately 8 days after the start of the infusion, ~100 µl of blood was drawn from the tail vein into heparinized capillary tubes for preparation of plasma for measurement of rhRLX by ELISA (see below).

Protocol 2—Recombinant human relaxin subcutaneous infusion: 0.05 µg/h

In this protocol, the male mice were 12-13 weeks of age at the time of surgery. The percent lesion closure was comparably high in Protocol 1 regardless of vehicle or rhRLX administration; therefore, we made adjustments in Protocol 2 in two major ways. First, we euthanized the mice 10-12 days instead of 11 weeks after making the cranial lesions and implanting the osmotic pumps, and we reduced the rhRLX infusion rate to reach a lower circulating concentration than that reached in Protocol 1. Otherwise procedures were the same as in Protocol 1.

Protocol 3—Recombinant human relaxin subcutaneous infusion: 0.20 µg/h

Male mice were 13-14 months of age at the time of surgery. The purpose of using older mice was to assess whether the rate of lesion closure might be retarded compared to younger mice, and consequently, more responsive to rhRLX treatment. In Protocol 3, we administered rhRLX by subcutaneous osmotic pump at a rate of 0.2 µg/h, in order to reach plasma

concentrations between those of Protocol 1 and 2. In addition, we euthanized the animals ~5 weeks after making the bilateral cranial lesions, which in this study were 3 mm in diameter. The purpose of making larger diameter lesions was to determine whether the extent of closure would be less extensive relative to the smaller diameter of ~1.5 mm, and consequently, more responsive to rhRLX treatment. Otherwise procedures were the same as in Protocol 1.

Protocol 4—Recombinant human relaxin locally administered by collagen scaffolds: 1.0 µg/scaffold.

Male mice were 13-14 months of age at the time of surgery, in which a unilateral 3 mm cranial lesion was made. The amount of rhRLX impregnated into each scaffold (Bio-Glide Geistlich Biomaterials, Princeton NJ) was based on an *in vitro* release assay (see below). The scaffolds containing rhRLX or vehicle were applied to the lesion, and then by applying a droplet or two of Vetbond Tissue Adhesive to the outer perimeter at several points, they were tacked to the bone. As in Protocol 3, we euthanized the mice ~5 weeks after surgery. Additionally, tail blood was obtained as previously described, in order to determine whether systemic levels of rhRLX would be detectable after local application by scaffold. Otherwise procedures were the same as in Protocol 1.

Bone Processing

In Protocol 1, mice were deeply anesthetized with isoflurane, and then after thoracotomy and heart puncture, 2-3 ml heparinized saline were injected into the ventricle followed by aspiration until the mouse was exsanguinated. The mouse was next perfused with 4% PFA, and the calvarium and the right femur (an uninjured long-bone) harvested and placed in 4% PFA at 4°C for 24 hours, washed 3 times with 1X PBS, and then stored in 70% ethanol for high-resolution three-dimensional (3D) microcomputed tomography (µCT, see below). Following µCT (3), the

parietal bones encompassing the bone defect lesions were decalcified, dehydrated in increasing concentrations of ethanol and embedded in paraffin. Paraffin blocks were sectioned at 5 μm using Accu-Cut SRM 200 Sakura microtomes (Sakura Finetek Europe B.V, Zoeterwoude, The Netherlands). Tissue sections were then stained with H&E, coverslipped, and examined under a microscope Olympus BX43 (Olympus Center Valley, PA, USA). In addition, an histomorphometric evaluation of angiogenesis was performed on the bone defects sections by labelling blood vessels with an specific blood vessel marker using immunohistochemistry techniques (see below).
For all measurements, observers were blinded to treatment.

In Protocols 2-4, mice were deeply anesthetized by isoflurane and then decapitated. After dissecting and cleaning the calvarium, it was placed in 4% PFA at 4°C for 24 hours, washed 3 times with 1X PBS, and then stored in 70% ethanol until analysis as described above.

