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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 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 is 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 $\mu$g/scaffold); however, again, the lesion closure was comparable in the relaxin- and vehicle-treated mice (~80%) each. Consistent with this finding again is 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.

**15. SUBJECT TERMS**

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# Potential Therapeutic Use of Relaxin in Healing Cranial Bone Defects

## W81XWH-15-1-0162

**PI:** KP Conrad MD

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## 1. Introduction

This DOD Discovery Award addresses the general problem of failed or delayed healing of craniomaxillofacial fractures. The 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. 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.

To recap, 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 (2017-18 reporting period), 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.

## 2. Keywords

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

## 3. Accomplishments

### A. Major Goals

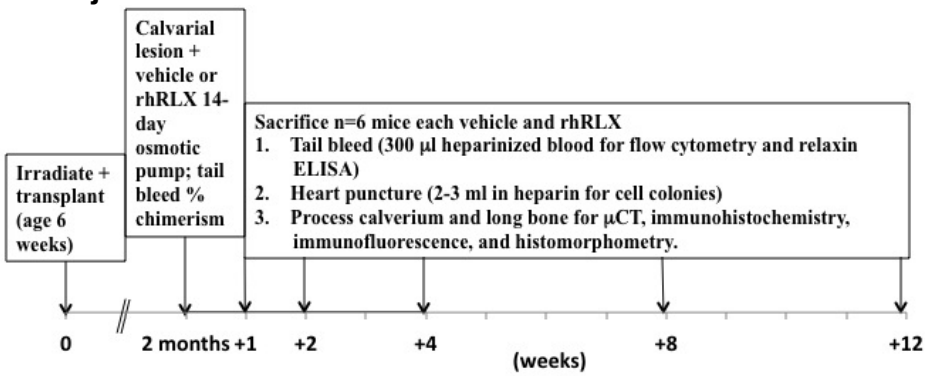


**Figure 1. Potential therapeutic use of relaxin in healing cranial bone defects: Experimental Design**

***Major Tasks***

**1.** Mouse manipulations

A. Subtasks

(i) Calvarial lesion

(ii) Osmotic pump implantation for systemic rhRLX delivery

(iii) Collagen scaffold for local rhRLX delivery

(iv) Tail bleed for measurement of rhRLX

**2.** Necropsy

A. Subtasks

(i) Bone harvest

(ii) Bone processing and fixation

**3.** Bone analyses

A. Subtasks

(i) Three dimensional computed tomography

(ii) Bone decalcification

(iii) Bone immunohistochemistry

**4.** Assays

(i) In vitro collagen scaffold release assay

(ii) Relaxin ELISA

**5.** Data analysis and statistics

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

***i. Major Activities***

**a.** Because the first experiments performed in years 01 and 02 yielded negative results, and the procedures for making chimeric mice with GFP+ bone marrow are time-consuming, laborious, and additional stress to the mice, we elected to forgo this initial step. The idea was that if we were eventually successful in accelerating bone lesion closure with rhRLX in subsequent experiments, then in future studies we would repeat the study using a smaller cohort of chimeric mice with GFP+ bone marrow, in order to quantitate bone marrow-derived progenitor cells at the lesion site, as proof of principle. In year 03 (current reporting period), after 3.0 mm cranial defects were created, vehicle or relaxin was administered systemically or locally for 10-12 days by osmotic pump or collagen scaffold, respectively. The mice were euthanized 10-12 days after the cranial defect and initiation of vehicle or rhRLX treatment. For these 2 cohorts of vehicle or rhRLX-administered mice, all Subtasks were completed under Major Task 1 Mouse Manipulation, and Major Task 2 Necropsy. For Major Task 3, (i) 3-D micro-computed tomography was completed. Major Task 4 was completed, and Major Task 5 was initiated and will be completed by 04/19/19 at the latest culminating in manuscript submission.

***Specific Objectives***

**a.** The specific objectives of the third study consisting of two protocols conducted in year 03 were to: 1. reproducibly create parietal defects of comparable 3.0 mm diameter; 2. chronically administer relaxin or vehicle by osmotic pump at 0.20 μg/hr for 10-12 days and to measure circulating concentrations of relaxin during the infusion by ELISA; 3. apply rhRLX locally to bone defects using collagen scaffolding (1.0 μg/scaffold, dose determined by in vitro release assay); 4. sacrifice the mice at 10-12 days after cranial defect and initiation of rhRLX treatment; 5. fix the bones for 3-D micro-computed tomography and measure cranial defect closure by 3-D micro-computed tomography.

***ii. Significant Results***

**a.*1 Recombinant human relaxin subcutaneous infusion: 0.20 μg/h (Protocol 3)***

The intermediate infusion rate used in Protocol 3 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**).

***a.2 Recombinant human relaxin locally administered by collagen scaffolds: 1.0 μg/scaffold (Protocol 4)***

Because systemic rhRLX administration was ineffective, we also 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. 0.5 and 5.0 μg rhRLX were tested. The cumulative release over a period of 10 days was comparable between the doses (% 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 an imtermediate dose of 1.0 μg. The treatment was confined locally, 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**).

**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 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 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 μg** | | | | | | | |
| 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 μg** | | | | | | | |
| 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.

**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 |
| Series 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 |
| Series 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).

**a. *iii. Other Achievements***
Nothing to Report

**C. What opportunities for training and professional development has the project provided?**

Nothing to Report

**D. How were the results disseminated to communities of interest?**
Manuscript in preparation and almost completed.

**E. What do you plan to do for the next reporting period to accomplish the goals?**

1. By the Final Report due 04/19/19, I will have finished revising the manuscript and submitting for publication to Physiological Reports.

**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.**
NA

**B. Actual or anticipated problems or delays and action or plans to resolve them.**
NA

**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.

**Julie Bailes**
Role: Technician
Research ID: NA
Nearest person month work: 4.0
Contribution to project: assisted the PI in coordinating the research efforts of contributing Co-Is and Staff; animal husbandry; transport of mice to the various laboratories for procedures; organizing the tail bleeds with the Animal Care Services Veterinary Technicians; assisting Dr. Conrad with the surgeries.

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

Nothing to Report