Optimization of a Biomechanical Countermeasure for Disuse Osteoporosis:

Final Report

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Summary

The human body undergoes roughly 50 changes while in a weightless environment, eight to ten of which also occur in aging bodies here on Earth. Of those, osteoporosis, the progressive loss of bone density and strength which cripples tens of millions here on our planet, distinguishes itself as perhaps the greatest physiologic obstacle to an extended human presence in space. This NASA funded proposal examined the potential of high frequency, low magnitude mechanical stimuli as a countermeasure for disuse osteopenia. The work over the period of the proposal served to demonstrate that these low-level mechanical signals are strongly osteogenic, and that they effectively serve to minimize the bone loss of disuse, where similar periods (10 minutes) of normal weightbearing failed to do so. Importantly, this work has led to a NASA funded study, currently in the "definition" phase, to determine if this biomechanical intervention could work on the ISS to minimize bone loss in astronauts.

Overview

A principal goal of the National Aeronautics and Space Administration is to implement long-term human exploration of space. Whether this will be in the near future, by habitation of the International Space Station, or within the next few decades by settling a permanent manned Moon base and embarking on a mission to Mars, it is clear that we must develop a better understanding of the ability of humans to tolerate an extended exposure to microgravity. As defined by the National Research Council report entitled: A Strategy for Research in Space Biology and Medicine in the New Century (Osborn, 1998), there are several areas of fundamental scientific investigation which must be addressed to make long-term space exploration not only feasible, but safe. Summing up their recommendations, which echo those made by previous reports published by the National Research Council's Space Studies

Board (e.g., Goldberg et. al., 1987; Smith et. al., 1991; Dutton et. al., 1992), *the principal physiologic hurdle to man's extended presence in space is the osteopenia which parallels reduced gravity*. The magnitude and rate of bone loss is staggering; astronauts lose bone mineral in the lower appendicular skeleton at a rate approaching 1.6% per month (LeBlanc et al., 1998; Ruff et al., 1999). Thus, in a 2.5-year return-trip to Mars, half of an astronaut's bone density may vanish, severely jeopardizing their health and well-being. While the astronauts are at risk of renal lithiasis during flight (Rambaut and Johnson, 1979), the most significant consequences may only be realized on return to planetary gravitational fields (Smith et al., 1977; Stupakov et al., 1984). To compound this problem, it is apparent that full recovery of bone mass may *never* occur (Rambaut and Goode, 1985; Shackelford et al., 1999), potentiating skeletal complications later in the astronaut's life (LeBlanc and Schneider, 1991).

The magnitude and rate of the loss is staggering; astronauts lose bone mineral in the lower appendicular skeleton at a rate approaching 1.6% per *month* (LeBlanc et. al., 1996; Ruff, et. al., 1999). Thus, in a 2.5-year return-trip to Mars, *half* of an astronaut's bone density may vanish, severely jeopardizing their health and well-being. Addressing this concern, NASA has developed a Critical Path Roadmap (CPR; 2001) to define its bioastronautics research priorities towards reducing or eliminating the risks to astronaut health and safety during and after spaceflight. This CPR emphasizes musculo-skeletal deterioration in general, and osteoporosis in particular, as research areas of highest priority. Considering the similarities between space and aging induced bone loss (Schultheis, 1991), an indisputable benefit of such a countermeasure would be its potential as a treatment for the osteoporosis which plagues 25 million people in the U.S. (NIH Consensus Development Conference, 2000).

Therapies that increase bone formation are highly desirable, but unfortunately, not nearly as well developed as antiresorptive interventions. Anabolic agents currently under investigation, including parathyroid hormone (PTH), fluoride, and insulin-like-growth factor-I (IGF-I), have important and significant disadvantages. For example, PTH must be administered by subcutaneous injection, may cause hypercalcemia and hypercalciuria, and concerns about osteosarcoma persist. Fluoride may cause gastrointestinal side effects and there is evidence of increased skeletal fragility and microfractures with increasing doses. IGF-I administration is associated with hypoglycemia, and has not been well studied as a therapeutic agent for bone. Even dismissing these concerns, such pharmacologic interventions seem inappropriate for long-term spaceflight, as they – in essence – ignore the etiologic basis for the disease, the removal of gravity, and may place otherwise healthy astronauts at undue risk. Further, the administration of a drug, as well as its pharmacokinetics, may be hampered in microgravity. With the removal of gravity considered the basis for the osteopenia, it is reasonable to examine the potential of mechanical stimuli (e.g., exercise) as a countermeasure.

