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Original Article

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A novel pharmacological approach of musculoskeletal losses associated with simulated microgravity

S.M. Wimalawansa, S.J. Wimalawansa

Department of Internal Medicine, General Medicine and Endocrinology, The University of Texas Medical Branch at Galveston, USA

Abstract

Exposure to microgravity (weightlessness) is known to cause rapid bone and muscle losses. We have used the hind limb-suspended (HLS) rat model to simulate microgravity-induced musculoskeletal losses in order to assess resulting hormonal changes and to develop a novel pharmacological countermeasure. Previously, we demonstrated significant decreases in circulatory hormonal levels [serum thyroxin, 1,25(OH)2 vitamin D ($p<0.05$), and serum testosterone ($p<0.001$)] in HLS rats. Both thyroxin and 1,25(OH)2 vitamin D levels returned to normal soon after removal from HLS, while testosterone levels matched normal levels only after a further 3-4 weeks. However, even by day 42, bone mineral density (BMD) remained significantly lower, although serum hormones were back to normal. Because serum testosterone levels become undetectable in HLS rats, we hypothesized that the replacement of testosterone during HLS could prevent musculoskeletal losses. Based on these data, an intervention study was carried out to assess the efficacy of testosterone and synthetic anabolic steroid, nandrolone decanoate (ND), in prevention of weightlessness-induced musculoskeletal losses. HLS rats (control) had a significant reduction of muscle volume (42.9 ± 3.0, versus 56 ± 1.8 in ground control rats; $p<0.01$). Both testosterone and ND treatments prevented this muscle loss (51.5 ± 2 cm^3 and 51.6 ± 1.2, respectively; a 63% improvement, $p<0.05$). Similarly, BMD of the placebo-treated HLS rats was significantly lower than that of ground control rats (0.416± 0.011 versus 0.354 ± 0.014, $p<0.05$), and testosterone and ND prevented this bone loss (0.404 ± 0.013 versus. 0.409 ± 0.011, respectively). These data suggest that both testosterone and ND therapy can minimize the musculoskeletal losses associated with exposure to simulated weightlessness. Experiments using the combination of bisphosphonate and testosterone demonstrated complete protection of both muscle and bone in these HLS rats. Therefore, considering that: 1) testosterone is anabolic to osteoblasts and muscle cells and also decreases the rate of bone turnover, 2) serum testosterone levels are markedly suppressed in simulated weightlessness, and 3) testosterone replacement therapy prevented musculoskeletal losses in HLS rats, we propose that the musculoskeletal losses observed in this animal model (i.e., simulated microgravity) are related to their testosterone deficiency. Since serum sex hormones levels are markedly reduced in this model of simulated microgravity, androgen replacement with a bisphosphonate seems to be a rational counter.

Keywords: Osteoporosis, Microgravity, Bone Turnover, Bone Mineral Density, Androgen, Disuse Atrophy

Introduction

Decrements in bone and muscle mass are major concerns in extended spaceflight[1-5]. Immobilization (e.g., bed rest or restricted movement of limbs) can also cause rapid loss in muscle or bone mass[3, 6-8]. In addition, these conditions lead to negative calcium balance and loss of bone mineral density (BMD)[9-12]. Similar reductions in musculoskeletal mass have been reported associated with immobilization of extremities seen in external bandaging, casting, or neural re-sectioning [7, 10, 13-17]. Both mineralization and collagen metabolism seem to be impaired in animals during the first few days of space flight[18]. Reductions in muscle forces led to decreases in bone formation and BMD in the os calcis and an increase in urinary loss of calcium, but no loss of BMD in the radius and ulna in Skylab crew members after an 84-day orbital flight[19]. Bone mineral losses during hind limb suspension (HLS, tail-suspension) or space flights are more prevalent in weight-bearing bones and are due mainly to decreases in bone formation[5, 20, 21]. It has also been reported that mechanical unloading may also account for the differential loss in

Corresponding author: Sunishka M. Wimalawansa, Department of Internal Medicine, General Medicine and Endocrinology, The University of Texas Medical Branch at Galveston, 8.104, Medical Research Building, Galveston, Texas, 77555-1065, USA, Tel:(409) 772-7646, Fax:(409) 747-1861, E-mail : swimalaw@utmb.edu