High-resolution three-dimensional (3D) microcomputed tomography (μCT)

The parietal region of the calvaria and right femurs were scanned *ex vivo* with a Bruker Skyscan 1172 μCT (Kontich, Belgium) to determine the effects of rhRLX on lesion closure and bone morphometry remote from the injury site, respectively, using our protocols that abide by guidelines of the American Society of Bone and Mineral Research (1-3). Briefly, calvaria from Protocols 1-4 were scanned at 50kVP/200μA with a 0.5 mm aluminum filter, 2k camera resolution, 10 μm voxel 0.7° rotation step, and 180° tomographic rotation. Cross-sectional images were reconstructed using a filtered back-projection algorithm (NRecon, Kontich, Belgium). A region of interest (ROI) that encompassed the entire lesion area was determined (CTAn, Konitch, Belgium) (**Supplemental Figure 1A**). Bone volume (BV, mm^3) and bone volume fraction (BV/TV, %) were calculated within this ROI. Volumetric bone mineral density (vBMD) and tissue mineral density (vTMD) were also calculated, following calibration with hydroxyapatite phantoms. Models of parietal calvaria were generated in CTVox (Bruker, Kontich, Belgium) and images were exported

to Image J (NIH) for 2D analysis of lesion closure, defined as bone area per original lesion area (%). Original lesion area was determined by assessing photomicrographs and micrometer-based measurements that were acquired at surgery. Additionally, left and right parietal lesions within the same animal were examined across groups to verify reproducibility of the lesion area and closure parameters. For Protocol 4, two separate ROIs were developed that included bone within the lesion area and bone within the lesion area plus that surrounding the scaffolding area because the scaffolding extended into areas surrounding the original lesion (**Supplemental Figure 1B**).

Femurs (Protocol 1 only) were scanned in an identical manner at 7.2 μm voxel. Images were reconstructed, as described above. The cancellous ROI at the distal femoral metaphysis began 0.75 mm proximal to the growth plate and encompassed 1 mm. The cortical ROI at the femoral diaphysis encompassed a 1 mm region at 45% of the femoral length, to avoid the third trochanter. 2D and 3D morphometric measurements at the distal femoral and femoral diaphysis ROIs included: cancellous bone volume (BV/TV, %), trabecular number (Tb.N, #/mm), trabecular separation (Tb.Sp, mm), trabecular thickness (Tb.Th, mm), trabecular pattern factor (Tb.Pf, #/mm), total cross-sectional area inside the periosteal envelope (Tt.Ar, mm^2), cortical bone area (Ct.Ar, mm^2), medullary area (Ma.Ar, mm^2), cortical area fraction (Ct.Ar/Tt.Ar, %), and 3D cortical thickness (Ct.Th, mm) (3).

Quantification of blood vessels in the calvarial defects

An evaluation of angiogenesis was performed during the healing phase of the calvarial defects. Blood vessel number (#/mm^2), blood vessel area (%) and blood vessel perimeter (mm) were assessed in the area of the calvarial defects by labelling blood vessels in the histologic sections with a specific marker of blood vessels using immunohistochemistry techniques. For this purpose, paraffin-embedded sections (5 μm) of decalcified transverse sections of calvariae were deparaffinized and rehydrated through graded alcohols. Endogenous peroxidase was quenched

by treatment with 3% hydrogen peroxide in methanol for 10 min. Heat-mediated antigen retrieval was performed by incubating slides for 25 min at 96°C in DAKO Target Retrieval Solution pH 6.0 (Clara, CA, USA). Sections were then blocked for 20 min in 10% goat serum in tris-buffered saline with 1% Tween, rinsed, and blocked with Avidin/Biotin blocking kit (Vector Labs, Burlingame, CA) for 30 min. Slides were incubated overnight at 4°C in a humidified chamber with a purified rat anti-mouse Pan Endothelial Cell antibody (MECA-32; BD Pharmingen, Cat #550563) at a concentration of 1.56 µg/ml in antibody diluent (Zymed Laboratories Inc). The secondary antibody was a biotinylated goat anti-rat IgG antibody, mouse absorbed (1:100 dilution; Vector Laboratories Cat#BA-9401). The antigen was visualized with Vectastain ABC Elite kit (Vector Laboratories, Burlingame, CA, USA). Diaminobenzidene (DAB) was used as the chromogen. Negative controls in sections incubated without primary antibody demonstrated absence of signal. Slides were counterstained with hematoxylin (QS; Vector Laboratories), dehydrated, cleared in xylene, mounted in Permount (Fisher Scientific), and examined by light microscopy.

