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EFFECTS OF GLYCYRRHETINIC ACID ON BONE LOSS INDUCED BY MECHANICAL UNLOADING

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Abstract:

Introduction: The societal factors in bone loss are numerous, ranging from old age to space flight. Although several treatment themes are available for bone loss, there continues to be a demand for new ideas as well as a better understanding of the topic. A novel treatment approach to attenuate bone loss has emerged in gap junction manipulation, specifically the knockout of bone specific connexin 43 proteins which make up gap junctions. Glycyrrhetinic acid (GA) has been shown as a non-specific gap junction inhibitor, and may also be an anti-inflammatory. Thus, we hypothesized that GA may be an effective tool in attenuating bone loss during periods of bone unloading, as in bed ridden patients or spaceflight. We hypothesized that GA administration at 20mg/kg would block gap junction function, and attenuate bone loss induced by hind limb suspension (HLS) unloading.

Design: A randomized controlled trial was designed with twenty five male wild-type C57C1/BJ mice of skeletally mature age (6 months), and placed into 5 groups: ground control, ground control GA treatment, HLS no treatment, HLS vehicle, and HLS GA treatment. Experimental protocol utilized mechanical unloading based on a 3 week duration of HLS and GA intervention via subcutaneous injection; the ground control groups were not mechanically unloaded.

Assessments: Animals were evaluated primarily using MicroCT scan to evaluate physical characteristics of bone in the femur. Mechanical testing based on three point bending, and monitoring of basic body composition were also evaluated.

Conclusions: The original hypothesis predicted that GA treatment during HLS would result in an attenuation of bone loss. HLS alone does cause significant changes in several microarchitecture parameters, such as a 60% decrease in trabecular BV/TV, when compared to ground control 38%. However, our results did not show significant changes in MicroCT results between suspended control and suspended GA treatment groups; changes in bone characteristics were similar across all unloaded mice. GA ground control mice did not show significant differences from ground controls not receiving GA. Our hypothesis that GA would attenuate bone loss during unloading was not supported.

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Chapter 1: Introduction

Examining bone characteristics, along with the physiological and environmental variables that affect them has been an important theme in research for many years. There are many societal reasons for conducting bone research; decreases in bone density associated with age, particularly following menopause in women has been an important topic to understand. Furthermore, age independent issues with bone loss surrounding unloading examples such as extended bed rest, or in space flight, are also important themes [1]. Indeed, unloading presents significant complications for patients following extended periods of complete bed rest. Patients were significantly more likely to experience a fracture following a period of being bed ridden, especially in the first year after the unloading period had occurred[2, 3]. With less societal impacts, space flight also remains an equally challenging task to mediate bone loss in astronauts [4]. While research continues examining scenarios of both loading and unloading of bone, a novel concept more specific to the unloading of bone manifests with gap junctions. The knock-out (KO) of gap junctions made up of the connexin 43 protein (Cx43), specific to bone cells, has been identified as playing a significant role in bone loss during bouts of unloading. Contrary to initial suspicions, osteocytes and osteoblasts with a KO of the Cx43 protein, rendering them essentially gap junction deficient, have been shown to significantly attenuate the amount of trabecular bone loss using a mouse model of unloading [5]. While genetic manipulation of gap junctions proves to have substantial effects on bone, other research has examined the effects of glycyrrhetinic acid (GA), an isolate of licorice root, which is a known reversible gap junction function blocker [6]. A review of literature was undertaken to explore the viability of using GA as a means blocking gap junction function, mimicking a gap junction KO and thus attenuating bone loss during bouts of unloading.

Bone responds constantly to the mechanical influences of the environment in accordance to Wolff's law, expressing osteoclast activity in response to a decrease in load, and osteoblast activity in response to an increase in load [7]. However, despite playing an important structural role, the changes of bone structure in response to changes in environment or stress are not fully understood. Several models have examined the effects of unloading, or a decreased demand, and have yielded several potential mechanisms for bone adaptation; three mechanisms suggested for bone adaptation include mechanical strain, streaming potentials, and fluid shear stress, respectively [8]. Specifically, the precise role of gap junctions in any of the previously mentioned mechanisms for bone changes is not fully understood; however many of the individual functions of gap junctions are well known.

Gap junctions are protein structured channels between adjacent cells that allow cell to cell communication. Gap-junctional intercellular communication can occur between two like cells such as osteoblast to osteoblast, or different cells such as osteoclast to osteoblast [9]. Intercellular communication is achieved by exchange of small molecules, less than 1 kDa, from one cell cytoplasm to another [10]. Similarly, cell to extracellular matrix communication is achieved through hemi-channels, which are similar to gap junctions in structure. By far the most abundant protein making up gap junctions as well as hemi-channels in osteocytes, osteoblasts, and osteoclasts is connexin 43 [11]. Six connexin subunits form to produce a channel called a connexon in the lateral cell plasma membrane in the case of gap junctions [11], or in the basal aspect of the plasma membrane in the case of hemi-channels. When two connexons in adjacent cells are docked next to each other, they form a gap junction, or intercellular channel. Although Cx43 is responsible for the vast majority of gap junctions displayed in bone, other connexin proteins in the form of connexin 45 and connexin 46 have also been reported [12]. In addition to

being the principal connexin protein in bone cells, Cx43 is also expressed abundantly in other tissues, most notably cardiac cells, hepatic cells, and cells in the eye [11].

