FINAL REPORT

PHARMACOLOGICAL INTERVENTION TO PREVENT DISUSE OSTEOPENIA

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INTRODUCTION

This project was designed to investigate the potential of pharmacological intervention as an approach to prevent bone loss during long-duration spaceflight. The critical path roadmap had not yet been developed when this project was submitted. Nevertheless, the project is directly relevant to several critical questions. These include: 2.03 Will bone mass loss continue unabated for missions greater than six months in duration?; 2.24 How can animal models be used to better understand the risk of fractures and impaired fracture healing; 2.05 Is there an additive or synergistic effects of estrogen deficiency and prolonged exposure to hypogravity; 2.06 What pharmacological agents will most effectively minimize the decrease in bone mass with hypogravity? Are anabolic as well as anti-resorptive agents required?; 2.08 Is there an optimal combination of exercise and a pharmacological countermeasure to minimize decrements in bone mass in hypogravity?; 2.12 What are the signal transduction pathways allowing bone cells to sense gravity and loading on bone?; 2.13 Does hypogravity affect the size, viability or differentiation of precursor bone cell populations?; 2.15 Are there important other mechanisms for bone loss with hypogravity that are critical to developing effective countermeasures; 2.07 What are the specifics of the optimal exercise regimen to be followed during exposure to hypogravity to minimize decreases in bone mass with regard to workout duration, intensity, frequency?

Our working hypothesis is that weight bearing acts on bone target cells via a cascade mechanism. We propose that the initial step of the cascade is initiated by dynamic loading and involves mechanoreceptor-transduced regulation of expression of a small number of immediate response genes (possibly nuclear proto-oncogenes) which code for transcription factors and other cell cycle regulatory factors. These factors are postulated to regulate expression of genes which code for skeletal signaling molecules (e.g., growth factors). These, in turn, are capable of influencing the expression of a large number of genes critical to cell proliferation, metabolism and expression of differentiated bone cell function. Thus, only a few genes are directly regulated by loading; most of the profound effects of dynamic weight bearing on bone metabolism are indirect and are mediated by the signaling molecules.

We postulate that disuse osteopenia results in a large measure from disturbed expression of cell signaling molecules. These signaling molecules are not exclusively regulated by weight bearing, they serve as intermediates in the signal transduction pathways for many physiological regulators of bone metabolism (e.g., calcium regulating hormones). If this hypothesis is correct, it may be possible to bypass the mechanoreceptor step in the signal transduction pathway for mechanical loading. This goal could be accomplished by pharmacological agents which modulate expression of skeletal cell signaling molecules. We postulate that normalizing expression of signaling molecules would be sufficient to maintain near normal bone balance in the absence of dynamic weight bearing.

Intensive investigation has revealed that decreased bone formation is the principal cellular mechanism leading to disuse osteopenia in normal male rats. This mechanism fails to account for the excess bone loss induced in ovariectomized rats during spaceflight; an observation which emphasizes the important role of the endocrine system in modulating the
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skeletal response to weight bearing. Ground-based studies have revealed that estrogen replacement prevents disuse osteopenia and regulates expression of some of the skeletal signaling molecules that are regulated by weight bearing. Similarly, human parathyroid hormone (hPTH) was shown to modulate genes regulated by estrogen and weight bearing. We proposed to take advantage of the overlap in the signal transduction pathways for weight bearing, estrogen, and hPTH to design novel interventions to mitigate or reverse the detrimental skeletal effects of disuse.

The specific aims of the proposed research in male rats were to:

1. Determine the efficacy of SERMs (selective estrogen receptor modulators) treatment to prevent cancellous osteopenia and maintain normal bone turnover, architecture, and mechanical properties in an unweighted limb.

2. Determine if the skeletal efficacy of combined treatment of unweighted rats with clomiphene and hPTH is superior to monotherapy.

3. Determine if there is a sex difference in the skeletal response to mechanical unloading.

MATERIALS AND METHODS

The studies were performed in male and female rats, with an emphasis on adults. In a few cases, studies were performed using cultured human and rat cells. Hindlimb unweighting was used to mechanically unload tibia. We also performed additional analysis on tissues and cells from earlier spaceflight experiments. Measurements included bone histomorphometry and gene expression analysis. The detailed methods are described in the publications listed in “Other Information and Materials.”

RESULTS

Published and Submitted Studies

The results of the published studies will be briefly described. The details can be found in the publication. Relevance to specific Critical Path Roadmap questions will also be noted.

1. Cavolina, et al: This study in ovariectomized rats demonstrated that bone loss during spaceflight over and above that caused by ovariectomy is due entirely to increased bone resorption. This study is relevant to Critical question 2.05 in the Critical Question Roadmap.