The skeleton's structural success can be largely attributed to bone tissue's ability to rapidly accommodate changes in its mechanical environment, ensuring that sufficient skeletal mass is appropriately placed to withstand the rigors of functional activity (Wolff, 1892). Bone's sensitivity to functional loading suggests that appropriate mechanical stimuli should be capable of providing a site-specific, exogenous treatment regimen for the inhibition and/or reversal of bone loss. Consistent with the adaptive potential of bone, extensive, long-term exercise has been shown to increase skeletal mass (Jones et. al., 1977; Snow-Harter et. al., 1992). Nevertheless, the efficacy of lengthy bouts of strenuous exercise as a countermeasure

for microgravity-induced osteopenia remain unclear (Shackleford et. al., 1999), and is certain to erode valuable crew time and may even provoke injury (e.g., stress fractures). It has been argued that one limiting factor of optimizing an exercise-based countermeasure is that the "regulatory" component of mechanical loading has yet to be identified.

Appealing as a mechanical prophylaxis may be, controversy arises with attempts to identify those components of the functional regimen that govern the bone modeling and remodeling processes. Our own work has led us to the hypothesis that extremely small strains, if induced at a sufficiently high frequency, are strong determinants of bone morphology (Rubin & McLeod, 1996). In essence, this hypothesis has evolved from the demonstration that the spectrum of *in vivo* bone strain extends up through 40 Hz (Fritton et. al., 2000), and that a strong interdependence of strain magnitude and cycle number exists, such that bone mass can be maintained either with few cycles of large strain events, or hundreds of thousands of cycles of extremely low magnitude (Qin et. al., 1998). Given this wide range of mechanical "input," it is reasonable to conclude that retention of bone mass and morphology depends as much on the persistent, low magnitude strains which arise through postural muscle activity as it does on the relatively large, rarely occurring strain events which arise from vigorous activity.

Therefore, the bone wasting that occurs in space may result *not only* from the diminished high-level impact of intense actions, but the *sarcopenia* that arises in microgravity (Desplanches et. al., 1990) will inevitably suppress the high-frequency, low magnitude strains concomitant to activities such as standing. Should this be the case, "reintroducing" this high frequency, low magnitude strain back into the skeleton, through vibration, may serve to inhibit the bone loss that normally parallels space flight. While it is generally presumed that the larger strains generated by vigorous functional activity (e.g., >2000 microstrain) are the most influential in this regard, the work which originally led to this NASA study (and has progressed extensively in parallel with this study) has demonstrated that short durations (<20 minutes) of high frequency (10-100 Hz), extremely low magnitude (<10 microstrain) mechanical signals are strongly anabolic (Fig. 1). Animal models have demonstrated that these mechanical signals, induced non-invasively into the skeleton through ground-based vibration, can and increase both the stiffness and strength of trabecular bone by as much as 25%.

***Figure 1**. Three-dimensional reconstructions of 1cm^3 region of trabecular bone harvested from the medial femoral condyle of a control animal (left) and an animal subject to the low level mechanical stimulation for a period of one year. These images, made from stacked μCT images, reflect the increases in trabecular density observed in the proximal femur determined by static and dynamic histomorphometry (Rubin et. al., 2000a).*

While it is clear from the previous studies in "normal" animals that these low-level mechanical stimuli were anabolic, it remains unclear if they are effective in inhibiting the osteoporosis that parallels disuse. We have adopted the rat-tail model of disuse osteopenia, as developed by Morey-Holton and colleagues (1981), as an effective ground-based model to examine the impact of disuse, and this low-level mechanical stimulus, on bone remodeling activity. This approach was split into three phases: *I)* to determine which parameters of the mechanical stimulus were osteogenic in the normal weight-bearing rat, *II)* to determine the extent to which alterations in histomorphometry as caused by tail-suspension influenced the expression of both known and novel genes in the bone tissue, and *III)* to determine if anabolic mechanical stimuli isolated in phase I could effectively influence both the remodeling state and transcriptional activity as perturbed by disuse.