As mentioned previously, the connexin proteins along with the gap junctions they form have many different functions; in vitro gap junctions are responsible for contributing to the differentiation of progenitor cells into respective osteoblasts or osteoclasts [12, 13]. In vivo, non-specific knock out of Cx43 during fetal development is embryonically lethal, as cardiac failure was observed at birth and resulted in death [14]. Furthermore, non-specific inhibition of gap junction communication appears to play a role a role in attenuating disease progression. Several studies have indicated that blockage of gap junctions slows the progression of breast cancer cells as well as progression of amyotrophic lateral sclerosis [15, 16]. However, although there is an abundance of research surrounding gap junctions in vitro, there remains a significant gap in the understanding of gap junctions and their mechanisms in vivo; more specifically how the gap junction influences changes in bone. Gap junctions and the implications of deficiency continue to be a topic of current research.

Glycyrrhetinic acid is an aglycone derivative of its sweeter counterpart glycyrrhizic acid, which is an extract from licorice root (Glycyrrhizia Glabra). It is commonly known in two major forms, alpha-Glycyrrhetinic acid (AGA), beta-Glycyrrhetinic aid (BGA), as well as the glycyrrhizic acid mentioned above. GA has been indicated to display anti-inflammatory effects [6, 17-19]; the anti-inflammatory effects could be explained by the apparent affinity of GA to mineralcorticoid receptor, as well as glucocorticoid receptors to a lesser degree [6]. GA is not a steroid, but displays steroid like structure, which explains its ability to interact with steroid associated receptors. In addition to its suspected anti-inflammatory effects, perhaps most notably GA has also been identified as a potent gap junction inhibitor in vitro [6, 17]. Although it is not

clear exactly the mechanism that mediates GA's ability to inhibit gap junctional communication, it has been noted that it is not through the relationship with mineral-corticoid or gluco-corticoid receptors mentioned above [6]. GA represents a reversible gap junction blocker, which works on established gap junctions as well as newly constructed junctions in vitro [6]. As a gap junction inhibitor, AGA appears to be a more effective blocker than BGA, as well as less cytotoxic at effective levels [6, 17, 20]; as an anti-inflammatory, AGA also appears to have more pronounced effects than BGA. Interestingly, in a study by Takeuchi et al examining the effects of gap junction suppression on lateral sclerosis [15], the novel compound used to block gap junctions was indeed a blocker with a synthetic structure based on GA. Glycyrrhetinic acid also shows anti-bacterial properties, as shown in a study examining GA effects on *staphylococcus aureus* [18]. Although GA did not have a direct effect on bacterial growth, it did impact the amount of toxins produced [18]; the effects of GA appear to be dose dependent in many cases. Furthermore GA had a significant effect on periodontal bone loss, as demonstrated in a study by Sasaki et al [19]; notably, the GA attenuated bone loss appeared to be independent of the interleukin –10, and glucocorticoid mechanisms.

Research surrounding gap junctions and research surrounding GA have found common ground with recent findings that suggest gap junction deficiency attenuates bone loss during unloading. A knockout model of bone-specific Cx43 in mice demonstrated an attenuation of trabecular bone loss over control mice following three weeks of unloading via hind limb suspension [5]. The same study showed less suppression of cortical bone formation during the unloading period in knockout mice, resulting in the cortical bone levels maintaining closer to baseline levels versus the wild type mice [5]. These results suggest gap junctions may play a significant role in unloading-induced bone loss. Likewise, in a similar study examining the

effects of muscle paralysis on bone, cortical bone again did not experience the amount of loss in Cx43 knock-out mice as was seen in wild type mice [21]. As a compliment to these studies, GA acid was discussed earlier as an effective, albeit general gap junction blocker; GA was also suggested to attenuate bone loss in a gap junction independent manner when bone was exposed to a pathogen (Porphyromonas gingivalis) [19], and appears to have effects on other inflammatory processes that could play a role on bone remodeling [22]. This draws a correlation between GA as a means of attenuating bone loss because of its gap junction blocking qualities, and gap junction deletion in osteocytes and osteoblasts being shown to attenuate bone loss.

Bone density, and bone density loss are important topics in society for a variety of reasons from age related degradation, effects of immobilization, and space flight amongst others. Although there are several therapies for combating bone loss, a novel approach has been introduced with the manipulation of gap junctions, specifically connexin 43 which is the most prevalent gap junction protein in osteocytes. Glycyrrhetinic acid is a well known in vitro gap junction inhibitor, and thus in vivo may play a role in bone density loss. If effective, GA could represent a natural, pharmacological approach to treating patients at risk of experiencing bone loss. Therefore, the purpose of this study was to investigate the effects of GA on the changes of bone characteristics in unloaded groups, when compared to non-treatment control groups using a mouse hind limb suspension model.

Hypothesis:

HA: The administration of Glycyrrhetinic acid will result in reduced loss of bone during a period of unloading in comparison to the vehicle control group.

HO: The administration of Glycyrrhetinic acid will have no effect on bone loss, or show an increase in bone loss during a period of unloading in comparison to the non-treatment group.

Chapter Two: Experimental Methods and Results

Methods:

The study employed a hind limb suspension (HLS) model developed by Morey-Holton and Globus at the National Aeronautics and Space Administration Ames Research Center [23]. The HLS utilized represents a well validated in-vivo mouse simulation for the environments of micro-gravity and bed rest unloading; the HLS model also incorporates a cephalic fluid shift as experienced by astronauts [4, 23]. Furthermore, this model has been employed by our lab with success to conduct HLS research for several years[5, 24]. As a result of our experience, this study adopted the modifications from the original HLS model, explained by Lloyd et al as having a 30 degree angle of elevation, keeping the forelimbs adequately stressed and minimizing strain on the animals [5], shown in Figure 1. The HLS unloading period lasted three weeks, a period previously shown as adequate to demonstrate significant loss of trabecular and cortical bone [5].