***In Vitro* rhRLX release assay**

Bio-Glide collagen sheets were cut into 3-4 mm diameter circular disks. This size completely absorbed 10 µl fluid. Stock rhRLX was diluted accordingly with DPBS without calcium/magnesium containing penicillin/streptomycin and 0.1% BSA to yield 0.5 and 5.0 µg rhRLX. After sterilization by autoclave, the small disks were suffused with 0.5 or 5.0 µg rhRLX each in 10 µl volume, and allowed to dry for 2 hours. The disks containing rhRLX were next placed into wells of a 24-well plate with 0.4 ml DPBS without calcium/magnesium containing penicillin/streptomycin and 0.1% BSA. The conditioned medium was harvested after 1, 2, 3, 4, 6, 8, and 10 days at 37^0C in a cell culture incubator. Recombinant human relaxin was measured in the conditioned medium using the R&D ELISA as described below. Based on this *in vitro* release assay, a dose of 1.0 µg/scaffold was used in Protocol 4 above (see Results).

Relaxin ELISA

Plasma or conditioned medium rhRLX concentration was measured in duplicate using a commercially available human ELISA also validated for mouse plasma (R&D Systems, Minneapolis, MN). The lowest standard in the assay was 7.8 pg/ml. According to the manufacturers specifications, the intra- and inter-assay precision ranged from 2.3 - 4.7 and 5.5 - 10.2 %, respectively (average CV of variation). Recovery after spike was 91 – 104 % and linearity 105 – 111 % of expected concentrations. As expected, plasma rhRLX was undetectable in vehicle-treated mice (data not shown).

Statistical analysis

Data were expressed as mean ± SE for each group. For Protocols 1-3, paired samples t-tests were used to assess differences in initial lesion area and lesion closure for the bilateral (left vs right) lesions in the same animal. Right and left bilateral lesion volumes were significantly correlated with no differences between sides (see Results). As such, right and left lesion parameters were averaged within each animal, and the average lesion values were compared between groups using independent samples t-tests. Femoral parameters (Protocol 1) and lesion values (Protocol 4) were compared between groups using independent samples t-tests because bilateral values were not present for these outcomes. The blood vessel parameters were also compared between the relaxin and vehicle treatment groups using independent sample-t tests. *P* values less than 0.05 were considered to be statistically significant.

Results

Representative photographs of the cranial lesions are depicted in **Supplemental Figure 2.** Overall, the baseline left and right lesion widths were 1.78 and 1.79 mm, respectively (N=28, p=0.947) with a correlation coefficient of 0.97 (p<0.001). Similarly, the baseline left and right lesion heights were 1.91 and 1.94 mm, respectively (N=28, p=0.435) with a correlation coefficient of 0.95 (p<0.001). Overall, the left and right lesion closures were also comparable—63.7 and 61.3%, respectively (p=0.401) with a correlation coefficient of 0.60 (p=0.001). Left and right calvarial BMD, TMD, BV and BV/TV were also not statistically significant from each other (p=0.43-0.87; data not shown). Thus, we averaged the left and right lesion values for all subsequent analyses.

Protocol 1—Recombinant human relaxin subcutaneous infusion: 1.0 µg/h

There were no significant differences between rhRLX and vehicle treatments for lesion closure (%), bone volume (mm^3), bone volume fraction (BV/TV%), bone mineral density (BMD g/cm^3) and tissue mineral density (TMD; g/cm^3) (**Table 1**). Plasma rhRLX was 53 $\pm$ 9 ng/ml [range 33-89].

Protocol 2—Recombinant human relaxin subcutaneous infusion: 0.05 µg/h

Because rhRLX can have a biphasic dose response curve in vivo (9, 11), we also explored a lower infusion rate that yielded an average circulating level of 1.5 $\pm$ 0.5 ng/ml [range 0.35-3.41]. Nevertheless, we did not observe significant differences between rhRLX and vehicle treatments for lesion closure (%), BV (mm^3), BV/TV (%), BMD (g/cm^3) and TMD (g/cm^3) (Table 1).

Protocol 3—Recombinant human relaxin subcutaneous infusion: 0.20 µg/h

An intermediate infusion rate produced a plasma rhRLX concentration of 4.9 ± 1.3 ng/ml [range 2.0-7.9]. However, rhRLX administration again failed to improve lesion closure or other bone parameters (Table 1).

