

Program: Biomedical Research and Countermeasures

Type: Ground

Element: Biomedical Countermeasures

Biomechanical Countermeasure for Disuse Osteopenia

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Tasks Info:

- **Responsible Center:** NSBRI
- **Solicitation:** NSBRI
- **Project Start:** 02/01/2001
- **Project End:** 01/31/2004
- **Joint Agency Participation:** None
- **Number of Post Docs:** 1
- **Number of PhDs:** 4
- **Number of Graduate Students:** 4
- **Number of Undergraduate Students:** 2

• Task Abstract/Description:

Osteoporosis, the progressive loss of bone density and strength which cripples tens of millions on our planet, distinguishes itself as perhaps the greatest physiologic obstacle to an extended human presence in space. The principal objectives of this proposal are to establish the efficacy of a unique, biomechanical countermeasure to inhibit bone loss in an animal model of disuse osteoporosis, and correlate this regulatory influence to the expression patterns of several genes critical to bone formation and resorption. Using a ground based model of microgravity, the tail-suspended rat, we have shown that brief exposure (10 minutes) to extremely low magnitude (0.25g, engendering < 5 microstrain), high frequency (30-90 Hz) mechanical signals will inhibit the bone loss which typically parallels disuse, even though 10 minutes of full weightbearing failed to curb this loss. Longer-term experiments in sheep have shown this stimulus to be strongly anabolic, increasing bone mineral density, trabecular number and connectivity, and improving bone strength. In a series of four specific aims, we will use several morphometric assays on the mouse model of tail-suspension to rigorously establish the efficacy of a specific mechanical signal (10 minutes at 30Hz, 0.3g; parameters being used in clinical trials to inhibit bone loss in the elderly) to inhibit and/or reverse 28 days of disuse osteopenia. In an effort to understand the mechanisms by which this signal is anabolic, we will also monitor the temporal and spatial expression of nine genes, each indicative of a specific process of

bone formation or resorption. The use of the mouse will facilitate many aspects of the protocol, including comprehensive genomic profiling and expedited access to spaceflight. Considering that many flight opportunities are brief and thus do not permit long term morphologic adaptations in bone to occur, combining the molecular with the tissue level strategies will facilitate establishing countermeasure efficacy even following short term exposure to microgravity. In essence, this work represents a critical step in establishing a physiologically based, non-pharmacologic, non-invasive treatment for osteoporosis, for use on earth or in space.

- **Task Progress:**

In a series of four specific aims, we are using several morphometric assays on the mouse model of tail-suspension to rigorously establish the efficacy of a specific mechanical signal (10 minutes at 30Hz, 0.3g; parameters being used in clinical trials to inhibit bone loss in the elderly) to inhibit and/or reverse 28 days of disuse osteopenia. In an effort to understand the mechanisms by which this signal is anabolic, we will also monitor the temporal and spatial expression of nine genes, each indicative of a specific process of bone formation or resorption. The use of the mouse will facilitate many aspects of the protocol, including comprehensive genomic profiling and expedited access to spaceflight. Considering that many flight opportunities are brief and thus do not permit long term morphologic adaptations in bone to occur, combining the molecular with the tissue level strategies will facilitate establishing countermeasure efficacy even following short term exposure to microgravity. In essence, this work represents a critical step in establishing a physiologically based, non-pharmacologic, non-invasive treatment for osteoporosis, for use on earth or in space.

2. Key findings of the project: Thus far, the project has demonstrated that the low-level mechanical intervention is powerfully anabolic, and can effectively inhibit the bone loss which parallels disuse. As importantly, using three distinct strains of mice, we have determined that the response of the skeleton to disuse is strongly dependent on the genomic makeup of the animal, and that the responsiveness of the skeleton to the signals are dependent on the animal strain. 3. Impact of these findings: These findings point to a unique, non-pharmacologic, non-invasive means of controlling bone loss in a microgravity environment. This biomechanical intervention may potentially displace the need for time consuming (and relatively ineffectual) exercise regimens, or replacing the need for pharmacologic countermeasures (and the potential long-term side effects that they may cause). Importantly, promising results from preliminary clinical trials on post-menopausal women, girls with osteoporosis, and children with cerebral palsy, also indicate that this therapy may work for the 20 million people on earth who suffer from osteoporosis. This work may also contribute to identifying the genetic basis for those at greatest risk of the diseases.

4. Plans for the coming year: Studies will continue to identify the strain-specific sensitivity of the skeleton to disuse and/or mechanical stimulation, and efforts will begin to determine those genes that are involved in regulating the process. Work funded by NASA has begun, in the "definition" phase, to determine if this biomechanical intervention can be used effectively on astronauts in the ISS.