Figure 1. HLS apparatus with two mice suspended per cage at an angel approx. 30˚.

We examined 25 male C57C1/BJ wild type (WT) mice of a skeletally mature age of 6 months; skeletal maturity in mice is regarded as four-six months, while six months is consistent with previous research in our lab [5]. Mice were randomly assigned to one of five groups with five animals per group. The five groups consisted of control (normally loaded), control with GA

intervention, HLS with GA intervention, HLS with vehicle (olive oil) intervention, and HLS with no intervention. Both HLS and control mice were housed 2 per cage, as per cage design for HLS. Animals were fed standard Harlan 18% protein 2018 rodent chow, and the HLS mice were additionally supplemented with bacon softies made by Bio-Serv in an effort to mitigate some of the HLS associated weight loss. Temperature was maintained at a constant 25 degrees Celsius, on a 12 hour light/dark cycle during all portions of the experimental procedure. As standard acclimatization, mice were moved to cages and room used for experiment one week prior to start, then randomly placed into groups on the starting day of the experiment. All animal protocols were approved by the Institutional Animal Care and Use Committee at the Penn State College of Medicine (protocol 2012-033).

Of the two ground control groups, one group consisted of mice normally loaded without any form of suspension or GA intervention. The second ground control group received subcutaneous injections of GA (20 mg/kg of body weight suspended in olive oil at 2mg/ml) every other day (48 hour intervals) at 10:30am [19, 25]. The HLS GA group was also given subcutaneous injections of GA suspended in olive oil at the same dosage; treatment schedule is a reflection of both the reported effective and half-life time for GA, as well as previous treatment protocols reported [6, 17, 19, 25]. One of the remaining HLS groups received olive oil injections with no GA on the same schedule as other injection groups, while the final suspension group underwent HLS with no treatment or pharmacological intervention.

Evaluation of the results was facilitated by in vivo MicroCT scan (Scanco Medical AG, Bruttisellen, Switzerland). Scans were performed on baseline day 0, and at the conclusion of the study, day 21. The right hind limb femur was used for scanning, as is consistent with previous scanning procedure [5]; the hind limb was extended and held in an immobilization device

produced by Scanco. The animals were anesthetized using a 2% mixture of isoflurane during a 30 minute scan. Trabecular microarchitecture was quantified based on a 72 slice of distal femur, immediately proximal to the epiphyseal growth plate. Cortical microarchitecture was quantified based on a 22 slice region at the femoral mid-shaft. MicroCT scan settings were done in 151 slice batches of 55 kVp, 145μA with 200ms integration time and conebeam mode. 2D images were reconstructed as a matrix of 2048x2048x76 isotropic voxels measuring 10.5μm, with 27.5 threshold used to remove surrounding soft tissue. Periosteal and endosteal borders of cortical bone were segmented using Scanco semi-automated edge detection software following manual outlining. The 72 slice trabecular regions were manually segmented out of 151 total slices by an evaluator blinded to treatment groups. The minimum of two MicroCT scans were performed to minimalize any radiation related effects on bone during the experiment.

We reported several bone microarchitecture parameters based on previously published guidelines [26]. Trabecular data parameters included bone volume fraction (BV/TV), trabecular number (Tb.N), trabecular thickness (Tb.Th), trabecular separation (Tb.Sp), and connectivity density (Conn.D). Cortical parameters reported include cortical bone volume (BV/TV), cortical thickness (Ct.th) and an approximated bending moment of inertia (BMOI) based on the cortical parameters at femur mid-shaft.

Biomechanical testing was completed on the same right hind limb femur used for MicroCT scanning. Femurs were isolated following the final MicroCT scan, cleaned of soft tissue, and stored in PBS at -80 degrees Celsius until testing. The femurs were tested using standard three point bending technique using a MTS MiniBionix 858 testing apparatus. Femurs were consistently oriented to receive the strain in the medial to lateral direction. Lower supports were spaced at 8mm, and load was applied through the mid-shaft of the femur at a rate of

1mm/min until bone failure. Using displacement (*d*) and force (*F*) collected by Minibionix device, total energy to failure (area under the curve) was calculated. Ultimate bending stress was also approximated by normalizing the shape of the femur at mid-shaft to a cylinder, and calculating bending moment of inertia using the inside and outside diameter of cortical bone structure. The equation used to approximate ultimate bending stress was $\sigma = yFL/4I$, where y = D/2, F = max force shown during bending, L = length between breaking supports (0.008m), and I = $\pi(D^4 - d^4)/64$, where D = the outer cortical diameter at mid-shaft, and d = inner cortical diameter at mid-shaft [27-29].

Statistical analysis was conducted using GraphPad Prism Version 5.0. All data results are expressed as average ± standard deviation. Statistical significant was assessed by one way ANOVA when comparing multiple groups (percent change from baseline), and Newman Keuls post test to assess differences between groups. Data were considered significantly different when $p<0.05$ for all comparisons. When necessary, power calculations were conducted using GraphPad StatMate; when comparing two groups to predict n values that would yield significance, the average standard deviation between the two groups was used.

All animal protocols were reviewed and approved by Penn State IACUC under protocol number 94-120. Any animals poorly tolerating experimental conditions were removed.

After starting with 5 sample groups, we decided to combine the HLS and HLS Ve groups in order to achieve a larger sample size to compare to the ground controls; the HLS and HLS Ve groups did not show any significant difference between them. Therefore, the final groups were GC (n=4), GC GA (n=4), HLS (n=7), and HLS GA (n=3). One outlier was removed from the HLS group based on being greater than 2 SD's from the average data for several parameters.