Protocol 4—Recombinant human relaxin locally administered by collagen scaffolds: 1.0 µg/scaffold

Because systemic rhRLX administration was ineffective, we next tried local application by collagen scaffolds permeated with rhRLX. In order to establish a dose of rhRLX for delivery by the collagen scaffolds, we first performed an *in vitro* release assay (5, 29). 0.5 and 5.0 µg rhRLX were tested. The cumulative release over a period of 10 days was comparable between the doses (% of initial dose ~11.5%; **Table 2**). However, the rhRLX concentrations in the conditioned media differed markedly. For the 5.0 µg dose, it ranged from 815 ng/ml on day 1 to 3.4 ng/ml on day 10. The concentrations for the 0.5 µg dose were 77 and 0.4 ng/ml, respectively. Because the 5.0 µg dose generally produced pharmacological concentrations especially in the first 2 days, and the 0.5 µg dose yielded concentrations after 6 days that were generally low, we selected a dose of 1.0 µg. The treatment was indeed locally confined, because circulating rhRLX was undetectable (below the lowest ELISA standard of 7.8 pg/ml). Once again, however, there were no significant differences between rhRLX and vehicle-infused collagen scaffolds for the % lesion closure, BV, BV/TV, BMD or TMD whether bone in lesion only, or bone in lesion and scaffolding of surrounding area was analyzed for BV and TMD (**Table 3**).

Influence of relaxin (or vehicle) administration by subcutaneous osmotic pump on cancellous and cortical morphometry of the femur

The femur was harvested in Protocol 1, in order to determine whether rhRLX might have effects on uninjured bone remote from the cranial lesion. Interestingly, trabecular thickness (mm) and trabecular pattern factor (1/mm) were significantly higher and lower, respectively, in the

cancellous bone of the relaxin-treated mice (both $p<0.05$ vs vehicle; **Table 4**). The bone volume fraction showed a borderline significant increase with relaxin administration ($p=0.094$).

Quantification of blood vessels in the calvarial defects

No differences were observed in blood vessel area (%), blood vessel number (#/mm^2) and blood vessel perimeter (mm) in the area of the calvarial defects between VEH and RLX mice after 11 weeks of treatment (Protocol 1; **Fig. 2**). Similarly, no differences were observed in these parameters between VEH and RLX mice at 10-12 days post-treatment (Protocol 2; **Figure 3**). However, independent of the treatment group, mice at 10-12 days post-treatment (protocol 2) had greater blood vessel area (%) and blood vessel number (#/mm^2), but not blood vessel perimeter (mm) compared to mice after 11 weeks of treatment (protocol 1; compare Figs. 2 and 3).

Discussion

A major impediment to bone healing is the disruption of osseous blood flow. Hence, therapeutic agents that will improve bone perfusion may accelerate healing of fracture lesions. To this end, we reasoned that relaxin may be potentially a therapeutic molecule, because the hormone possesses biological attributes that theoretically-speaking could improve (i) local angiogenesis, (ii) vasculogenesis and osteogenesis by mobilizing and activating BMPC, (iii) blood flow via vasodilation, (iv) bone formation, and (v) reduce inflammation (Fig. 1; 4, 6, 7, 12, 16, 18, 24). Moreover, relaxin has a good safely record based on human clinical trials for other indications (14, 23).

Our pre-clinical study in mice did not support a beneficial role for relaxin in bone lesion healing whether administered systemically or locally (Tables 1-3). Despite implementing a rhRLX dose response reaching mean plasma concentrations which spanned the physiological to supra-physiological range (1.5, 4.6 and 53 ng/ml), testing 2 different sizes of lesions (~1.5 and 3.0 mm), imposing different intervals of time from imposition of cranial lesions to euthanasia (1.6, 5 and 11 weeks), and investigating different mouse ages (3-4 and 13-14 months), we were unable to elicit a salutary role for relaxin in bone lesion healing. Consistent with this lack of response in accelerating the closure of the parietal defects, was the absence of any beneficial effect on vascularity in the bone lesions by relaxin. One possible explanation for the lack of beneficial effect is that the relaxin receptor, RXFP1, is not expressed in all blood vessels, and perhaps those relevant to perfusion necessary for healing of the calvarium are devoid of receptors (15). However, independently of the experimental group, the angiogenic process was more active at the earlier time point after implementing the cranial bone lesion (10-12 days), than after 11 weeks in support of bone healing (Figs. 2, 3).