Phase One

In the first phase of this work, we used a clustered factorial design based on the interdependence of time, intensity and frequency to determine if short periods (10, 20 & 80 minutes) of low level mechanical stimuli (0.25 & 0.5g), induced non-invasively at distinct frequencies (22, 45 & 90Hz), were effective in stimulating bone formation in the normal weight-bearing rat (Rubin et. al. 1999a). When animals were subject to the mechanical stimulus, they were not removed from their cage, but instead the cage was simply placed on the oscillating plate. No visible changes in animal behavior were noticed during the stimulus periods. In essence, the goal was to identify several stimulus regimens that were anabolic, as well as several that failed to influence skeletal remodeling/modeling activity. It is important to point out that all of the data reported are on female Sprague-Dawley retired breeders, individually housed, 6m at the beginning of the protocol (young adult), and that the histomorphometric data are compared both to long-term control (LTC) and zero-time control (ZC).

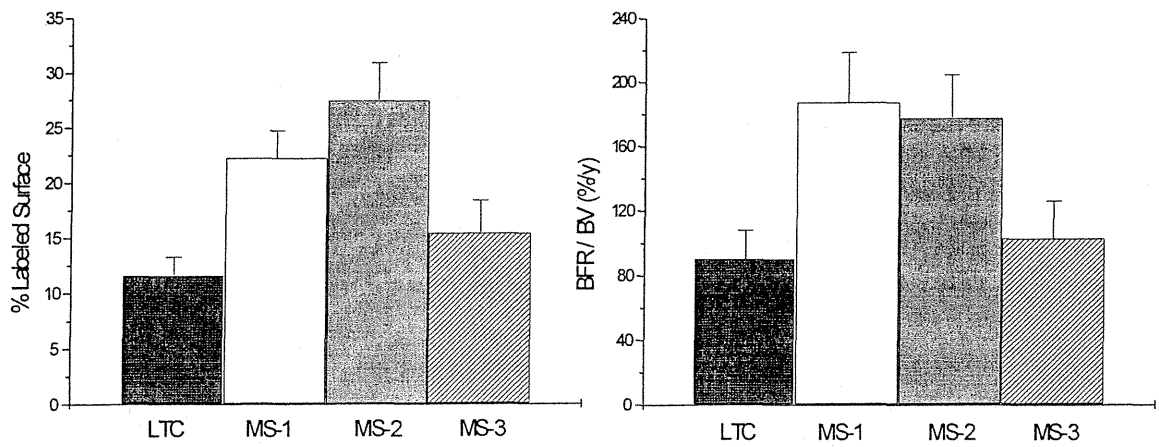


Fig. 2. *In phase one, following 28 days of unrestricted weight bearing (LTC; n=12), or weight bearing plus 10 minutes per day of a 0.25g mechanical intervention, histomorphometry was performed on the proximal tibial metaphysis. Both the 90Hz (MS-1; n=15) and 45Hz (MS-2; n=14) groups showed a significant increase in LS and BFR/BV over LTC ($p<0.02$). The 22Hz (MS-3; n=12) stimulus was NSD from LTC.*

A single element strain gage, attached longitudinally to the tibial diaphysis of rats used for calibration showed that, similar to the sheep and turkey studies described above, the mechanical intervention, at around 0.3g, generated strains less than 5με. After 28 days of ten minutes per day, five days per week, exposure to a 0.25g mechanical stimulus, both of the higher frequency signals (45Hz & 90Hz) stimulated significant increases in both labeled surface and mineral apposition rate (Fig. 2).