2. Westerlind, et al: These spaceflight and ground-based studies show that estrogen regulates the rate of bone turnover in female rats but that bone balance is modulated by prevailing mechanical strain. This study is relevant to critical questions 2.05 and 2.08.
3. Dobnig and Turner: The purpose of this research was to develop a method for delivering parathyroid hormone to rats flown in space. In addition, we investigated possible detrimental side effects of parathyroid hormone treatment. These studies are relevant to critical question 2.06.

4. Ritman, et al: This manuscript describes a novel synchrotron-based high resolution micro-CT system that can be used to investigate 3D bone architecture. We have successfully used this system to investigate changes in bone quality in women undergoing treatment for postmenopausal osteoporosis (Published abstract #7).

5. Westerlind, et al: This manuscript demonstrates that resistance exercise can increase bone mass in rats. This study is relevant to critical question 2.08.

6. Zhang and Turner: This manuscript investigated the effects of spaceflight on gene expression in the skeleton of ovariectomized rats. This study is relevant to critical question 2.12.

7. Evans, et al: This manuscript demonstrated that spaceflight results in bone compartment and gene-specific effects on mRNA for bone matrix proteins in rat femurs. This study is relevant to critical question 2.13.

8. Turner, et al: This study demonstrated our ability to deliver parathyroid hormone in hindlimb unloaded rats using implanted osmotic pumps. These studies demonstrated the efficacy of parathyroid hormone in stimulating bone formation in hindlimb unloaded rats. This study is relevant to critical question 2.06.

9. Ritman, et al: This manuscript describes our further efforts in developing a novel high resolution of micro-CT system.

10. Kostenuik, et al: This manuscript reports resistance of osteoprogenitor cells isolated from bone and culture in vitro to parathyroid hormone and insulin-like growth factor I from animals that had been hindlimb unloaded.

11. Turner: This manuscript reviews the relationship between mechanical loading and postmenopausal osteoporosis. This manuscript is relevant to critical question 2.05.

12. Harris, et al: This manuscript demonstrates that immortalized fetal human osteoblastic cells grow normally during spaceflight. However, they demonstrate alterations on expression of selected cytokine following their return to earth.

13. Sibonga, et al: This manuscript demonstrates the feasibility of reversing severe osteoporosis in aged animals using a pharmacological approach. This study is relevant to critical questions 2.05 and 2.19.
14. Sibonga, et al: Manuscript #13 was highlighted by J Gerontol for editorials by two distinguished researchers. This manuscript documents our reply.

15. Luo, et al: This manuscript describes a mathematical model for predicting the effects of estrogen and mechanical usage on bone architecture. This study is relevant to critical questions 2.05 and 2.08.

16. Turner: This manuscript reviews the effects of spaceflight on bone.

17. Sibonga, et al: This manuscript describes spaceflight and hindlimb unloading studies which suggest that mechanical unloading does not influence the rate of longitudinal bone growth but may alter growth plate matrix chemistry.

18. Colleran, et al: This manuscript demonstrates that hindlimb unloading results in alterations in skeletal perfusion which may play a role in the detrimental effects of spaceflight on bone remodeling. This study is relevant to critical question 2.15.

19. Turner, et al: This manuscript reviews animal models for osteoporosis; including disuse models. This paper is relevant to critical question 2.24.

20. Turner, et al: This manuscript describes a theoretical approach for investigating structure to function relationships in the skeleton.

21. Buhl, et al: This manuscript demonstrates that aged bone displays an increased responsiveness to low-intensity resistance exercise. This study is relevant to critical question 2.07.

22. Turner: This manuscript critically reviews the hypothesis that skeletal adaptation to external loads results in optimal mechanical properties.

23. Lotinun, et al: This manuscript investigates the mechanisms that lead to the beneficial and detrimental effects of parathyroid hormone on bone metabolism. This study is relevant to critical question 2.06.

24. Maran, et al: This manuscript describes a method for assaying gene expression in bone specimens following high resolution μ-CT analysis.

25. Hefferan, et al: This manuscript demonstrates that disuse exaggerates the detrimental effects of alcohol on cortical bone.

26. Hefferan, et al: This manuscript demonstrates that the mechanisms of bone loss are similar in male and female adult rats.
Unpublished Studies

Unpublished Study 1. The effects of combination treatment with parathyroid hormone and tamoxifen on bone metabolism and architecture in hindlimb unloaded rats.