Results:

The average weight of the wild type (WT) C57B1/6J mice entering as HLS mice was 29.83g ± 1.29SD, while the average weight of WT mice entering as ground controls was 30.16g ± 2.55SD. Post study, the average weight of the WT HLS mice was 29.17g ± 1.11SD while the average weight of the WT ground control mice was 31.5g ± 2.23SD. Mice in all the HLS groups lost an average of approximately 1% body mass at the end of the three weeks; mice in both GC groups gained an average of approximately 9% body mass. Weights recorded at the 7th day for HLS groups showed average weight of 27.65g ± 2.00SD, a loss of approximately 9% from baseline. This characterizes a suspected period of stress before adaptation or acclimatization, where the mice likely did not feed as much initially following suspension, followed by a regulation and rebound in body mass. Mice weights throughout the study are summarize in Table 1, and depicted in Figure 2.

Group	Weight Day 0	Weight Day 7	Weight Day 21	ABS Change (0/21)	% Change (0/21)
GC	30.1g ± 1.8SD	29.9g ± 1.8SD	31.3g ± 1.5SD	+1.2g	4.0% ± 5.9SD
GC GA	30.2g ± 3.6SD	30.6g ± 3.6SD	31.8g ± 3.1SD	+1.6g	5.9% ± 2.1SD
HLS	30.0g ± 1.1SD	27.6g ± 2.4SD	29.1g ± 1.0SD	-1.0g	-2.9% ± 3.9SD
HLS GA	29.5g ± 1.6SD	27.7g ± 1.0SD	29.3g ± 1.4SD	-0.2g	0.56% ± 1.9SD

Table 1. Average weights for experimental groups on day 0, 7, and 21. Weight is expressed in grams.

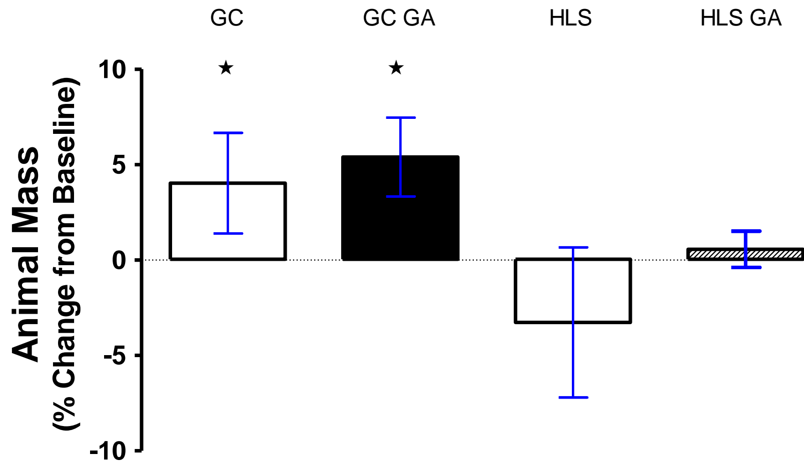

Figure 2. Comparison of percent change in mouse body weight. ★ Represents significance compared to HLS group.

Over the three week protocol, HLS caused significant changes in WT mice bone architecture compared to regularly loaded WT mice. GA intervention did not protect against trabecular bone loss. Typically the most drastically altered parameter during previous HLS experiments, trabecular bone volume/total volume, was not significantly affected by the GA intervention. The HLS GA group showed an average percent change (APC) of trabecular bone volume/total volume of -54% ± 10%SD, while the HLS showed percent change of -60% ± 8%SD; the GC group showed an APC of -38% ± 6%SD, while the GC GA group showed APC of -29% ± 7%SD (Figure 3A). Trabecular number (Tb.N) HLS GA results showed a decrease in trabecular number, with an APC of -24% ± 3.5%SD, while the HLS group showed a percent change of -25% ± 3.8%SD; GC group showed an APC of -12% ± 3.5%SD, and the GC GA showed an APC of -10% ± 3%SD (Figure 3B). Trabecular thickness data displayed HLS GA group experiencing the least amount of change from baseline, with an APC of -20% ± 9%SD (Figure 3C); compared to the HLS APC of -33% ± 7.6%SD, the change was not significant. GC trabecular thickness showed an APC of -16% ± 6.5%SD, and GC GA showed APC of -7% ± 15%SD (Figure 3C). Trabecular separation increases between HLS groups were very similar

with no significant difference between groups, with HLS GA showing APC of 36% ± 7%SD, and HLS APC of 38% ± 8%SD, shown in Figure 3D; GC groups were also very similar, with GC APC of 17% ± 6%SD, and GC GA APC of 15% ± 3%SD (Figure 3D). Connectivity density results for trabecular bone were not significant between any of the HLS or GC groups, due to a large standard deviation. The percentage change for connectivity density HLS GA group was -55% ± 3%SD, HLS was -42% ± 18%SD, GC was -33% ± 7%SD, and GC GA was -33 ± 11%SD (Figure 4).