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musculoskeletal masses of small animals flown in space flights. Over the past few years, several countermeasures have been examined to prevent the loss of musculoskeletal mass during exposure to microgravity, including pharmacological therapies such as calcitonin and bisphosphonates, and several active and passive exercise regimens, but positive outcomes have been limited. The hindlimb elevation model (HLS; tail-suspended model) in rats has been shown to simulate the bone turnover and muscle changes seen in growing rats in space flight[18, 20, 22]. This model mimics microgravity affecting musculoskeletal systems, metabolic changes altering bone formation, renal function; electrolyte disturbances, and muscle mass changes comparable to those recorded for biosatellite animals. We previously demonstrated that there is a significant decrease in serum testosterone levels in HLS rats and that this is probably independent of the changes in serum cortisol levels[23, 24]. We hypothesized that this marked decrease in testosterone levels at least in part contributes to the observed decrement in musculoskeletal mass.

Testosterone as well as the synthetic anabolic androgen analogue, nandrolone decanoate (ND) has also been shown to decrease bone turnover and increase BMD in animals and humans[15, 25]. Therefore, this study was designed to assess whether hindlimb suspension-induced reduction in musculoskeletal mass can be prevented with testosterone or ND. Unlike in humans and monkeys, in adult rats, mice and rabbits, the inguinal canal remains open. Because of this, it has been shown that when male rats and mice are tail-suspended (or when in spaceflight), their testes periodically move into the abdominal cavity. Due to the increased temperature in this new environment, the testes undergo several changes including a dramatic reduction in spermatogenesis[13]. Marked changes occur in the histology in the testis and epididymis in tail-suspended rats without inguinal canal ligation[16]. Therefore, in the present study in both control and tail-suspended rats, a loose ligature of nonabsorbable suture was placed around the inguinal canal under anesthesia, in order to prevent upward movement of the testes.

Methodology and Results

Basal studies to understand the pathophysiology of musculoskeletal interactions

In the first study 24 adult male rats were assigned to two groups with 12 rats being HLS for 12 days, and the remaining 12 rats serving as ground controls. Each weight-bearing group was fed the average daily amount of food consumed by its corresponding tail-suspended group of rats (i.e., pair-fed).

On days 0, 6, and 12, blood samples were taken to estimate circulating hormone levels. HLS rats had significant reductions in testosterone, 1,25 $(OH)_2$ vitamin D and thyroxin levels by day 6 ($p<0.01$); their testosterone levels were almost undetectable by day 12 ($p<0.001$) (Fig. 1). Serum cortisol levels in these rats were elevated on day 6 ($p<0.02$) but returned to normal levels by day 12. No changes were observed with serum-ionized calcium and other hormones examined. Body weights and weights of thymus, heart and brain were also unchanged[24]. During this

