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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

Nothing listed

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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. 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 to next use a lower dose.

Therefore, in year 02, we repeated the experiment using a lower dose of 0.05 ng/ml. This report will detail the methods and results of this study. In addition it will include analyses from the first batch of mice that were not run until year 02: % blood vessel area and #blood vessels/mm^2; bone and tissue mineral densities; % bone/tissue volume; as well as 3-D microcomputed tomography of femoral bones (an unmanipulated bone control for the cranial lesions).

2. Keywords

GFP^+ chimeric mice, cranial defect closure, relaxin, angiogenesis, vasculogenesis, bone marrow-derived progenitor cells, 3-D microcomputed tomography, immunohistochemistry, immunofluorescence, flow cytometry

3. Accomplishments

A. Major Goals

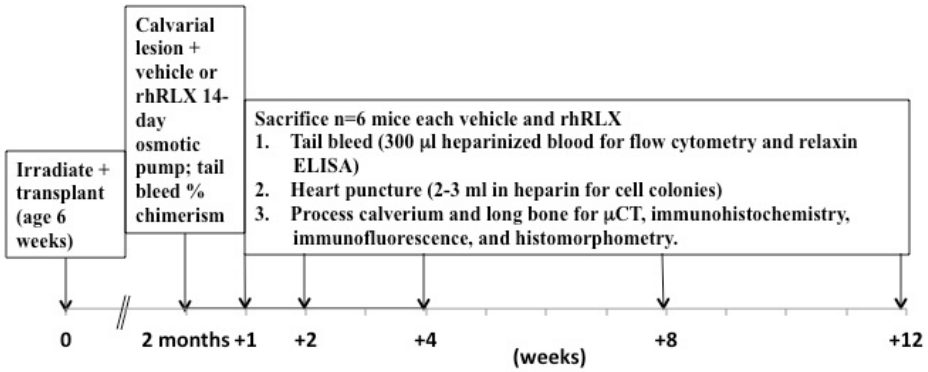

Figure 1. Potential therapeutic use of relaxin in healing cranial bone defects: Experimental Design

Major Tasks

1. Mouse manipulations

A. Subtasks

(i) Calvarial lesion

(ii) Osmotic pump implantation

(iii) 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 histomorphometry and immunhistochemistry

4. Data analysis and statistics

B. What was accomplished under these goals?

i. Major Activities

a. As stated in the Introduction, further analysis of the cranial lesions by 3-D computed tomography in the first group of chimeric mice with GFP+ bone marrow were conducted in year 02: bone volume/tissue volume (%); bone and tissue mineral densities (g/cm^3). The immunocytochemistry analysis of the cranial lesions and the 3-D computed tomography of the femurs (an unmanipulated bone control for the cranial lesions) for this first group of mice tested in year 01 were also performed in year 02.

b. Because the first experiment performed in year 01 was unsuccessful 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. If we are 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. After bilateral cranial defects were created, vehicle or relaxin was administered for 10-12 days in 6 mice each. They were euthanized 10-12 days after the cranial defect. 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 and (ii) bone decalcification were completed, while Subtask (iii) bone histomorphometry and immunocytochemistry is planned. Major Task 4 was completed.

Specific Objectives

- **a.** To complete the 3-D micro-computed tomography and immunocytochemistry analyses of the cranial lesions, and the 3-D micro-computed tomography of the femurs (an unmanipulated bone control for the cranial lesions) for the first group of mice tested in year 01.
- **b.** The specific objectives of the second series of experiments conducted in year 02 were to: 1. reproducibly create bilateral, parietal defects of comparable diameter; 2. chronically administer relaxin or vehicle by osmotic pump at 0.05 μg/hr for 10-12 days and to measure circulating concentrations of relaxin during the infusion by ELISA; 3. sacrifice the mice at 10-12 days after cranial defect; 4. fix the bones for 3-D micro-computed tomography and measure cranial defect closure by 3-D micro-computed tomography; 5. to decalcify the bone for histomorphometry and immunohistochemistry.

ii. Significant Results

a.*1* *Immunocytochemistry analysis of the cranial lesions from the first study conducted in year 01.*

The % blood vessel area (mean±SEM) was 1.49±0.22 and 1.31±0.31 in relaxin and vehicle infused mice, respectively. The number of blood vessels/mm^2 was 310±72 and 256±38 in relaxin and vehicle infused mice, respectively. Neither of these variables were significantly different between the 2 cohorts.