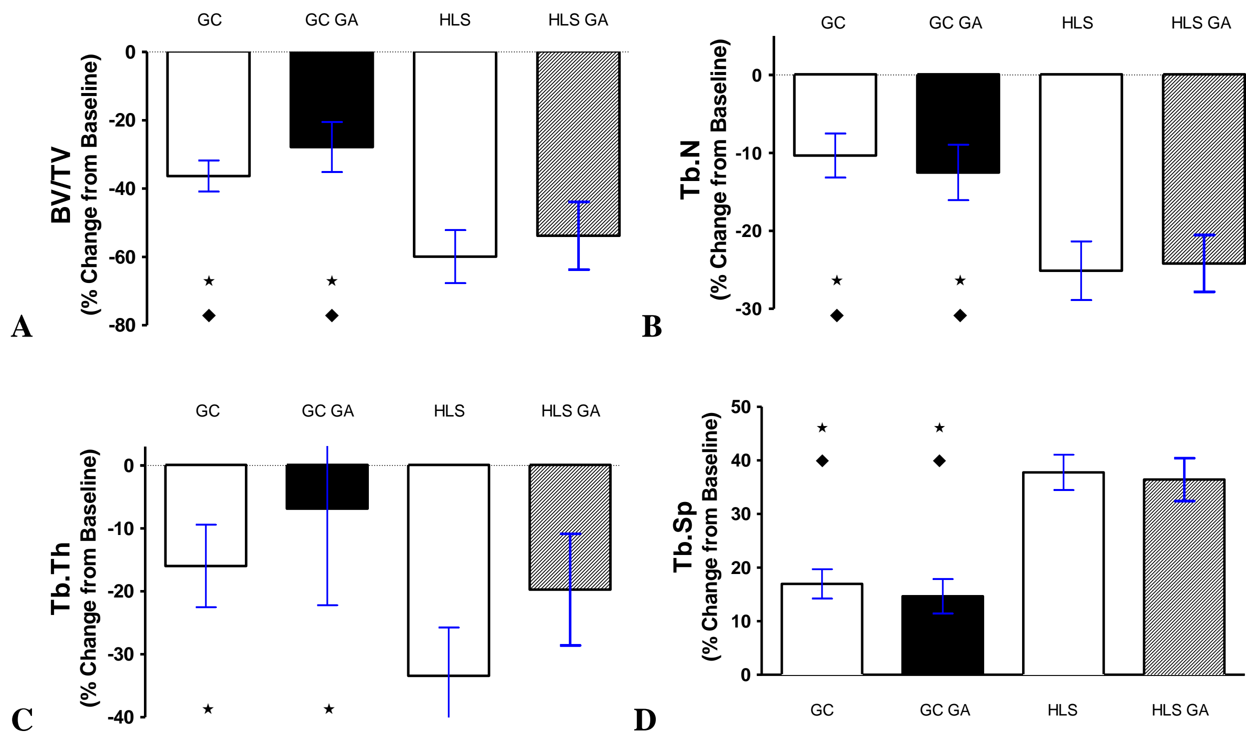

Figure 3. Comparison of trabecular output parameter percent changes from baseline for the 4 experimental groups (n=3-8 per group). ★ Represents significance compared to HLS, ◆ represents significance compared to HLS GA. There was no significance when comparing between HLS groups, and comparing between GC groups for all parameters. All bars represent SD.

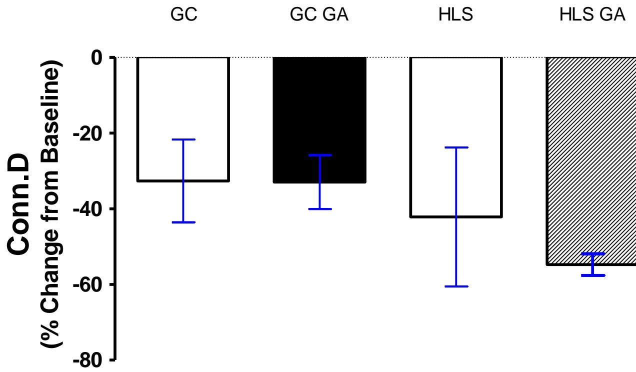

Figure 4. Comparison of the percent change in connectivity density from baseline between the 4 experimental groups (n=3-8 per group). There was no significant difference between groups. All bars represent SD.

Groups Compared	Significance/P < 0.05?				
	BV/TV	Tb.N	Tb.Th	Tb.Sp	Conn.D
HLS vs GC	Yes	Yes	Yes	Yes	No
HLS vs GC GA	Yes	Yes	Yes	Yes	No
HLS vs HLS GA	No	No	No	No	No
HLS GA vs GC	Yes	Yes	No	Yes	No
HLS GA vs GC GA	Yes	Yes	No	Yes	No
GC vs GC GA	No	No	No	No	No

Table 2. A summary of significance between groups for trabecular outputs measured.

Cortical data also supports that HLS groups were significantly affected by unloading when compared to the ground control groups. Cortical bone volume over total volume was characterized by an APC of -13.5% ± 8.5%SD for the HLS GA group, while the HLS showed an APC of -15% ± 4%SD; GC GA group displayed an average loss of -2% ± 2%SD, while GC showed an average loss of -3.5% ± 1.5% (Figure 5A). Cortical thickness was not as drastically changed as trabecular values were, with HLS GA group showing an APC of -20% ± 6.5%SD, HLS group average change of -17% ± 5%SD, HLS group average change of -15.7% ± 4%SD, GC GA group showed APC of -1.8% ± 4%SD, and GC group average of -1.7% ± 3%SD (Figure 5B.) Significance between each group for cortical data reported is summarized in Table 3.