Phase Two

In phase two of the protocol, our goal was to identify both known and novel genes upregulated in bone tissue by disuse. The data reported here reflect two distinct experiments; the transcriptional activity is examined at 10 days (to determine the early response genes to disuse), and the histomorphometry evaluated at both 10 and 28 days (to allow correlation to the long term mechanical studies described above; only the 28d histomorphometry are described here). Animals were individually housed and suspended by their tails, following the published routines and personal communication with Dr. Morey-Holton. Following sacrifice, the right tibia was prepared for histomorphometry of the proximal metaphysis. The left tibia was immediately frozen in liquid nitrogen to be used for the extraction of RNA, which was isolated using the acid-guanidinium thiocynate-phenol-chloroform extraction method (Chomczynski & Saachi, 1987). The differential expression of genes was analyzed using the differential mRNA display method (DD-PCR; Liang & Pardee, 1992) utilizing polymerase chain reaction (PCR) and RNA image kits (GenHunter Corp.). Any bands clearly present under one condition (i.e., disuse vs. control) and absent in the other, was subsequently excised from the gel and reamplified, cloned and sequenced using standard procedures in order to determine its identity. Northern analysis was used to confirm that such cDNA fragments were differentially expressed and their relative expression determined using densitometry (normalized to GAPDH). For the gene identification protocols performed at 10d, six animals were evaluated in each group, and each animal was evaluated independently (RNA was not pooled).

At 28d, as compared to long-term controls, the tail-suspension caused LS to drop 26% (LTC: 11.7% ±1.6%; DIS: 8.6% ±2.4%; $p<0.05$), and BFR/BV (%/y) to drop –52% in the tibia (LTC: 90% ±18%; DIS: 42.9% ±17.4%; $p<0.05$). These data are similar, but not identical, to that observed at 10d (Fig. 3). DD-PCR of RNA from the tibiae of the tail suspended animals successfully identified several gene candidates with increased expression following 10d of disuse, as compared to long term controls. Discussed here are two specific genes, one known, and one unknown. S-14 was first isolated from liver (Liaw & Towle, 1984), and is believed to participate in adipose synthesis. S-14 has not been shown to be expressed in bone (Fig. 4). The second, OPO-1 (Osteoporosis-1) is a novel gene, and the full-length clone has been isolated and sequenced (1.8 Kb). It is important to note that, while DD-PCR is an effective way of identifying both known and novel genes, it is inherently limited by the small scale in which is can be used, a potential limitation in the undoubtedly complex biological processes of formation and resorption. To more comprehensively address molecular basis of bone remodeling, we recently begun using cDNA microarrays (Iyer et. al., 1999), discussed in detail in the experimental methods section.

Fig. 3. Dynamic histomorphometry was used to quantify the bone tissue response to ten days of tail suspension. Both labeled surface (left) and bone formation rate per bone volume (right) were reduced significantly by disuse (P<0.05).