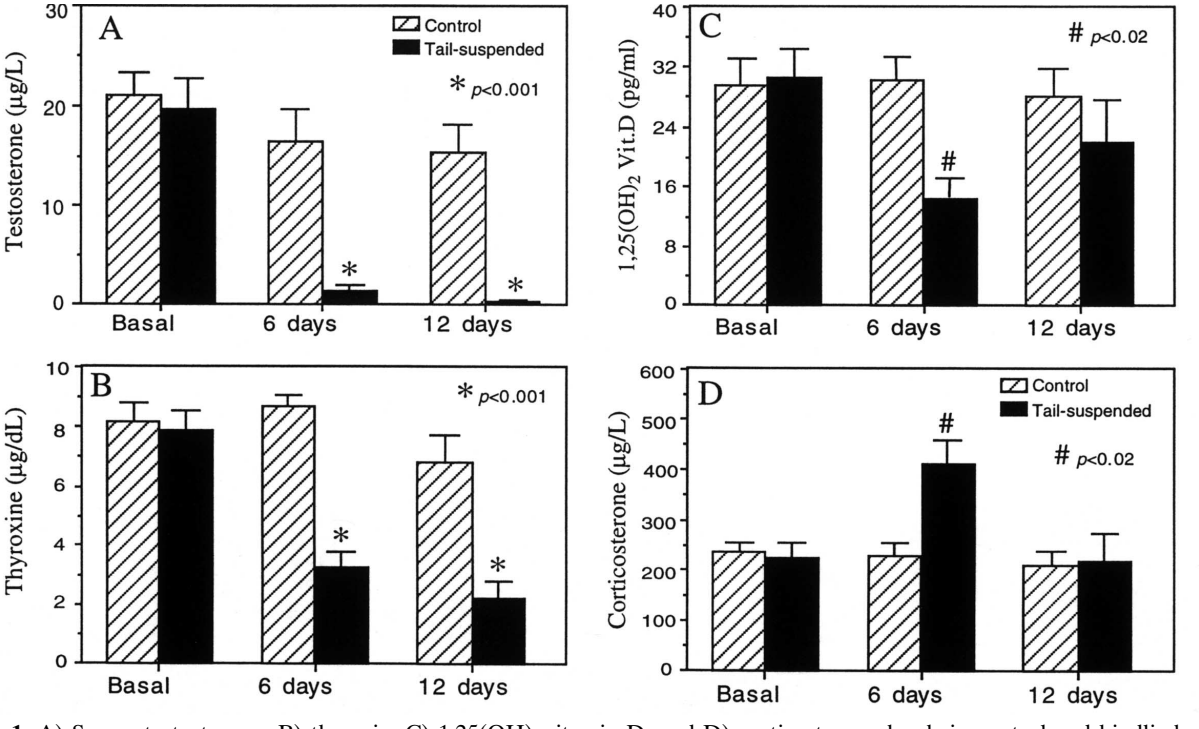

Figure 1: A) Serum testosterone, B) thyroxin, C) 1,25$(OH)_2$ vitamin D, and D) corticosterone levels in control and hindlimb suspended (HLS) rats prior to, and during 12-day HLS (n=12 each group, #$p<0.02$, *$p<0.001$) (published with permission from Endocrine; Wimalawansa et al, 1999; 10: 253-60).

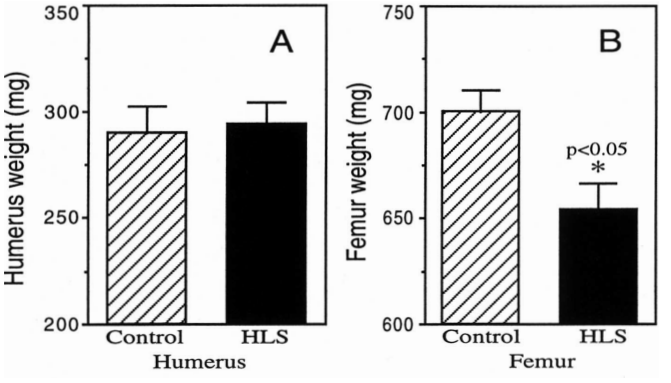

Figure 2: Mean (± SEM) of. A) femur weights, and B) humeri weights on day 12, of the HLS rats in compared to ground control rats (n=12 each group, *p<0.05) (published with permission from Endocrine; Wimalawansa et al, 1999; 10: 253-60).

12-day period of weightlessness these HLS studies consistently showed significant decreases of femur weights (Fig. 2B), but not weights of humeri (Fig. 2A). Dual energy X-ray absorptiometry (DXA) studies showed 12-14% decrease of hone mineral density (BMD) during 12-day hindlimb suspension of the affected bones (Fig. 3A). Nuclear magnetic resonance imaging (NMR/MRI) showed that rats exposed to HLS lost 28.5% of leg muscle mass within the 12 day experimental period ($p<0.005$) (74.5 ± 3.6 cm^3 in control versus. 53.3 ± 4.8 cm^3 in hindlimb suspended rats) (Fig. 3B). In the second study, 8 rats were ground controls while an additional 8 rats were HLS for 12 days before being removed from tail-suspension and maintained for a further 30 days. Blood samples were collected every 6th day for 42 days.