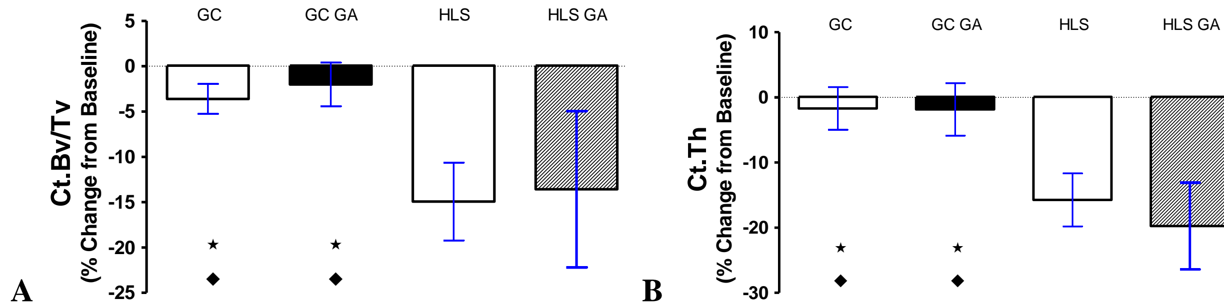

Figure 4. Comparison of cortical output parameter percent changes from baseline between the 4 experimental groups (n=3-8 per group). ★ Represents significance compared to HLS, ◆ represents significance compared to HLS GA. All bars represent SD.

Groups Compared	Significance/P < 0.05? BV/TV	Ct.Th
HLS vs GC	Yes	Yes
HLS vs GC GA	Yes	Yes
HLS vs HLS GA	No	No
HLS GA vs GC	Yes	Yes
HLS GA vs GC GA	Yes	Yes
GC GA vs GC	No	No

Table 3. A summary of significance between groups for cortical outputs measured.

Femurs scanned for MicroCT data were also harvested for biomechanical testing. Bending stress was calculated for each group, HLS GA showed average stress (in mega Pascals) of 62 ± 12SD, HLS group 59 ± 5.3SD; the two ground control groups showed a higher average stress of 71 ± 6.5SD for the GC group, and 74 ± 6SD for the GC GA group. There was significance when comparing results for stress between the HLS group and GC groups; GC groups show a trend of higher stress, summarized in Figure 5A. Ultimate force averaged (in Newtons) for the HLS GA group was 11.3 ± 2.5SD, HLS showed average ultimate force of 11 ± 1SD, while the GC showed ultimate force average of 13.5 ± 1%SD and GC GA average was 13.7 ± 2SD; ultimate force values between all groups was not significant, shown in Figure 5B. Bending energy, or area under the stress/strain curve, also did not show significance between HLS groups, and showed significance ($P<0.05$) when comparing HLS to the GC group. Average bend energy for the HLS GA group was 1509±280SD, HLS was 1576 ± 502SD, while the

ground control groups were 2603 ± 693SD for the GC, and 2121 ± 339SD for the GC GA group. Bending energy is shown in Figure 5C; an example of a curve used to determine energy is shown in Figure 5D and displays a typical stress/strain curve for bone [30].

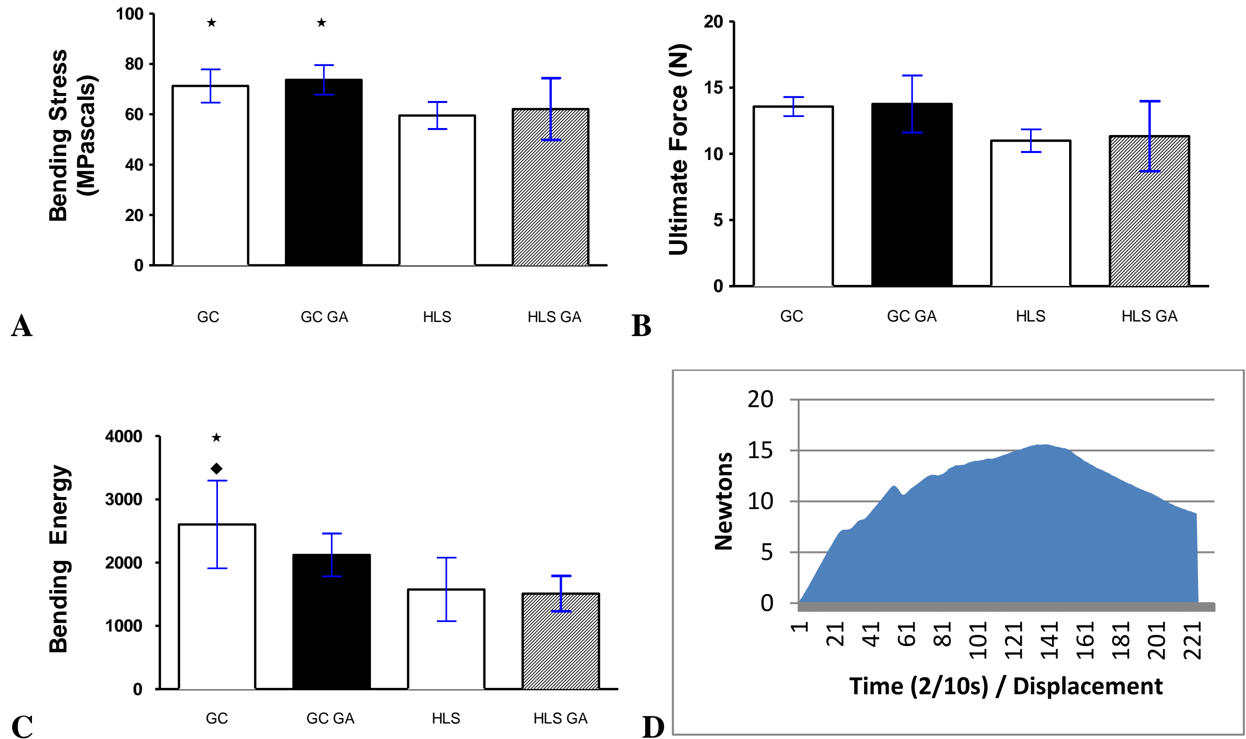

Figure 5. Comparisons of averages for mechanical testing results for 4 experimental groups (n=3-8 per group). (A) Shows the average stress, a resultant of the maximal force normalized to bone size and structure, in mega-Pascals. (B) The average of the maximal force (Newtons) shown during breaking procedures. (C) The average energy absorbed by bones during breaking procedures. (D) A visual representation of the stress/strain curve used to determine energy during a breaking sequence. ★ Represents significance compared to HLS, ◆ represents significance compared to HLS GA. All bars represent SD.