Introduction:
Exercise is likely to be the most important countermeasure to weightlessness but that approach might not be sufficient. A pharmacological approach may also be necessary. PTH and SERMs have been shown to preserve bone mass in disuse models. However, they act by different cellular mechanisms; PTH stimulates bone formation whereas SERMs inhibit bone resorption. Skeletal unloading results in decreased bone formation as well as increased bone resorption. Combination treatment with PTH and SERMs may be more effective than monotherapy in preventing alterations in bone architecture during spaceflight. The goal of this research was to test this possibility in hindlimb unloaded rats.

Materials and Methods:
6-month-old intact male Fisher 344 rats were obtained from Charles River (Wilmington, DE). The rats were maintained at a 12 hour light and dark cycles. The room temperature was maintained at 78 degrees. All rats had free access to food and water ad libitum. The control rats were pair-fed to the hindlimb suspension (HLS) group to control for weight changes.

A total of 60 rats were weight matched and divided into individual cages. Control weight bearing group N=10 (vehicle s.c. injection and placebo pellet), tamoxifen N=10 (10 mg/pellet 21 day release), PTH N=10 (daily s.c. injection 80 μg/kg), combination N=10 (tamoxifen 10 mg/pellet and daily s.c. injection of 80 μg/kg PTH), placebo N=10 (s.c. injection with vehicle and 10 mg placebo pellet). The HLS groups were suspended according to Morey-Holton protocol. At the start of the experiment all groups received a fluorochrome label via perivascular tail injection of calcein (Sigma, St. Louis, MO) (20 mg/kg). A baseline group N=10 were sacrificed 24 hours later to establish initial bone parameters. The remaining animals received fluorochrome labels: tetracycline 920 mg/kg) obtained from Sigma 7 and 12 days before sacrifice. The animals were anesthetized by CO2 gas followed by decapitation. Trunk blood was collected and spun down and froze for future analysis. The seminal vesicles were weighed and the wet weight recorded. Both tibiae were fixed in 70% ETOH for histomorphometry analysis. The femurs were frozen and fixed for mRNA analysis and biochemical testing.

Results:
The seminal vesicle weights in the baseline animals were significantly higher when compared to all other groups. Tamoxifen resulted in a significant loss of final body weight and seminal vesicle weight when compared to the weight bearing control. The combination treatment resulted in a significant reduction in the final body weight when compared to both the placebo and weight bearing controls (data not shown).
Cancellous Bone Volume: There was significant bone loss in the placebo (HLE) rats vs. the weight bearing control. All HLE treatment groups were significantly different from the placebo HLE group. Only PTH treatment resulted in a significant increase when compared to the control weight bearing group (Figure 1).

Structural Indices: Tb.Th was significantly increased in the combination and PTH groups when compared to either control or placebo rats. Tb.N; tamoxifen resulted in a significant increase when compared to the placebo group (Figure 2).

Labeled Surface/Bone Surface: There was a significant increase in labeled surface in the combination and PTH group when compared to either control weight bearing group or placebo (Figure 3).

Cancellous Bone Formation: There was a significant increase in bone formation in the combination and PTH treated groups when compared to either the placebo HLE or the control group (Figure 4).

Conclusion:
As expected, hindlimb unloading caused bone loss in male rats compared to the weight bearing controls.

PTH and tamoxifen were effective in preventing bone loss. This prevention of bone loss occurred however, through different mechanisms. PTH is an anabolic agent that prevented the bone loss by creating thicker trabeculae. Tamoxifen is an antiresorptive agent and caused an increase in Tb.N.

The combination of PTH and tamoxifen was effective in preventing bone loss. However, 80 μg/kg of PTH over-stimulated bone formation compared to weight bearing rats, leading to an increase in Tb.Th.

The results of the present short-term study were encouraging. However, further long-term investigation into using a combination treatment of anti-resorptive agent and anabolic agent is needed to establish the efficacy of this approach for preventing turnover and spaceflight-induced bone loss and maintaining normal bone architecture.

Unpublished Study 2. The effects of a submaximal anabolic dose of parathyroid hormone and raloxifene on hindlimb unloaded rats.

Introduction:
In unpublished study 1, we demonstrated that the combination of PTH and tamoxifen was effective in preventing bone loss in hindlimb unloaded rats. However, 80 μg/kg of PTH over-stimulated bone formation compared to weight bearing rats resulting in an increase in Tb.Th. The purpose of this study was to investigate the combined effects of a submaximal anabolic dose
of parathyroid hormone (PTH) (20 μg/kg) and a SERM (raloxifene) on bone architecture and metabolism in hindlimb unloaded rats. We chose to substitute raloxifene for tamoxifen in this study because the former drug has received approval for treatment of osteoporosis.