A somewhat incidental finding is that relaxin may have stimulated trabecular bone growth in an uninjured bone (femur), as evidenced by higher Tb.Th and lower Tb.Pf (Table 4). This finding is consistent with previous reports that relaxin may stimulate osteoblast activity, resulting in bone

formation (12). However, a larger study utilizing histomorphometry is necessary to confirm whether relaxin may have produced a bone anabolic or anti-resorptive effect in the cancellous bone of the femur. It is possible that relaxin may have an effect in skeletal bones with endochondral ossification, but not in craniofacial bones with intramembranous ossification and periosteal bone formation.

Significant strengths of this work include the overall study design, which was carefully and thoughtfully, constructed and implemented; state-of-the-art μCT approaches for quantifying bone lesion closure volume and density; and implementation of appropriate protocol changes as dictated by the results as described above. Potential weaknesses include the limited number of mice per group. Even though for the majority of experimental protocols, we met the power analysis criteria based on Wang and colleagues (28), this work should still be considered a pilot study. Another possible drawback is that we had sufficient funding to investigate only one sex. We chose males, because to date, the majority of front-line combatants at risk for bone trauma are males. Moreover, we have not observed any differences in the physiological responses of males and females to relaxin administration at least in the cardiovascular system, and the focus of this work was mainly on improving osseous blood supply (10, 11). Nevertheless, future investigations definitely need to include female animals. Perhaps relaxin would prove beneficial in repairing bone in females.

In summary, relaxin did not accelerate bone lesion closure in a mouse model. However, this study should not dissuade future investigation of a potential salutary role for relaxin by testing different animal species and experimental models of bone fracture, as well as the female sex.

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Disclosures

KPC discloses use patents for relaxin. The other authors have declared that no conflict of interest exists.

References

1. **Beck DT, Yarrow JF, Beggs LA, Otzel DM, Ye F, Conover CF, Miller JR, Balaez A, Combs SM, Leeper AM, Williams AA, Lachacz SA, Zheng N, Wronski TJ, and Borst SE.** Influence of aromatase inhibition on the bone-protective effects of testosterone. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 29: 2405-2413, 2014.
2. **Beggs LA, Ye F, Ghosh P, Beck DT, Conover CF, Balaez A, Miller JR, Phillips EG, Zheng N, Williams AA, Aguirre JI, Wronski TJ, Bose PK, Borst SE, and Yarrow JF.** Sclerostin Inhibition Prevents Spinal Cord Injury Induced Cancellous Bone Loss. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research*, 2014.
3. **Bouxsein ML, Boyd SK, Christiansen BA, Guldberg RE, Jepsen KJ, and Muller R.** Guidelines for assessment of bone microstructure in rodents using micro-computed tomography. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 25: 1468-1486, 2010.
4. **Brecht A, Bartsch C, Baumann G, Stangl K, and Dschietzig T.** Relaxin inhibits early steps in vascular inflammation. *Regul Pept* 166: 76-82, 2011.
5. **Charles LF, Woodman JL, Ueno D, Gronowicz G, Hurley MM, and Kuhn LT.** Effects of low dose FGF-2 and BMP-2 on healing of calvarial defects in old mice. *Exp Gerontol* 64: 62-69, 2015.
6. **Conrad KP.** Maternal vasodilation in pregnancy: the emerging role of relaxin. *Am J Physiol Regul Integr Comp Physiol* 301: R267-275, 2011.
7. **Conrad KP.** Unveiling the vasodilatory actions and mechanisms of relaxin. *Hypertension* 56: 2-9, 2010.
8. **Conte SM and Vale PR.** Peripheral Arterial Disease. *Heart Lung Circ* 27: 427-432, 2018.
9. **Danielson LA and Conrad KP.** Time course and dose response of relaxin-mediated renal vasodilation, hyperfiltration, and changes in plasma osmolality in conscious rats. *J Appl Physiol* 95: 1509-1514, 2003.
10. **Danielson LA, Kercher LJ, and Conrad KP.** Impact of gender and endothelin on renal vasodilation and hyperfiltration induced by relaxin in conscious rats. *Am J Physiol* 279: R1298-R1304, 2000.
11. **Debrah DO, Conrad KP, Danielson LA, and Shroff SG.** Effects of relaxin on systemic arterial hemodynamics and mechanical properties in conscious rats: sex dependency and dose response. *J Appl Physiol* 98: 1013-1020, 2005.
12. **Ferlin A, De Toni L, Sandri M, and Foresta C.** Relaxin and insulin-like peptide 3 in the musculoskeletal system: from bench to bedside. *Br J Pharmacol* 174: 1015-1024, 2017.
13. **Fischmeister G, Kurz M, Haas OA, Micksche M, Buchinger P, Printz D, Ressmann G, Stroebel T, Peters C, Fritsch G, and Gadner H.** G-CSF versus GM-CSF for stimulation of peripheral blood progenitor cells (PBPC) and leukocytes in healthy volunteers: comparison of efficacy and tolerability. *Annals of hematology* 78: 117-123, 1999.
14. **Ghosh RK, Banerjee K, Tummala R, Ball S, Ravakhah K, and Gupta A.** Serelaxin in acute heart failure: Most recent update on clinical and preclinical evidence. *Cardiovasc Ther* 35: 55-63, 2017.
15. **Jelinic M, Leo CH, Post Uiterweer ED, Sandow SL, Gooi JH, Wlodek ME, Conrad KP, Parkington H, Tare M, and Parry LJ.** Localization of relaxin receptors in arteries and veins, and region-specific increases in compliance and bradykinin-mediated relaxation after in vivo serelaxin treatment. *FASEB J* 28: 275-287, 2014.
16. **Jeyabalan A, Shroff SG, Novak J, and Conrad KP.** The vascular actions of relaxin. *Adv Exp Med Biol* 612: 65-87, 2007.