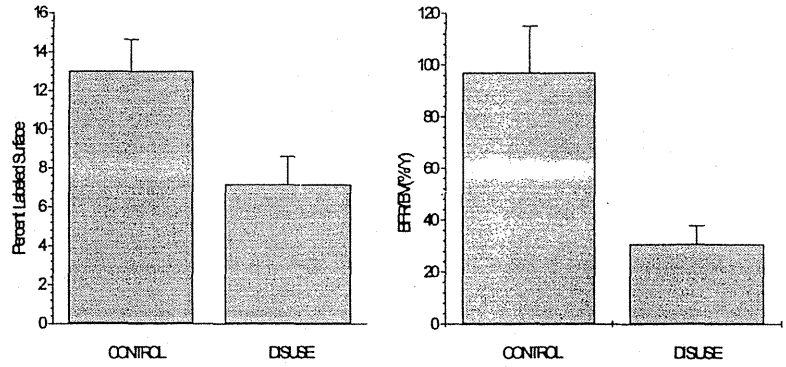
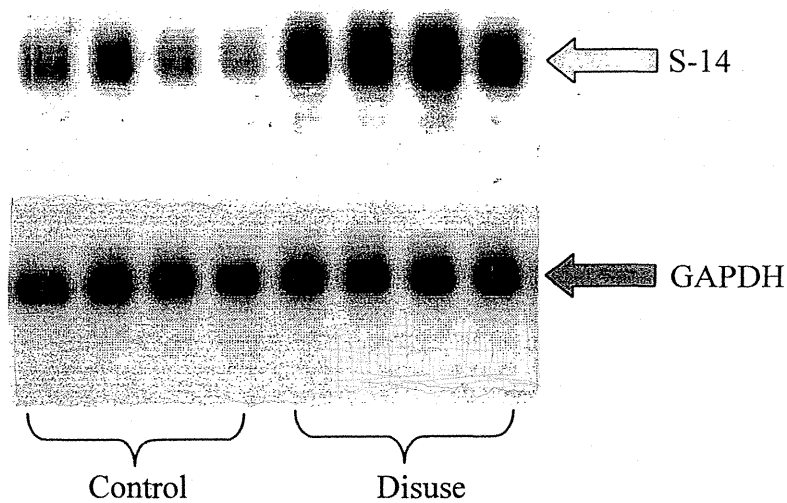


Fig. 4. Northern analysis was used to confirm all DD-PCR gene candidates. In this case, northerns were performed to confirm the upregulation of S-14 in the disuse animals. The left four lanes are from four different long-term (10d) control animals, and the right four lanes are from four animals subject to 10d disuse. Using optical density methods and normalized to GAPDH, S-14 was upregulated over six-fold when following ten days of disuse.



Using RNA extracted from the phase one animals, the relative transcriptional activity following the osteogenic activity relative to control, was examined (Table 1). Interestingly, while MS-1 failed to influence the activity of S-14 relative to control, MS-2 downregulated expression by 45% (Rubin et. al., 1999b). Conversely, MS-1 downregulated the activity of OPO-1 by 74%, but MS-2 failed to influence expression. MS-3, which failed to influence parameters of bone remodeling relative to long term control, failed to influence transcriptional activity of OPO-1 relative to control, while S-14 expression was *increased* by 33%, thus trending towards disuse activity (Fig. 5).

	Control	Disuse	MS-1	MS-2	MS-3
S-14	+ +	+ + + +	+ +	+	+++
OPO-1	+ +	+ + +	+	+ +	++

Table 1. *Relative expression of two genes, compared to control, following 28d of disuse or disuse plus 10 minutes per day of three distinct low-level mechanical stimuli. This gene expression (e.g., S-14 increases 6-fold in disuse; OPO-1 increases 60%) parallels the increased resorptive activity observed histomorphometrically, and to a certain degree, is inversely proportional to the anabolic potential of the mechanical stimulus.*

In contrast to systemic, pharmaceutical intervention such as estrogens, bisphosphonates, parathyroid hormone or calcitonin, the attributes of such mechanical prophylaxes are that they are native to the bone tissue, safe at low intensities, incorporate all aspects of the remodeling cycle, will ultimately induce lamellar bone, and the relative amplitude of the signal will subside as formation persists (self-regulating and self-targeting). Preliminary evidence indicates that these low level mechanical signals can be used prophylactically; they can serve to *maintain* bone as well as rebuild lost bone. Especially at the onset of osteopenia, where the benefits of a pharmacological intervention may not supercede the risks to the patient, a mechanical treatment modality represents a long-term strategy with a minimal danger to the patient.

The anabolic potential of low-level, high frequency mechanical stimuli points to their potential as a unique intervention for disorders and injuries of the musculoskeletal system. Even in the reconsideration of Wolff's Law presented here, the widespread use of low-level mechanical stimuli in the treatment of skeletal disorders will inevitably, and understandably, be delayed until we achieve a better understanding of both the physical and biological mechanisms by which they act. Nevertheless, even in this post-genomics era and the great promise of "drugs-by-design", the impact of environmental stimuli, the premise of Wolff's Law, can not be ignored.