Methods:
The methods were identical to the previously described unpublished study with the following exceptions. The raloxifene was delivered by s.c. implantation of a controlled release pellet designed to release 7 mg of drug in 21 d. The PTH-treated rats received 20 μg/kg/d. The controls received carrier only daily and were implanted with a drug-free pellet.

Results:
The effects of hindlimb unloading, PTH and raloxifene on cortical bone histomorphometry, body weight, seminal vesicle weight and serum testosterone and tabulated in Table 1. All treatments reduced body weight. Seminal vesicle weight decreased with age. Serum testosterone was reduced by hindlimb unloading and was reduced in weight bearing rats by raloxifene.

The effects of hindlimb unloading, PTH and raloxifene on cancellous bone histomorphometry are shown in Table 2. hindlimb unloading resulted in cancellous bone loss, and decreases in indices of bone formation (LS/BS, MAR and BFR/BS). There were minimal changes in bone architecture during this short-duration study. PTH treatment and combination treatment (PTH and raloxifene) prevented bone loss and increased bone formation compared to the weight bearing controls.

Conclusion:
A submaximal anabolic dose of PTH is sufficient to prevent bone loss in adult hindlimb unloaded rats. Further studies should focus on longer duration experiments to prove efficacy.

DISCUSSION
We have shown, for the first time, that bone resorption is increased in rats during spaceflight (1) and simulated spaceflight (8,26). The failure to detect increased bone resorption in earlier studies was likely due to the use of growing animals. Furthermore, we have shown that in older animals spaceflight and simulated spaceflight results in true bone loss. These findings contrast to those in rapidly growing animals in which the osteopenia is due to the suppression of bone growth.

We have shown that estrogen interacts with mechanical loading to modulate bone turnover in female rats (1,2,6,15,22,26). Our results suggest that there is partial overlap between estrogen signaling and mechanical signaling. This overlap provides a rationale for using estrogen analogs as an intervention to prevent spaceflight-induced bone loss. In this regard, we have shown (see Unpublished Studies) that the SERMs tamoxifen and raloxifene reduce bone loss in hindlimb unloaded rats by preventing the expected increase in bone resorption.
We investigated the effects of gender on the skeletal response to hindlimb unloading in adult rats. We observed bone loss that was due to a combination of increased bone resorption and decreased bone formation in both genders, suggesting that similar mechanisms mediate the bone loss in males and females having normal gonadal function (26). Additional studies, however, are necessary to determine whether the magnitude of bone loss and response to restoration of normal weight bearing are gender independent.

We have demonstrated the efficacy of PTH as an anabolic agent to prevent the inhibitory effects of short duration (1-2 w) simulated spaceflight on bone formation (8) (Unpublished studies). Furthermore, our studies suggest that combination treatment with an anabolic and anti-resorbing agent may be more effective than either treatment alone in maintaining normal bone architecture as well as bone turnover during long-duration spaceflight. These encouraging results need to be confirmed in longer term studies.

In the process of developing a novel method to deliver PTH to rats during spaceflight using sc implanted osmotic pumps (3,8,10) we realized a similar method could be used to investigate the mechanisms for the detrimental skeletal effects of chronic hyperparathyroidism. These investigations have revealed that platelet-derived growth factor A (PDGF-A) is a likely causative factor in the etiology of parathyroid bone disease (23). Furthermore, treatment with PDGF-A signaling antagonist is likely to prevent and/or reverse parathyroid bone disease. These findings are relevant to patients with renal osteodystrophy and age-related secondary hyperparathyroidism as well as patients with primary hyperparathyroidism.

*a reference refers to publication #.
<table>
<thead>
<tr>
<th>Group</th>
<th>Cross section Area (mm²)</th>
<th>Cortical bone area (mm²)</th>
<th>Medullary area (mm²)</th>
<th>Peri BFR (x10^-3 mm²/day)</th>
<th>Peri MAR (μm/day)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline WB (N=10)</td>
<td>4.29±.05</td>
<td>3.53±.05</td>
<td>.75±.03</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTH HLU (N=9)</td>
<td>4.33±.085</td>
<td>3.57±.07</td>
<td>.76±.03</td>
<td>1.10±.19 a</td>
<td>1.01±.10</td>
</tr>
<tr>
<td>PTH &amp; Raloxifene HLU (N=9)</td>
<td>4.36±.11</td>
<td>3.60±.09</td>
<td>.76±.03</td>
<td>1.04±.15 a</td>
<td>.97±.10</td>
</tr>
<tr>
<td>Control HLU (N=10)</td>
<td>4.31±.08</td>
<td>3.51±.06</td>
<td>.79±.03</td>
<td>.67±.17 a</td>
<td>.80±.06 a</td>
</tr>
<tr>
<td>Raloxifene WB (N=10)</td>
<td>4.23±.10</td>
<td>3.43±.08</td>
<td>.79±.03</td>
<td>2.85±.58 b</td>
<td>1.03±.08 b</td>
</tr>
<tr>
<td>Control WB (N=10)</td>
<td>4.25±.08</td>
<td>3.50±.06</td>
<td>.75±.03</td>
<td>2.90±.4</td>
<td>1.02±.04</td>
</tr>
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</table>