17. **Lew TA, Walker JA, Wenke JC, Blackbourne LH, and Hale RG.** Characterization of craniomaxillofacial battle injuries sustained by United States service members in the current conflicts of Iraq and Afghanistan. *Journal of oral and maxillofacial surgery : official journal of the American Association of Oral and Maxillofacial Surgeons* 68: 3-7, 2010.
18. **Masini E, Nistri S, Vannacci A, Bani Sacchi T, Novelli A, and Bani D.** Relaxin inhibits the activation of human neutrophils: involvement of the nitric oxide pathway. *Endocrinology* 145: 1106-1112, 2004.
19. **Matsumoto T, Kawamoto A, Kuroda R, Ishikawa M, Mifune Y, Iwasaki H, Miwa M, Horii M, Hayashi S, Oyamada A, Nishimura H, Murasawa S, Doita M, Kurosaka M, and Asahara T.** Therapeutic potential of vasculogenesis and osteogenesis promoted by peripheral blood CD34-positive cells for functional bone healing. *Am J Pathol* 169: 1440-1457, 2006.
20. **Moon JS, Kim SH, Oh SH, Jeong YW, Kang JH, Park JC, Son HJ, Bae S, Park BI, Kim MS, Koh JT, and Ko HM.** Relaxin Augments BMP-2-Induced Osteoblast Differentiation and Bone Formation. *Journal of bone and mineral research : the official journal of the American Society for Bone and Mineral Research* 29: 1586-1596, 2014.
21. **Novak J, Parry LJ, Matthews JE, Kerchner LJ, Indovina K, Hanley-Yanez K, Doty KD, Debrah DO, Shroff SG, and Conrad KP.** Evidence for local relaxin ligand-receptor expression and function in arteries. *FASEB J* 20: 2352-2362, 2006.
22. **Otsuru S, Tamai K, Yamazaki T, Yoshikawa H, and Kaneda Y.** Circulating bone marrow-derived osteoblast progenitor cells are recruited to the bone-forming site by the CXCR4/stromal cell-derived factor-1 pathway. *Stem Cells* 26: 223-234, 2008.
23. **Said S and Mukherjee D.** Serelaxin: new investigational treatment in acute heart failure. *Cardiovascular & hematological agents in medicinal chemistry* 11: 243-248, 2013.
24. **Segal MS, Sautina L, Li S, Diao Y, Agoulnik AI, Kielczewski J, McGuane JT, Grant MB, and Conrad KP.** Relaxin increases human endothelial progenitor cell NO and migration and vasculogenesis in mice. *Blood* 119: 629-636, 2012.
25. **Sherwood OD.** Relaxin. In: *The Physiology of Reproduction* (Second Edition ed.), edited by Knobil E, Neill JD, Greenwald GS, Markert CL and Pfaff DW. New York: Raven Press, 1994, p. 861-1008.
26. **Singer A, Exuzides A, Spangler L, O'Malley C, Colby C, Johnston K, Agodoa I, Baker J, and Kagan R.** Burden of illness for osteoporotic fractures compared with other serious diseases among postmenopausal women in the United States. *Mayo Clinic proceedings* 90: 53-62, 2015.
27. **Wade AL, Dye JL, Mohrle CR, and Galarneau MR.** Head, face and neck injuries during operation Iraqi Freedom II: results from the US Navy and Marine Corps Combat Trauma Registry. In: *Naval Health Research Center Technical Report*. San Diego, 2006, p. 1-17.
28. **Wang XX, Allen RJ, Jr., Tutela JP, Sailon A, Allori AC, Davidson EH, Paek GK, Saadeh PB, McCarthy JG, and Warren SM.** Progenitor cell mobilization enhances bone healing by means of improved neovascularization and osteogenesis. *Plast Reconstr Surg* 128: 395-405, 2011.
29. **Wen B, Karl M, Pendrys D, Shafer D, Freilich M, and Kuhn L.** An evaluation of BMP-2 delivery from scaffolds with miniaturized dental implants in a novel rat mandible model. *J Biomed Mater Res B Appl Biomater* 97: 315-326, 2011.