Bone mineral density was (mean±SEM) 0.43±0.02 and 0.44±0.02 g/cm^3 for relaxin and vehicle infused mice, respectively. Tissue mineral density was 0.96±0.01 and 0.97±0.01 g/cm^3 for relaxin and vehicle infused mice. The bone/tissue volume was 34.3±2.3 and 35.3±1.5%, respectively. Again, there

were no significant differences in these bone parameters between the 2 cohorts.

a.*2* *3-D micro-computed tomography analysis of femurs from the first experiment conducted in year 01.*
There were no significant differences in the multiple parameters of the femoral cancellous and cortical bone between the 2 mouse cohorts (data not shown).

b.*1* *To reproducibly create bilateral, parietal defects of comparable diameter.*
Using a dental burr and a variable speed drill, lesions of ~1.5 mm in diameter were reproducibly created under isoflurane anesthesia and sterile surgical conditions as in the first group of mice (see year 01 Annual Report).

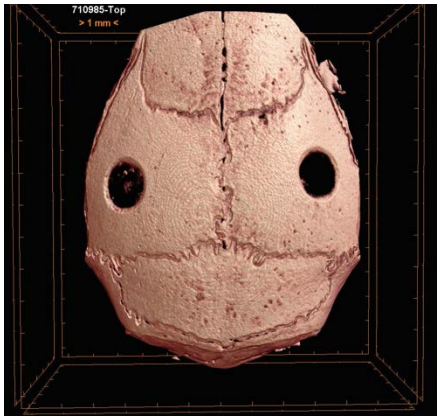

Figure 1. Representative 3-D micro-CT image of bilateral, parietal lesions in a mouse 10-12 days after surgery.

b.2 *To chronically administer relaxin or vehicle by osmotic pump for 10-12 days, and to measure circulating concentrations of relaxin during the infusion by ELISA.* Values of 0.35, 0.69, 1.61, 0.66, 1.99, and 3.41 ng/ml of relaxin were measured in the 6 mice that were administered recombinant human relaxin. We did not assay for circulating levels in the vehicle-infused mice, because in our previous work, none was detected. This makes sense, because the ELISA we use does not detect mouse relaxin, and relaxin is unlikely to circulate in non-pregnant rodents anyway.

b.3 *To harvest cranial bones (control).*
Completed.

b.4 *To fix the bones and to measure cranial defect closure by for 3-D micro-computed tomography.*
As documented in the year 01 Annual Report for the initial groups of mice, the lesions created on both the left and the right side in this second group also demonstrated comparable degrees of closure (data not shown). Therefore, lesions on the right and left side were both included in the analysis. In the present trial, the mice administered relaxin and vehicle showed similar degrees of lesion closure, too (**Fig. 2**).

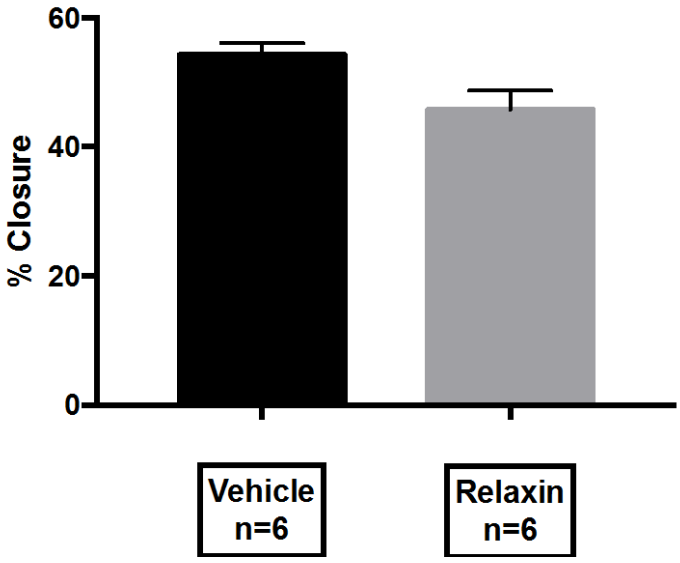

Figure 2. The percent closure was comparable after 10-12 days of relaxin or vehicle administration. Mean ± SEM. P=NS by unpaired t test.