In addition to the large amplitude strains typically associated with functional activity, smaller magnitude strain signals are evident in bone. These small strain signals persist over long durations, including passive actions such as standing, and therefore represent a dominant component of the bone's functional strain history. Not surprisingly, therefore, animal models, such as rat-tail suspension, remove this regulatory stimulus, and are therefore permissive to the uncoupling of formation and resorption, and facilitate resorptive activity. In the studies reported here, we have shown the anabolic potential of extremely low level, high frequency mechanical stimuli in several distinct animal models, as well as the first demonstration that these low level biomechanical signals effectively prevent osteoporosis from occurring, even when subject to 23 hours and 50 minutes of a strong stimulus for resorption (fig. 7). As these signals are induced non-invasively, and are so small in magnitude, their role in the prevention and/or treatment of skeletal disorders is promising.

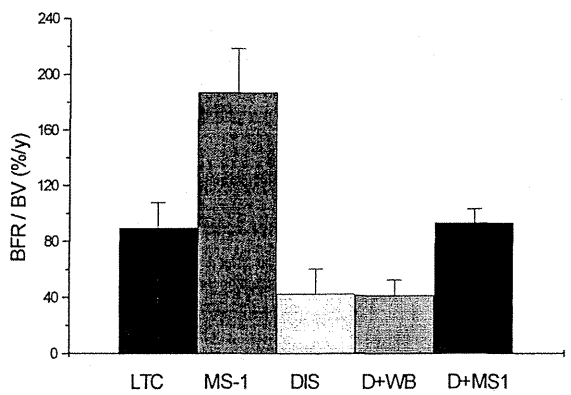
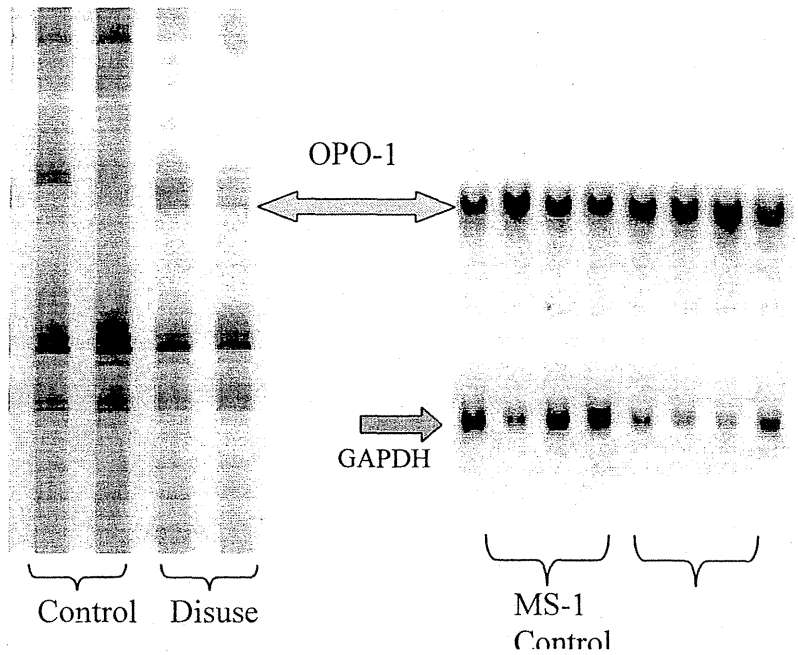


Fig. 7. *Bone formation rate per bone volume is used to compare distinct aspects of phases one, two and three. It is clear that the low level mechanical intervention, MS-1, is osteogenic in normal weightbearing conditions. Further, interrupting disuse (D) with 10 minutes per day of weightbearing (D+WB) fails to influence remodeling activity. Interestingly, when using MS-1, the anabolic stimulus, to combat disuse, the impact of the intervention only serves to "normalize" the response back to values seen in long-term control.*

