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Oral Administration of Hyaluronan Reduces Bone Turnover in Ovariectomized Rats

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ABSTRACT: The effect of oral hyaluronan (HA) on bone loss in ovariectomized (OVX) 3-month-old rats was measured using serum markers of bone turnover and bone mineral density. OVX rats were administered 1 mg/kg HA (OVX + HA) or phosphate-buffered saline (PBS) (OVX + PBS) by oral gavage (5 days/week for 54 days). Additional controls included sham ovariectomy with PBS gavage (Sham + PBS) and no treatment. Oral administration of HA resulted in approximately 50% ($p < 0.05$) increases in serum HA. Gel filtration analyses showed this was high molecular weight HA (300−500 kDa). Osteopenia was mild due to the young age of the animals. Thus, ovariectomy resulted in a 30% increase in serum collagen N-terminal telopeptides ($p < 0.001$), a 20% increase in serum nitrate/nitrite levels ($p = 0.05$), and a 5−6% decrease in femur bone mineral density/content ($p < 0.05$). HA gavage blunted the development of osteopenia in this model as determined by preventing the 30% increase in serum collagen N-terminal telopeptide levels ($p < 0.001$) and by reducing bone mineral content loss from 6 to 4%. These results show that oral supplements of HA (gavage solution, 0.12% solution) significantly reduce bone turnover associated with mild osteopenia in rats.

KEYWORDS: *hyaluronan, hyaluronic acid, bone turnover, oral gavage, osteopenia, ovariectomized rats*

INTRODUCTION

Osteopenia and osteoporosis are the most common bone diseases worldwide and are conditions that often result from genetic, age-related, and/or hormone-dependent causes.[1] The disease is increasing particularly in northern countries and is currently estimated to affect one in three women and one in eight men over age 50.[2] In the aged, osteoporotic fractures are a major cause of morbidity and mortality, which limits the effectiveness and outcome of orthopedic procedures and places a heavy strain on health care systems. Osteopenia and osteoporosis can result, to a lesser extent, secondarily from other diseases. For example, osteoporosis is a comorbid factor in diabetes, irritable bowel disorder, celiac disease,[3] some neurodegenerative diseases, arthritis, other inflammatory disorders, and cancers such as multiple myeloma.[1,4] Disease treatment, for example, use of tamoxifen in the management of breast cancer and androgen deprivation therapy for prostate cancer, as well as prescription drugs including thyroxine, glucocorticoids, thiazolinediones, anticonvulsants, and others[1−4] also predispose patients to osteoporosis.[1] Finally, otherwise healthy and young individuals can suffer from these bone diseases as a result of some forms of endurance sport activities,[5] and dietary choices including the consumption of cola beverages and low calcium and other cation intakes.[6] To date, replacement of bone loss resulting from osteoporosis has been difficult to achieve. Although dietary regulation of protein as well as calcium and hormone replacement therapy can retard bone loss,[6] the long-term compliance (e.g., >1 year) required for success of these chronic treatments remains a significant problem,[2,4] and new treatments are required.

A homeostatic rate of bone turnover, resulting from coupled osteoblast and osteoclast activity, is necessary for sustaining bone density.[4] Osteoporosis results when this process becomes uncoupled in favor of catabolism, which generally occurs when an enhanced rate of osteoclast activity exceeds basal osteoblast activity.[4] Coupling of osteoblast and osteoclast activity is regulated by parathyroid hormone, vitamins C/D, and estrogens/androgens. These factors regulate key signaling pathways including the osteoprogenin/RANKL/RANK pathway, vitamin D endocrine pathway, estrogen endocrine pathway, and the Wnt/β-catenin signaling pathway, which control osteoblast/osteoclast coupling. Consistent with experimental evidence for their role in bone health, systems analyses of these pathways have revealed that single nucleotide polymorphisms and copy number variations in at least 15 of the genes in these pathways are associated with susceptibility to osteoporosis.[4] In addition to the above factors, factors in the extracellular matrix such as the polysaccharide hyaluronan (hyaluronic acid, HA) contribute to bone turnover by fine-tuning osteoclast/osteoblast activity. HA primarily achieves this through the RANKL/RANK signaling pathway.[7−9] Thus engineered HA matrices, both alone and in combination with bone differentiation factors, promote human osteoblast bone matrix protein expression in vitro[10] and bone growth in vivo.[8,11−13] These effects are most striking when high molecular weight HA forms (e.g., >100 kDa) are utilized, whereas