Consistent with the analysis of % closure, there were no significant differences in bone mineral density (mean±SEM): 0.31±0.01 and 0.35±0.01 g/cm^3 for relaxin and vehicle infused mice, respectively. Tissue mineral density was 0.94±0.01 and 0.94±0.01 g/cm^3 for relaxin and vehicle infused mice were also comparable between the cohorts. The bone/tissue volume was 21.9±1.1 and 25.6±0.8%, respectively, was also not different.

b.5 *To decalcify the bone for histomorphometry and immunohistochemistry.*
The cranial bones have been decalcified.

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?
Nothing to Report

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

1. For the second study described herein, the plasma concentrations of relaxin were actually lower than we desired. Therefore, in the time remaining in the NCE, we will next infuse rhRLX at an intermediate dose of 0.2 μg/hr, in order to reach circulating concentrations of 10-20 ng/ml (n=4 rhRLX and n=4 vehicle).
2. In another cohort of mice, instead of administering rhRLX systemically, we will make a unilateral rather than a bilateral cranial lesion, and apply a 3 mm collagen disc (Bio Gide from

Geistlich Biomaterials) containing either rhRLX (n=4 mice) or vehicle (n=4 mice). Currently, we are testing the *in vitro* release rate of different amounts of rhRLX applied to the collagen scaffolding. The amount applied to the collagen discs for application to the cranial lesions will be based on the results of the *in vitro* experiment. The idea is that local rather than systemic administration of rhRLX might prove to be efficacious.

3. The closing of the 1.5 mm lesion was still quite rapid—~50% closure (Fig. 2)—even though the mice were euthanize only 10-12 days after making the lesion. Therefore, for the next group of mice, we will make a lesion of 3 mm using a larger dental burr. Perhaps we can detect differences in relaxin and vehicle treatments, if the % closure is not so extensive.
4. Finally, we will use older mice of >12 months of age rather than <6 months of age. Older mice are likely to have relatively impaired bone healing capacity compared to younger mice. Perhaps in this setting, relaxin may prove to be more efficacious.

4. Impact

A. What was the impact on the development of the principal discipline(s) of the project?

Nothing to Report

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.

As explained in B.1.b. What was accomplished under these goals? Major Activities, above, we did not make chimeric bone marrow GFP+ mice in the second group of mice.

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

2017 saw considerable unanticipated problems leading to delays in the project.

1. There was turnover in the Research Assistant position, which slowed progress. But, I have recently hired Julie Bailes, who should be with me until the end of the NCE.
2. There was a several month delay associated with IACUC and then ACURO approval for the various protocol modifications that I needed to make before proceeding (e.g., use of a bone scaffold to administer relaxin locally at the lesion site).
3. It also took several months to identify a suitable commercially-available collagen bone scaffolding. Several companies initially expressed interested in the project. But after my request made its way up the chain of command, the powers that be ultimately decided not to let me use their collagen scaffolding. In fact, Geistlich Biomaterials is the third company that I approached on the recommendation of a visiting scholar from the Netherlands. Fortunately, they were willing to sell me Bio-Gide, and I am currently performing *in vitro* relaxin release assays to inform the amount of relaxin I should apply to the scaffolding for *in vivo* use.
4. The X-ray source on the 3-D micro-computed tomography machine failed April 2017. However, the new part was approved for purchase by the VA, and recently acquired and installed, just in time for our final group of mice.

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 who committed at least 1 person month to the project)

Biswadeep Dhar
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.

Morgan Carson (replaced Mr. Dhar who found a higher paying position in another lab)
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.

N.B. Kirk P. Conrad MD PI and technician Julie Bailes BS; Mark S. Segal MD/PhD Co-I; Yanpeng Diao PhD Co-I; Joshua F. Yarrow PhD Co-I and technician (VA Medical Center, Gainesville, FL); Ignacio Aguirre PhD Co-I and technician contributed, but less than 1 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