This study showed that both serum thyroxin and $1,25(OH)_2$ vitamin D levels returned to normal levels soon after hindlimb weighting, while serum testosterone levels matched normal levels only after further a 3-4 weeks (Fig. 4). On day 12 in both studies, significant reductions were observed in the lumbar spine ($p<0.05$) and femoral neck ($p<0.01$) BMD in HLS rats. In the second study, HLS led not only to a significant decrease in BMD, but also BMD did not recover even by day 42 (Fig. 5). In this model of simulated weightlessness in rats, these studies showed significant decreases of serum testosterone levels and BMD in the affected weight-bearing bones (i.e., lumbar spine and the femur). Sex-steroid hormones are known to have an anabolic effect on osteoblasts and muscle cells. Taking together our recent findings and previously reported data, we conclude that in this model, markedly reduced serum testosterone levels may be contributing to these significant musculoskeletal losses.

Interventional studies for prevention of simulated weightlessness-induced musculoskeletal losses

Based on these data, we then designed a set of intervention studies to assess whether we could minimize musculoskeletal losses by replacement of the deficient sex-steroid hormone in this model. We hypothesized that this

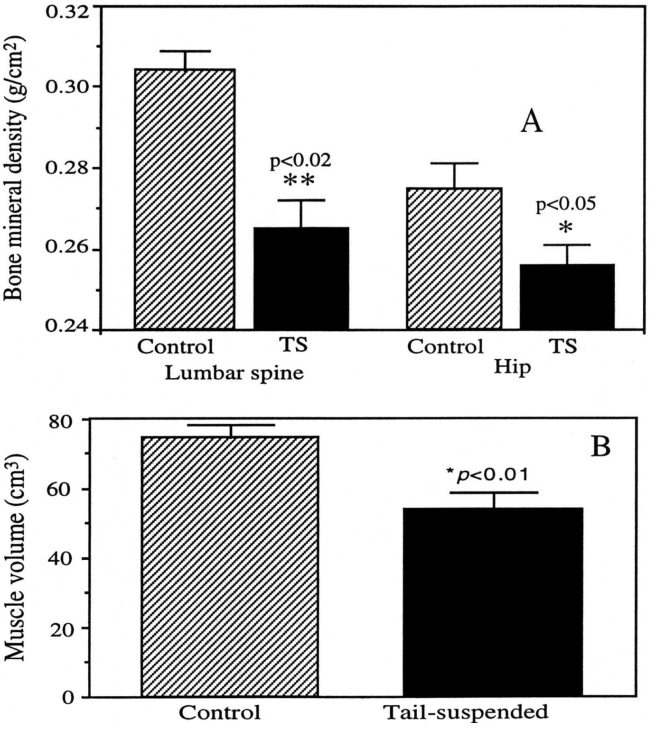

Figure 3: A) Percentage changes in bone mineral density (gr/cm^2) in the lumbar spine and proximal femur measured with DXA in 12-day HLS rats compared to ground control rats (n=12 each group, *p<0.05, #*p<0.02). B) Changes of muscle volume after 12-day HLS as measured by MRI (28.5% loss; p<0.0l) (published with permission from Endocrine; Wimalawansa et al, 1999; 10: 253-60).

musculoskeletal loss could be prevented by supplementation with testosterone and an anabolic steroid nandrolone decanoate (ND). In the 3rd study, 12 week old male Wistar rats were HLS, and administered either a vehicle (control), testosterone or ND (6 mg/kg body weight, as a single S.C. injection). Additional 20 rats were used as ground controls; of which half received testosterone. On day 12, rats were anesthetized and muscle volumes and bone masses were quantitated using MRI. HLS rats had a significant reduction of muscle volume (42.9 ± 3.0, versus 56 ± 1.8 in ground controls; $p<0.01$). Both testosterone and ND treatments prevented this muscle loss (51.5 ± 2 cm^3 and 51.6 ± 1.2, respectively; a 63% improvement, $p<0.05$) (Fig. 6). MRI studies demonstrated 85% improvement in BMD of femurs in the testosterone group (1.15 ± 0.04 versus 1.04 ± 0.04, $p<0.05$) and 76% improvement in the ND group (1.13 ± 0.07), while ground control rats had a BMD of 1.17 ± 0.03 (Fig. 7). These data suggest that in HLS rats, countermeasures using either testosterone or ND can abolish the muscle and bone losses. Following use of sex-steroid therapy in HLS, the beneficial effects are most pronounced in the muscles. Since serum testosterone levels are markedly reduced in this model of simulated microgravity, androgen replacement seems to be a rational countermeasure to prevent microgravity-induced musculoskeletal losses.