II. Project research objectives and activity

The principal responsibility of the skeleton is to support the loads and moments which arise during activity, resulting in mechanical strain in the bone tissue. The skeleton's ability to adapt to these functional signals was recognized well over a century ago and is now referred to as Wolff's Law. The premise of this "law" is that bone strives toward an optimized structure which caters to an individual's level of activity. Thus, each individual would tune the mass and morphology of their skeleton such that it could safely withstand the extremes of functional loading. While mechanical signals are, in general, recognized as strongly anabolic, identifying those specific components which drive the osteogenic response has proven difficult.

There is increasing evidence that extremely low magnitude (<100 microstrain) mechanical signals can be strongly osteogenic if applied at a high frequency (15 to 60 Hz).¹ Such high frequency low magnitude strains comprise a dominant component of a bone's strain history,² indicating that these mechanical events represent a significant determinant of bone morphology. With this in mind, we have been examining if small perturbations in high frequency loading, induced non-invasively into the lower appendicular skeleton, will stimulate an increase in bone mass without sacrificing bone quality. Short term animal studies provide evidence that very low intensity (<10 microstrain) mechanical stimuli are strongly anabolic if applied above 20Hz. Extremely low-level strains (80me), if induced at 20Hz, promote osseointegration.³ Longer term animal studies (one year), have shown that low level mechanical loading, inducing cortical strains on the order of 5 microstrain, can increase cancellous bone volume fraction,⁴ thicken trabeculae, increase trabecular number and enhance bone stiffness and strength.⁶ Considering these strain levels are far below (<1/1000th) those which may cause damage to the tissue, we believe these signals hold great potential as a mechanical prophylaxis for osteoporosis.

Importantly, this unique biomechanical intervention affords the ability to examine the molecular basis of an osteogenic signal, thus identifying novel targets for drug development. For example, osteoclast differentiation factor (ODF, or RANK-L) is a cytokine involved the recruitment and activity of osteoclasts,⁷ and in vitro studies have linked its upregulation to the absence of mechanical strain.⁸ In the first series of experiments, we used rats as a model to examine the osteogenic efficacy of low-level high frequency mechanical stimuli and their ability to reverse the bone loss which arises under microgravity⁹. We then hypothesized that the expression of RANK-L would be inversely related to altered tissue level bone formation rates.

Adult (6 month) female Sprague-Dawley rats were assigned to controls (n=30), mechanically stimulated (n=21), tail suspension related disuse (n=11), disuse interrupted by 10min/d of normal weight bearing (n=7), and disuse interrupted by 10min/d of 90Hz stimulation at 0.25g (n=19). All experimental procedures were applied for 28d. Mechanical stimulation consisted of whole body vibration at 90Hz (0.25g). All rats were given injections with demeclocycline prior to the beginning of the study and calcein on day 18 of the protocol to determine histomorphometric indices of bone formation. RANK-L mRNA levels were quantified in three animals of each group (except disuse plus normal weight bearing group) via Northernblots. RNA was extracted from whole left tibiae, including bone marrow and cartilage.

Figure 1. Tibial trabecular bone formation rates (BFR/BV) of age matched controls rats (LTC) and after 28 days of mechanical stimulation for 10 min/d at 90Hz (90Hz), tail suspension (Dis), disuse interrupted by 10min of weightbearing (Dis + WB), and disuse interrupted by 10 min of mechanical stimulation (Dis + 90Hz). b. Relative expression of RANK-L (ODF) in control, 90Hz stimulated, disuse, and disuse interrupted by 90Hz vibration rats (mean \pm SD).

Body mass of the rats did not change significantly in any of the groups during the course of the 28d study. Mechanical stimulation at 90Hz for 10 min/d proved to be a strong osteogenic stimulus as indicated by increased trabecular bone formation rates (+97%, Fig. 1a). Hindlimb suspension significantly decreased trabecular bone formation rates by 92% as compared to controls. This suppression was not significantly different from the animals subject to disuse for most of the day (23h, 50min) and then allowed to freely bear weight for 10 min/d (D+WB). In contrast, when low-level mechanical stimulation was applied for 10min/d to combat disuse, the countermeasure served to normalize bone formation rates back to control values. Mechanical stimulation for 10 min/d decreased RANK-L mRNA levels by 78%. Disuse increased the expression of RANK-L by 72% with respect to control values while disuse interrupted by 10 min of daily mechanical stimulation

decreased RANK-L levels by 49% (Fig. 1b). When linear correlation was used to relate bone formation rates to ODF expression levels across groups, the r^2 value was 0.79 (inverse correlation).

Fig. 2. Percent difference in bone formation rates (BFR/TV) between (a.) mechanically stimulated and age matched control mice and (b.) disuse and age matched control mice in the three genetically distinct strains of mice (mean \pm SD of the difference). Labels here refer to BALB/cByJ, C57BL/6J, and C3H/HeJ. It is clear that the genetic makeup of the animals helps define the extent to which they respond to anabolic and/or catabolic stimuli.