Groups Compared	Significance/P < 0.05?		
	Stress	Ultimate Force	Energy
HLS vs GC	Yes	No	Yes
HLS vs GC GA	Yes	No	No
HLS vs HLS GA	No	No	No
HLS GA vs GC	No	No	Yes
HLS GA vs GC GA	No	No	No
GC GA vs GC	No	No	No

Table 4. A summary of significance between groups for biomechanical testing results.

Chapter 3: Discussion and Conclusions:

Discussion:

Mechanical unloading by HLS method causes profound loss of trabecular bone microarchitecture, while also suppressing cortical bone formation in WT mice during suspension. Our results confirm that mechanical unloading via HLS results in significant changes in several bone microarchitecture parameters. Previous research has supported these effects are attenuated by knocking out bone specific Cx43 and thus rendering the majority of gap junctions in bone non-functional [5, 24]. However, these effects were not attenuated by the administration of GA, a known gap junction blocker in vitro [6]. With consistent baseline MicroCT data from all WT mice, GA administration did not result in any significant changes of any microarchitecture parameters evaluated following three weeks of HLS between either the ground control or suspended groups. Throughout all trabecular and cortical outputs determined, HLS GA group changes from baseline scans were nearly equal to both and HLS. In one instance, trabecular thickness, the HLS GA group showed a trend of protecting against bone loss when compared to the HLS group; although this was not a significant result, a power calculation estimates that a sample size of approximately 25 (difference of 21 mice) yielding similar results to our trabecular thickness data would produce a significant result. Regardless of the average value suggesting or refuting bone protection, there were no P values < 0.05 when the HLS GA group was directly compared to the HLS group.

Despite negative results in relation to our hypothesis that GA would protect against bone loss during unloading, our results validated previous findings in our lab surrounding mechanical unloading [5, 24]. While the HLS GA group did not show significant differences when

compared to the HLS, the HLS groups responded similarly to previous unloaded WT mice results; for example Lloyd et al reported a trabecular BV/TV change of -67% from baseline in WT HLS control mice [5], similar to an average -58% shown across the two HLS (WT) groups suspended here. However, for the BV/TV parameter, our GC mice did show greater loss compared to Lloyd et all, with our GC groups showing an average percent change of -34%, while Lloyd et al data showed less than -10% loss for WT GC group [5].

Quantitatively and qualitatively, the suspension and control mice responded to the mechanical unloading and regular loading as we predicted under normal conditions based on previous research [5, 24]; Figure 6 shows a qualitative representation of the microarchitecture bone changes between groups. Interestingly, ground control mice in both experiments underwent bone loss despite being normally loaded, although our findings indicated a larger percent change than Lloyd et al for several parameters [5]. The loss of bone microarchitecture associated with the ground control groups could be a result of both the isoflurane anesthesia used during scanning, and the radiation effects of the MicroCT scanner [31]. Additionally, the C57C1/BJ WT mice strain utilized are reported to begin naturally loosing bone as early as 5 months, which would contribute to the bone lose shown through the GC groups in this case [32]. Additionally, unpublished observations from our lab suggest short durations of anesthesia can have detrimental effects on mouse body weight.