A 4th study was conducted identical to the study 1 except

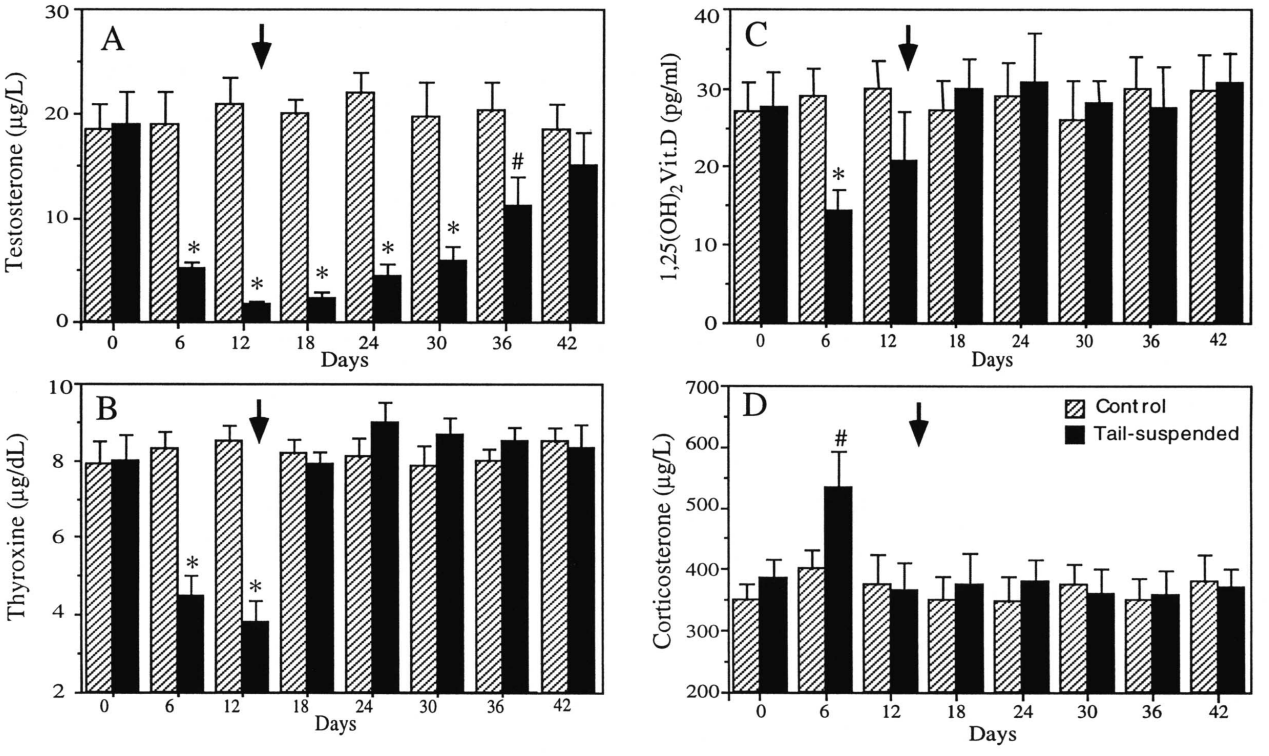

Figure 4: A) Serum testosterone, B) thyroxin, C) 1,25(OH)2 vitamin D, and D) corticosterone levels in the ground control and 12-day HLS rats. Data are presented at the basal (i.e., prior to HLS, day 6 and day 12 of HLS, and during the 30-days of post HLS (n=8/group; #p<0.01, *p<0.001). The arrows indicate the day of termination of HLS (published with permission from Endocrine; Wimalawansa et al. 1999; 10:253-60).