Tables

Table 1. Influence of relaxin (or vehicle) administration by subcutaneous osmotic pump on cranial lesion closure.

	Lesion Closure (%)		BV(mm^3)		BV/TV (%)		BMD (g/cm^3)		TMD (g/cm^3)	
	V	R	V	R	V	R	V	R	V	R
Protocol 1	73.5 ± 3.5	67.0 ± 3.4	0.172 ± 0.012	0.165 ± 0.012	35.3 ± 1.4	34.3 ± 1.8	0.441 ± 0.015	0.428 ± 0.021	0.973 ± 0.007	0.964 ± 0.008
p-value	0.216		0.709		0.696		0.635		0.424	
Protocol 2	54.3 ± 2.2	45.8 ± 3.6	0.128 ±0.011	0.104 ± 0.010	25.6 ± 0.9	21.9 ± 1.3	0.351 ± 0.009	0.310 ±0.015	0.942 ± 0.006	0.943 ± 0.005
p-value	0.072		0.143		0.039		0.039		0.903	
Protocol 3	70.3 ± 9.5	76.1 ± 5.1	0.440 ± 0.141	0.457 ±0.024	28.3 ± 6.0	33.3 ± 2.6	0.371 ± 0.072	0.428 ± 0.025	1.001 ± 0.030	1.002 ± 0.004
p-value	0.591		0.895		0.444		0.434		0.963	

Mean ± SEM. BV, bone volume; BV/TV, bone volume fraction; BMD, bone mineral density; TMD, tissue mineral density; V, vehicle; R, recombinant human relaxin.

Protocol 1: mice were euthanized ~11 weeks after implementing bilateral 1.5 mm cranial lesions and subcutaneous implantation of 14 day osmotic pumps containing recombinant human relaxin (rhRLX; 1.0 µg/h) or vehicle (n = 6 mice each for relaxin and vehicle treatments).

Protocol 2: mice were euthanized 11-12 days after implementing bilateral 1.5 mm cranial lesions and subcutaneous implantation of 14 day osmotic pumps containing recombinant human relaxin (rhRLX; 0.05 µg/h) or vehicle (n = 6 mice each for relaxin and vehicle treatments).

Protocol 3: mice were euthanized ~5 weeks after implementing bilateral 3.0 mm cranial lesions and subcutaneous implantation of 14 day osmotic pumps containing recombinant human relaxin (rhRLX; 0.2 µg/h) or vehicle (n = 4 relaxin and n = 3 vehicle treated mice).

Table 2. *In vitro* release of recombinant human relaxin from Bio-Gide collagen disks.

	Days						
	1	2	3	4	6	8	10
Bio-Gide Collagen containing:							
Relaxin 0.5 ∝g							
ng/ml	77.3	50.8	7.3	3.4	0.6	0.5	0.4
ng	30.9	20.3	2.9	1.4	0.25	0.18	0.15
Cumulative release (% initial dose)	6.2	10.2	10.8	11.1	11.2	11.2	11.2
Relaxin 5.0 ∝g							
ng/ml	815	508	57	29	5.8	4.3	3.4
ng	325.8	207.0	22.8	11.6	2.3	1.7	1.4
Cumulative release (% initial dose)	6.5	10.7	11.1	11.3	11.4	11.4	11.5

Recombinant human relaxin released from the collagen scaffolds was measured in the conditioned media for up to 10 days. See Methods for details.