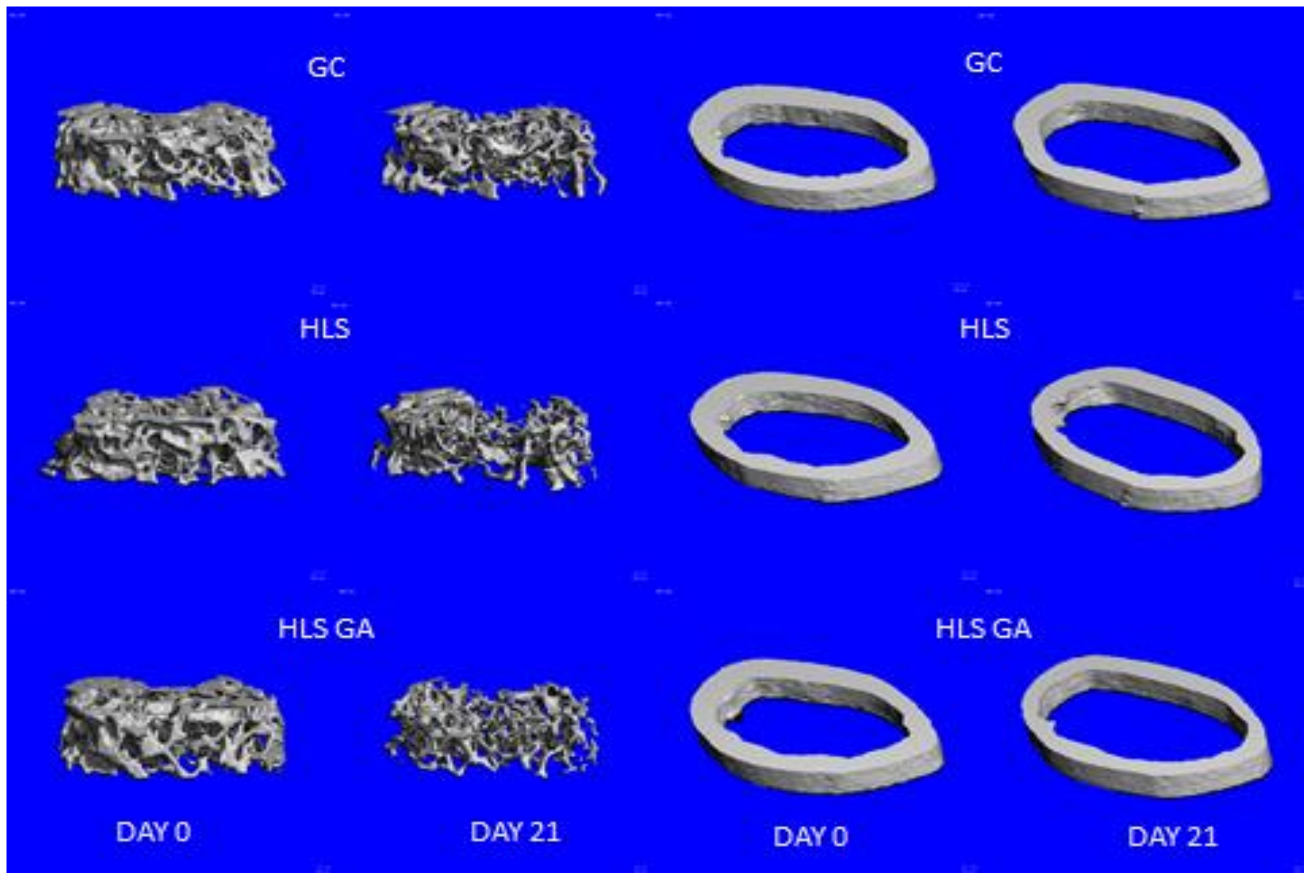

Figure 6. Qualitative representation of the bone changes occurring between ground control, hind-limb suspension, and hind-limb suspension GA groups. Left are trabecular changes between day 0 and end of study day 21, while the right shows less affected cortical changes.

Previous studies utilizing the HLS method dealt with weight loss issues for the mice undergoing suspension, and reported a challenge in maintaining the body weight of the unloaded mice [5, 23, 24]. These observations have outlined that some mice have tolerated the HLS well and maintain body weight within 10-15% of baseline; other mice do not tolerate the HLS well, and may lose in excess of 20% of body weight. While weight loss could be a combined effect of the anesthesia and unloading related environmental stress, in our case the weight loss was largely mitigated. The mice in the untreated HLS group appeared to tolerate the unloading relatively well, losing an average of weight loss of -3%. On the other hand, the GA treatment group gained +1% average weight across all mice; some mice in the HLS GA gained weight over the three weeks of unloading. This may have been due to the calories obtained via the olive oil carrier for

each injection; each injection of olive oil was approximately 0.2ml, which represents roughly 1.7 Calories. While mice require a range of 1-5+ Calories per day, 1.7 represents a large proportion of additional energy, and would explain the mitigated weight loss in the injected group. This also serves as a potential avenue for future research related to mechanical unloading, examining whether some of the bone loss can be attenuated with an increase in nutrients. Other protocols have experimented with high Calorie gels [5], and as in our case higher Calorie food pellets, in an effort to reduce weight loss. However, this is ultimately still left to the mouse physically eating the additional food; feeding may be reduced with the mouse in a new stress environment created by the HLS. A more aggressive approach of injecting a food source with appropriate macro-nutrient distribution may be an area for future research.

Perhaps the largest limitation we encountered when evaluating the effectiveness of GA was a way to measure GA activity in vivo. While GA has been shown to be effective in-vitro at blocking gap junctions, we were not able to confirm that gap junction blockage was occurring in-vivo in any cell, or more specifically at the osteocytes as we intended. Instead, we relied on potential differences in bone characteristics between the HLS groups to evaluate whether or not GA had been effective. While these results proved negative, the inability to properly evaluate the effect of GA in-vivo remains the paramount limitation. A theme for control studies may be to measure blood serum levels of GA following injections as a means of better understanding the circulating levels of GA at different doses; different injection techniques such as intra-peritoneal or tail vein could also be evaluated in comparison to sub-cutaneous for blood serum GA level, to determine if a more direct access to circulating blood would affect the availability of GA.

Another limitation to the evaluation of GA effectiveness was sample size. Five mice per group entered the experiment, however each of the HLS groups was reduced to four when two

mice died unexpectedly during HLS, and another escaped the HLS apparatus. Despite consistent results, only four mice per group limits the ability to identify and remove major outliers. The major comparisons between HLS GA and HLS groups showed data very close to each other, such that even major increases in sample size would not have yielded significant results. For example, a power calculation estimated that for the difference in trabecular BV/TV to be significant, it would require a sample size excess of 5000. Despite this, a realistic increase in sample sizes may have afforded the ability to flag some of the outliers and reduce the standard deviation between groups.

The results of this study show a largely unchanged response to mechanical unloading when treated with GA versus regular HLS. Despite not supporting our hypothesis, the data does support previous HLS findings from our lab. The inability to adequately monitor in-vivo responses to GA injection, along with small sample sizes proved to be main limitations. The olive oil vehicle for GA appears to be a viable means of mitigating the weight loss associated with HLS, and thus raises potential future experiments examining more aggressive techniques for attenuating weight loss, and perhaps bones loss along with it.

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