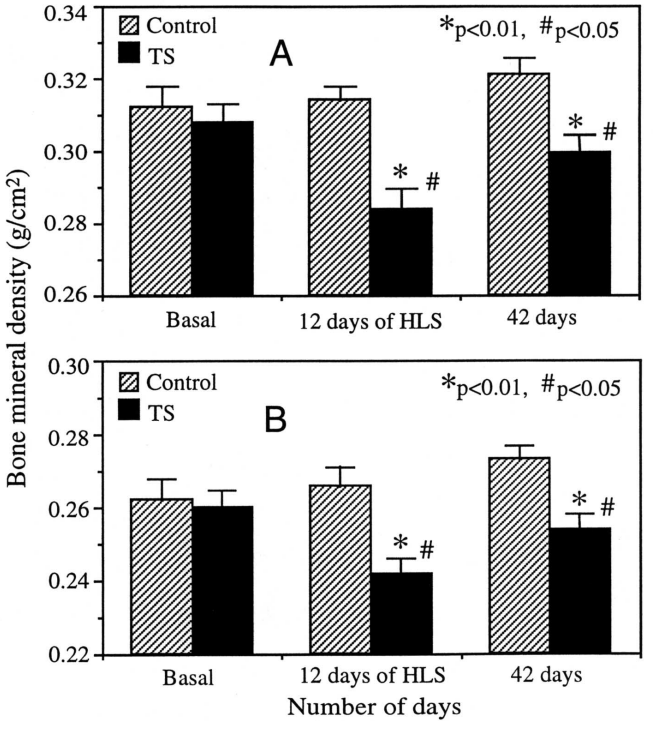

Figure 5: Bone mineral density (g/cm^2) at basal, 12 days after hindlimb suspension (HLS), and 30 days post-tail-suspension in the A) lumbar spine, and B) proximal femur measured with DXA (n=8/group;#p<0.05, *p<0.01). *Significant changes of BMD from control rats (unpaired t-test); significant changes from basal BMD (paired t-test).

that we used adult female rats instead of male rats. In this study, we also observed a significant reduction of circulating estrogen levels within the first few days of HLS, and this was associated with marked reductions in both muscle and bones. In the 5th study (the protocol was similar to the study 3), but treatment groups were different: consisting of a placebo group, a single dose of testosterone alone, alendronate alone, and the combination of testosterone and alendronate. The responses in BMD were: 75% improvement in the testosterone group, 65% improvement in the alendronate group, and 95% improvement in the combined treatment group.

Following this, we carried out another study (a protocol similar to study 3), but we used adult female rats. The treatment groups included a placebo group, estrogen treatment alone, alendronate alone, and the combination of estrogen and alendronate. In this study, the responses in BMD were: 68% improvement in the estrogen group, 74% improvement in the alendronate group, >95% improvement in the combined treatment group.

Statistics

Statistical analysis was performed using the statistical package SigmaStat (Jandel, San Rafael, CA). Differences between groups were analyzed by analysis of variance, followed by the Student-Newman-Keuls test for multiple group comparisons. Between group data were also evaluated first by Kruskal-Wallis one-way analysis to determine

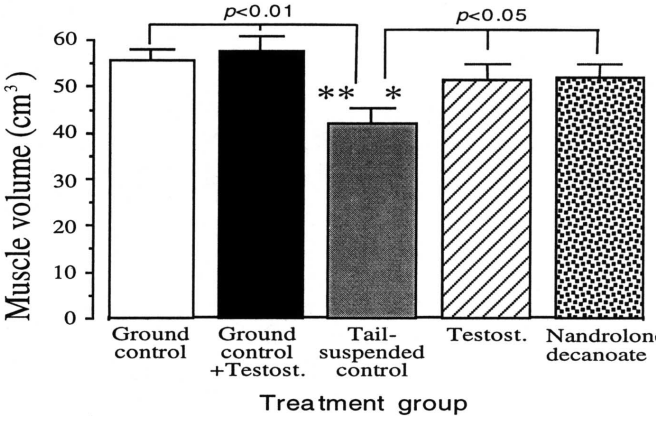

Figure 6: Changes in muscle volume (mm^3) measured by magnetic resonance imaging (MRI) in controls and rats subjected to 12-day HLS in response to therapy with testosterone and nandrolone decanoate 13 (ND) in comparison to untreated (n=10 each group, *p<0.05, **p<0.01) (published with permission from J Applied Physiol; Wimalawansa et al, 1999; 86: 1841-46).