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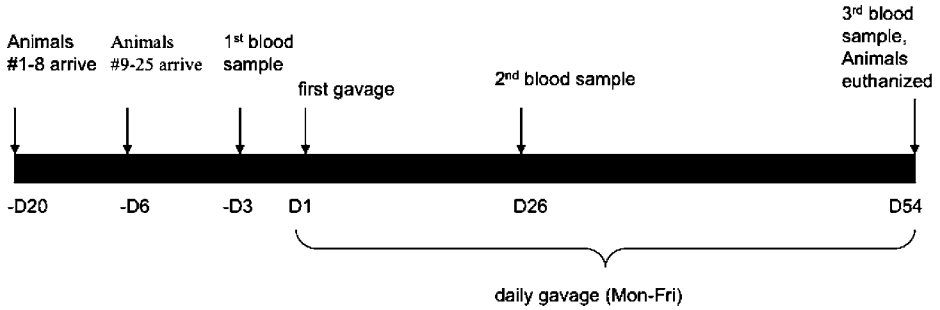

Figure 1. Treatment schedule. Animals 1−8 were shipped and arrived 20 days before the study began (-D20). Sham OVX's were performed on animals 1−5 at Veterinary Services (UWO) 2 weeks before study start date. Animals 6−8 were used as intact controls for weight gain (Figure 2). Animals were OVX at the Charles River Facility 2 weeks before the study start date and arrived at the UWO facility on -D6. Serum samples were taken from all animals at -D3, D26, and D54 of the study. Animals then received PBS or HA by gavage each weekday morning at the beginning of the study (D1).

intermediate HA fragments (e.g., 20−100 kDa) promote osteoclast functions.[9] HA can also exert indirect effects on homeostatic bone cell functions through its ability to control innate immunity. For example, high molecular weight, native HA polymers reduce bone resorption resulting from osteoarthritis by inhibiting prostaglandin E synthesis.[14] High molecular weight HA therefore also has the potential for reducing bone turnover associated with osteopenia.

Several previous papers have documented both systemic and localized bioavailability of orally administered unmodified HA and tagged HA.[14−16] One paper documented reduced osteopenia following oral administration of HA in ovariectomized rats.[16] An additional study showed that oral administration of high molecular weight HA reduced inflammatory chemokines in trafficking T cells, increasing the production of anti-inflammatory cytokines such as IL-10, which is associated with increased bone density and altered bone metabolism.[14,17,18] IL10 production results from an interaction between HA and TLR2,4, and mutations in these receptors are associated with altered bone density. Collectively, these studies provide a rationale for the use of oral HA supplementation to promote bone health. HA has an impressive safety record, can be taken as oral supplements like vitamins, and should therefore improve compliance as a long-term treatment regimen. Surprisingly, only one study has, to our knowledge, assessed the consequences of oral HA on osteopenia.[16] We therefore assessed the effects of oral dosing of a commercially available HA "syrup" with molecular weight polydispersity of 500 kDa−1.6 MDa HA on bone loss in a young rat model of osteopenia resulting from ovariectomy (OVX). We used surrogate serum markers for detecting early changes in bone resorption and also micro-CT/MRI that we have previously shown provides reproducible and sensitive quantification of bone density in rat cadavers.[19,20]

MATERIALS AND METHODS

Chemicals. HA (MHB3 syrup, >500 kDa, concentration = 12 mg/mL, viscosity ≈ 41K mPa s, MW range 500−750 kDa 80% and 750−1600 kDa 20%) was supplied by Cogent Solutions Group (Lexington, KY, USA). HA-ELISA kits (Echelon Biosciences, Salt Lake City, UT, USA) were used to detect serum HA. This kit utilizes a highly specific biotinylated recombinant HA binding protein to detect a wide range of HA molecular weights (6.4 kDa−2 MDa) in an ELISA format.[21] OSTEOMARK NTx Serum assay kits were used to detect serum collagen N-terminal telopeptides (Wampole Laboratories, Princeton NJ, USA) as described by the manufacturer. Serum nitrite/nitrate levels were measured as previously reported.[16]