Conclusions

As first described by Julius Wolff, it is well accepted that bone subscribes to "form follows function" principles, which ultimately helps to define the mass and morphology of the skeleton. As important as this Law of Bone Transformation is to the musculoskeletal disciplines, identifying the specific mechanical factors that regulate the bone response has proven difficult, and has impeded the development of effective means of applying these unique anabolic factors to treat skeletal injury or diseases, such as microgravity induced bone loss. The work presented here has summarized evidence that demonstrates bone's sensitivity to high frequency, low magnitude mechanical signals, and its ability to stop disuse osteoporosis. Arising from muscle contraction, these signals may be small, but they are omnipresent and represent a dominant signal in the bone's strain history. These low-level signals have great potential for direct clinical applications, such as in fracture healing, osseointegration, and the prevention and/or reversal of osteoporosis.

Fig. 5. In the left gel, DD-PCR of the RNA from the tibia of two control animals (left lanes), and two animals subject to 10d of disuse. Several bands were upregulated in the disuse animals, identifying both known and unknown gene candidates. In this case, OPO-1 was isolated and identified as upregulated in the disuse animals, increasing 60% over controls. When northern analysis was performed, comparing MS-1 animals to control, OPO-1 expression dropped 74% in the osteogenic condition (right gel).



Phase Three

In phase three of this study, signals demonstrated as osteogenic in phase one were evaluated in terms of their ability to inhibit the remodeling changes caused by the tail-suspension (Rubin et. al. 2000b). As described above, 28d of tail suspension (DIS) caused both LS and BFR/BV to drop precipitously, as compared to LTC. *This suppression was not significantly different from the animals subject to disuse for most of the day (23h, 50m) and then allowed to freely bear weight for 10 minutes per day (D+WB; Fig. 6).* However, 10m/d of either 90Hz (D+MS1) or 45Hz (D+MS2) loading normalized LS (+9%; +3%) and BFR/BV (+4%; +11%) to normal weightbearing levels as shown by long term control (LTC). Transcriptional activity from the bones of these animals is not yet evaluated. It should be noted that no significant weight differences from LTC were measured in any of the experimental groups.

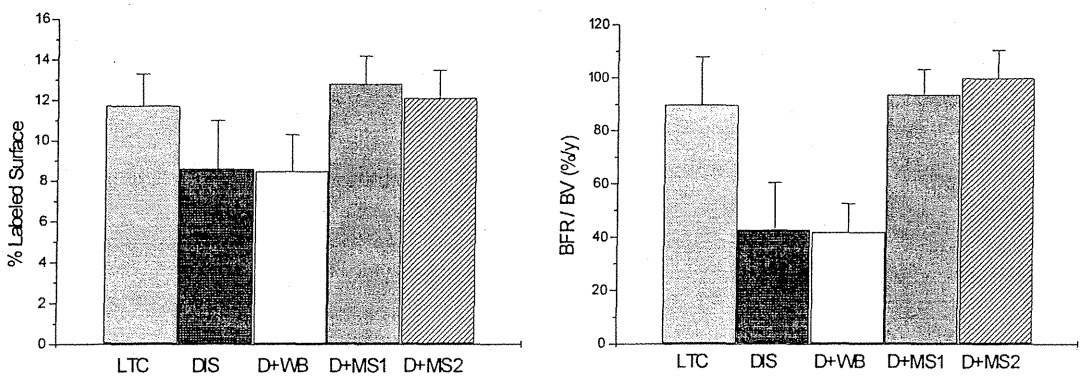


Fig. 6. *In phase three, following 28 days of tail suspension (DIS), or disuse plus mechanical intervention, the tibia were examined. Allowing 10 minutes per day of free ambulation failed to negate the influence of disuse (D+WB). A 10m/d intervention of either 90Hz (D+MS1) or 45Hz (D+MS2) restored bone parameters to those measured in long-term control (LTC). DIS nsd from D+WB; both significantly different from LTC, D+MS1 & D+MS2 ($p<0.05$); LTC nsd from D+MS1 and/or D+MS2. $N \geq 12$ for each group.*