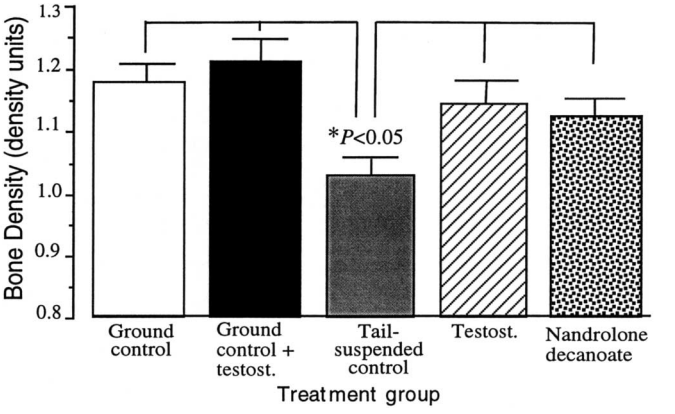

Figure 7: The effects of testosterone and nandrolone decanoate treatment on bone mass (density units) measured by MRI in HLS rats in comparison to placebo treated and ground control rats as measured by MRI (n=10/each group; *p<0.05) (published with permission from J Applied Physiol; Wimalawansa et al, 1999; 86: 1841-46).

whether differences existed, and then by the Newman-Keuls multiple group comparison test to determine if the groups varied significantly from each other. A value of $p<0.05$ was considered significant.

Discussion

These studies suggest that one of the fundamental causes of musculoskeletal losses in animals (and in human patients) exposed to weightlessness is a marked decrease in circulatory levels of testosterone in males and estrogen levels in females. We have previously observed a similar significant reduction of circulatory testosterone levels in human patients who were bed-ridden after suffering from a stroke (Wimalawansa, 1991, unpublished). In this model, testosterone replacement and ND therapy, both, were able to significantly decrease the expected muscle volume and bone mass loss. Furthermore, for the first time, musculoskeletal losses associated with the exposure to weightlessness could be abolished using this simple pharmacological intervention. Similar additive effects of hormone replacement therapy and a bisphosphonate were previously demonstrated by our group in two randomized double blind control clinical studies in humans[26, 27]. Muscle volumes and bone masses of these two treated groups of rats were not statistically different from that of the ground controls.

A negative calcium phosphate balance and decreased BMD in the peripheral skeleton such as calcaneum and radius bones have been reported in rats exposed to microgravity and simulated weightlessness[5, 22, 28]. Mechanical loading and muscle forces play an important role in the development and maintenance of skeletal tissues[5, 20, 28]. Subnormal mechanical stresses as a result of bed rest, immobilization or spaceflight can lead to disuse osteoporosis, whereas supranormal loads upon extremities results in increased bone masses[1-3, 4, 11-14, 17, 29]. The testes are the major source of testosterone, which serves both growth and maintenance functions for the musculoskeletal system [30, 31]. For example, castration in rats leads to skeletal muscle atrophy in the hormone sensitive levator ani, and in other striated muscles[32], whereas supra-physiological doses of testosterone increase muscle mass and strength[31]. Changes of serum hormone levels have been reported in 12-day HLS rats[23, 33], and in rats flown in the 14-day COSMOS 2014 mission[34]. Testosterone and anabolic steroids have positive effects on osteoblasts in bone formation[25], and the mechanisms of action of androgen on bone cells involves induction of TGF-β, and may also involve sensitization of bone cells to FGF and IGF-2[34].

Indeed, the increase in skeletal mass in androgen treated patients has been directly attributed to the effect of androgen on bone formation. Since testosterone is known to influence bone formation and maintenance of muscle cells, one could postulate that a marked reduction in testosterone may be at least in part responsible for the decreased musculoskeletal losses in HLS rats as well as humans exposed to microgravity, and its replacement should alleviate this. MRI is a very sensitive technique that can be used for measuring changes in muscle volume in this animal model (~2% loss of muscle volume/day). Here, we demonstrated that testosterone replacement therapy or treatment with the anabolic steroid, ND, significantly decreased expected muscle volume and BMD decrements in these tail-suspended rats[36].

We conclude that in these HLS rats, the marked reduction of serum testosterone levels may contribute to the observed reductions in muscle and bone masses, and replacement of this deficient hormone minimizes these losses of musculoskeletal mass. A combination of sex-steroid replacement therapy together and a bisphosphonate was able to abolish musculoskeletal losses in simulated microgravity.

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