Using the mouse as a model, it is also apparent that the genetic make-up of the animal is a strong determinant of their sensitivity to mechanical stimuli.^{10,11} Adult (16 week) female 16wk old C57BL/6J (low density), BALB/cByJ (medium density) and C3H/He (high density) mice were assigned to control, mechanically stimulated, and disuse groups (n=13 each). Mice in the mechanically stimulated group were placed on a vibrating plate (45 Hz, 0.25g) for 10 min/d. Disuse animals were subjected to tail suspension. Four animals per group were culled 4d into the protocol for determining gene expression levels (semi-quantitative RT-PCR) while the remaining animals were sacrificed after 21d for the assessment of bone formation. Disuse failed to affect histomorphometric indices in C57BL/6J mice (Fig. 2). In BALB/cByJ, mechanical stimulation increased bone formation rates by 34% ($p<0.02$), but bone volume was unaffected. This increase in bone formation rate was primarily achieved by an increase in the ratio of double labeled surface to single labeled surface (+101%, $p<0.001$). Disuse in the BALB/cByJ mice suppressed bone formation rates by 48% ($p<0.01$), the ratio of double labeled surface to single labeled surfaced by 47% ($p<0.01$), and mineral apposition rates by 45% ($p<0.03$), resulting in trabecular bone volume that was 43% smaller ($p<0.01$) compared to control BALB/cByJ's. In contrast to the responsiveness of the skeleton of C57BL/6J and BALB/cByJ mice, no significant effects of mechanical stimulation or disuse were measured in tibial trabecular bone of C3H/HeJ mice.

These tissue level results were essentially mirrored at the molecular level. The transcriptional levels of collagen type I, the most abundant protein in bone, were significantly reduced in tibiae of hindlimb suspended BALB mice but not in those of any other group (Fig. 3a). This further emphasized the differential response of these mouse strains. The lack of upregulation of type I collagen mRNA after 4 days of mechanical stimulation may reflect its late occurrence in the cascade of events leading to new bone formation. Inducible nitric oxide synthase was significantly down-regulated by a similar percentage in mechanically stimulated mice of those strains that had responded to mechanical stimulation at the tissue level (Fig. 3b).

Fig. 3. (a) Collagen type I was downregulated in BALB/cByJ mice subjected to disuse while the transcription of this gene was not affected in C57BL/6J or C3H/HeJ mice. (b) Inducible nitric oxide synthase mRNA levels. Only the two strains that, at the tissue level, responded to mechanical stimulation, demonstrated a similar molecular (mean + SD, n=4). These data demonstrate that trabecular bone from C3H/He, Balb/c and C57BL/6 mice is differentially mechanosensitive, and implies that, at the level of the human, some people may be more prone to osteoporosis, but that these individuals may be more responsive to biomechanically based interventions.

Testing the anabolic potential of this biomechanical intervention in the human, sixty-two healthy women, 3-8 years past the menopause, enrolled in a double-blind, placebo controlled pilot study.¹² 32 women underwent mechanical loading of the lower appendicular and axial skeleton for two ten-minute periods per day, through floor mounted devices that produced a 0.2g mechanical stimulus at 30Hz (TX). 32 women received placebo devices (PL) and underwent daily treatment for the same period of time. Linear regression of the change in BMD shows a -3.3% (± 0.83) loss of BMD in the

spine of the placebo group. Treatment reduces this loss to -0.8% (± 0.82), reflecting a net benefit of 2.5% ($p=0.03$). The trochanter of the femur shows a 2.9% (± 1.2) loss of BMD in the placebo group, while treatment stimulates a $+0.4\%$ (± 1.2) gain, reflecting a net benefit of 3.3% of treatment ($p=0.03$). Interestingly, the intervention was more beneficial in the group of women in the lower 50% of body mass, the same group that was most susceptible to bone loss. Subsequent studies in children with cerebral palsy,¹³ and girls, ages 10-13, in the lowest quartile of BMD,^{14,15} also demonstrate the anabolic nature of the signal.

III. Implications of project findings for future research

Evidence in both animals and humans, at the molecular, histomorphometric, densitometric and structural level shows that short exposure to extremely low-magnitude, high frequency loads are anabolic. Such a biomechanical intervention is self-targeting, endogenous to bone tissue, and auto-regulated. In essence, these studies lay the groundwork for a unique, non-pharmacogenic intervention for osteoporosis, based purely on the premise of "form follows function" in the skeleton, and that these low level signals can enhance both the quantity and quality of bone. These low level signals, perhaps providing a surrogate for the deteriorating musculature that occurs with age, implies that the persistent barrage of low-level signals provided by muscles during predominant functions such as standing may be as important as the high level signals, which occur far less often,¹⁶ in defining (and retaining) bone mass and morphology. Pivotal clinical trials are essential to determine if this late stage technology development can effectively treat osteoporosis.

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- **Earth Benefits:**

Understand how gravity influences the quantity and quality of bone, and how mechanical information may be used to prevent and/or reverse osteoporosis, a disease which effects over 20 million americans here on earth

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