Table 3A. Influence of local relaxin (or vehicle) application by Bio-Gide collagen scaffold on cranial lesion closure (lesion, only).

	Lesion Closure (%)		BV-1 (mm^3)		BV/TV (%)		BMD-1 (g/cm^3)		TMD-1 (g/cm^3)	
	V	R	V	R	V	R	V	R	V	R
Protocol 4	81.3 ± 2.8	85.8 ± 3.3	0.899 ±0.037	0.969 ± 0.122	34.7 ± 1.7	39.8 ± 2.3	0.451 ± 0.021	0.486 ± 0.027	1.013 ± 0.010	1.011 ± 0.009
p-value	0.335		0.604		0.132		0.334		0.888	

Mean ± SEM. BV-1, bone volume in lesion only; BMD-1, bone mineral density in lesion only; TMD-1, tissue mineral density of bone in lesion only; V, vehicle; R, recombinant human relaxin.
Protocol 4: mice were euthanized ~5 weeks after implementing 3.0 mm unilateral cranial lesions and applying scaffolds containing rhRLX (1.0 µg/scaffold) or vehicle (n = 4 mice each for relaxin and vehicle treatments).

Table 3B. Influence of local relaxin (or vehicle) application by Bio-Gide collagen scaffold on cranial lesion closure (lesion and scaffolding).

	BV-2 (mm^3)		TMD-2 (g/cm^3)	
	V	R	V	R
Protocol 4	1.383 ± 0.126	1.515 ± 0.191	0.967 ±0.019	0.964 ± 0.007
p-value	0.586		0.899	

Mean ± SEM. BV-2, bone volume in lesion and scaffolding of surrounding area; TMD-2, tissue mineral density of bone in lesion and scaffolding of surrounding tissue; V, vehicle; R, recombinant human relaxin. Protocol 4: mice were euthanized ~5 weeks after implementing 3.0 mm unilateral cranial lesions and applying scaffolds containing rhRLX (1.0 µg/scaffold) or vehicle (n = 4 mice each for relaxin and vehicle treatments).

Table 4. Influence of relaxin (or vehicle) administration by subcutaneous osmotic pump on cancellous and cortical morphometry of the femur.

	Cancellous Morphometry									
	BV/TV (%)		Tb.Th (mm)		Tb.N (1/mm)		Tb.Sp (mm)		Tb.Pf (1/mm)	
	V	R	V	R	V	R	V	R	V	R
Protocol 1	1.81 ± 0.19	2.74 ± 0.38	0.037 ± 0.001	0.044 ± 0.002	0.494 ± 0.049	0.626 ± 0.075	0.377 ± 0.030	0.379 ± 0.025	51.1 ± 0.65	42.8 ± 2.69
p-value	0.094		0.030		0.229		0.957		0.040	
	Cortical Morphometry									
	3D Ct.Th (mm)		Tt.Ar (mm^2)		Ct.Ar (mm^2)		Ma.Ar (mm^2)		Ct.Ar/Tt.Ar (%)	
	V	R	V	R	V	R	V	R	V	R
Protocol 1	0.194 ± 0.004	0.193 ± 0.002	1.554 ± 0.026	1.616 ± 0.048	0.705 ± 0.011	0.719 ± 0.010	0.849 ± 0.025	0.897 ± 0.041	0.454 ± 0.008	0.446 ± 0.009
p-value	0.897		0.313		0.360		0.363		0.560	

Mean ± SEM. V, vehicle; R, recombinant human relaxin.

Protocol 1: mice were euthanized ~11 weeks after implementing bilateral 1.5 mm cranial lesions and subcutaneous implantation of 14 day osmotic pumps containing recombinant human relaxin (rhRLX; 1.0 µg/h) or vehicle (n = 6 mice for relaxin and n = 4 mice for vehicle treatments). BV/TV, bone volume fraction; Tb.Th, trabecular thickness; Tb.N, trabecular number; Tb.Sp, trabecular separation; Tb.Pf, trabecular pattern factor; Ct.Th, cortical thickness; Tt.Ar, total area inside the periosteal envelope; Ct.Ar, cortical bone area; Ma.Ar, medullary area; Ct.Ar/Tt.Ar, cortical bone area fraction.