P<.05 compared with the a weight bearing control (WB); b hindlimb unloaded (HLU control)

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight Starting (g)</th>
<th>Body Weight Ending 9g</th>
<th>Seminal Vesicle Wet Weight</th>
<th>Testosterone Levels ng/dl</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline (N=10)</td>
<td>364</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>PTH HLU (N=9)</td>
<td>366</td>
<td>263 a</td>
<td>.681 ± .047</td>
<td>67.20± 12.27 a</td>
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<tr>
<td>PTH &amp; Raloxifene HLU (N=9)</td>
<td>368</td>
<td>249 a,b</td>
<td>.512 ± .035 a</td>
<td>44.00 ± 8.22 a</td>
</tr>
<tr>
<td>Control HLU (N=10)</td>
<td>371</td>
<td>273 a</td>
<td>.696 ± .055</td>
<td>42.14 ± 2.43 a</td>
</tr>
<tr>
<td>Raloxifene WB (N=10)</td>
<td>372</td>
<td>273 a</td>
<td>.665± .073</td>
<td>71.71± 29.50 a</td>
</tr>
<tr>
<td>Control WB (N=10)</td>
<td>362</td>
<td>295</td>
<td>.727± .124</td>
<td>118.40± 32.19</td>
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</table>

P<.05 compared with a weight bearing (WB) control; b hindlimb unloaded (HLU)
Table 2: Cancellous Bone Histomorphometry

<table>
<thead>
<tr>
<th>Group</th>
<th>BV/TV (%)</th>
<th>LS/BS (%)</th>
<th>MAR (μm/d)</th>
<th>BFR/BS (μm³/μm²/d)</th>
<th>Tb.Sp (μm)</th>
<th>Tb.Th (μm)</th>
<th>Tb.N (mm⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Baseline WB (N=10)</td>
<td>28.30±2.17b</td>
<td></td>
<td></td>
<td></td>
<td>189.73±18.19b</td>
<td>71.30±3.20</td>
<td>3.96±2.56b</td>
</tr>
<tr>
<td>PTH HLU (N=9)</td>
<td>27.72±1.58b</td>
<td>18.09±2.06a,b</td>
<td>1.15±0.06a,b</td>
<td>.22±0.03a,b</td>
<td>209.34±11.02</td>
<td>79.64±4.98</td>
<td>3.51±1.66b</td>
</tr>
<tr>
<td>PTH &amp; Raloxifene HLU (N=9)</td>
<td>25.05±1.58</td>
<td>21.69±2.68a,b</td>
<td>1.14±0.04a,b</td>
<td>.25±0.04a,b</td>
<td>214.12±11.60</td>
<td>70.71±4.45</td>
<td>3.54±1.36b</td>
</tr>
<tr>
<td>Control HLU (N=10)</td>
<td>22.57±1.68a</td>
<td>.094±.094a</td>
<td>.06±.06a</td>
<td>.001±.001a</td>
<td>229.23±15.84</td>
<td>64.96±3.64</td>
<td>3.46±1.66b</td>
</tr>
<tr>
<td>Raloxifene WB N=10</td>
<td>26.26±2.00</td>
<td>6.73±.98b</td>
<td>.78±.05b</td>
<td>.05±.01b</td>
<td>205.21±14.48</td>
<td>70.93±3.08</td>
<td>3.66±1.56b</td>
</tr>
<tr>
<td>Control WB N=10</td>
<td>26.70±.71</td>
<td>8.59±1.55b</td>
<td>.90±.05b</td>
<td>.08±.02b</td>
<td>198.52±7.87</td>
<td>72.34±3.50</td>
<td>3.73±1.36b</td>
</tr>
</tbody>
</table>

P<.05 compared with a weight bearing control (WB); b hindlimb unloaded (HLU) control.
PRESENTATIONS:


PUBLISHED ABSTRACTS:


PUBLICATIONS:


**UNDERGRADUATES**

Jason M. Cavolina

**POST-DOCTORAL FELLOWS**

Theresa E. Hefferan

Sutada Lotinun