Animals. Three-month-old Sprague−Dawley female rats (270 ± 15 g) were purchased from Charles River Laboratory. Ovariectomy was performed at the Charles River facility before shipping. Sham ovariectomy was performed at the Western Veterinary Services Facility to confirm the normal appearance of ovaries. Incisions were closed with 5.0 absorbent suture threads. Two rats were housed per cage with a 12 h light/dark cycle at room temperature (22 °C). Rat chow (Rodent Diet Extruded Global, Haarlan Teklad Laboratories' crude protein, 19%; fat (acid hydrolysis), 9.0%; crude fiber, 2.6%) was constitutively available, and cereal treats were given to rats after procedures. On weekday mornings, rats were weighed and gavage performed between 8:00 and 9:00 a.m. for a total of 54 days. PBS or HA gavage was adjusted to the weight of the animals so that each received 1 mg/kg/day. Animal handling was performed in accordance with guidelines established by the Animal Use Committee at the University of Western Ontario. Animal procedures used in this study were approved by the University Animal Use Committee (protocol UWO2006-129-12).

Treatment Schedule. The treatment schedule is outlined in Figure 1. Rats were divided into the following groups: rats 1−5 (Sham + PBS) were sham ovariectomized and administered PBS by gavage; rats 6−8 (intact control) were not operated on and did not receive gavage; rats 9−13 (OVX + PBS) were ovariectomized and administered PBS by gavage; and rats 14−25 (OVX + HA) were ovariectomized and administered 1 mg/kg/day of HA (12 mg/mL, diluted 1:10 in PBS, to reduce viscosity for measurement accuracy) by gavage. All rats were the same age (3 months) at the time the experiment was performed. Rats 1−8 (sham and intact animals) were shipped earlier than rats 9−25 (ovariectomized animals) to perform the sham operation at the Western Animal Facility at the same time as ovariectomy was performed at the Charles River Facility. All rats received cereal treats after each gavage procedure. The gavage dose was 1.0 mg/kg/day and was administered using an animal feeding needle (18Qx2). PBS, used as a gavage control, was administered in the same manner. Blood was harvested from tail veins (0.3 mL) at the three times indicated in Figure 1, allowed to clot, then centrifuged to obtain serum, and stored at −20 °C. Animals were euthanized by CO_2 following the final blood draw. Euthanized animals were subsequently frozen for bone mineral density analyses.[19,20]

Measurement and Assessment of Serum HA Amounts and Molecular Weight. Serum HA levels were measured by ELISA as per the manufacturer's instructions and as described under Chemicals. For molecular weight analysis, HA was precipitated from serum.[21] Serum fractions and Dextran blue molecular weight standards were applied to a Sephadex G-100 column (Sigma Chemicals), 0.5 mL fractions were collected, and the HA/fraction was quantified by ELISA. The void volume of columns was established using Dextran blue (10^6 Da), and the included volume was determined using methylene blue dye (200 Da). Dextran blue polymers of various sizes (50−400 kDa) were used as reference standards. HA present in the fractionated serum was detected using the above ELISA. The molecular weight of the HA used

for the experiments was confirmed by this method to be >500 kDa (results performed in duplicate for each batch of HA used, data not shown).

Serum Collagen N-Terminal Telopeptide Levels. N-Terminal collagen telopeptides were used as surrogate markers for bone remodeling associated with osteopenia.[20] These were measured using an OSTEOMARK NTx Serum kit (Wampole Laboratories), a competitive inhibition enzyme-linked immunosorbent assay for quantifying serum collagen N-terminal peptides. Assays were conducted according to the manufacturer's instructions. Data were analyzed using Vernier Graphical Analysis 3.0.

Bone Mineral Content and Density Analysis. Frozen, euthanized rats were analyzed for bone density following euthanization at day 54 using magnetic resonance imaging (MRI) and micro-computed tomography at the Robarts Imaging facility as described.[19]

Statistical Analysis. The two control groups (intact and sham operated) were first compared for statistical differences using a two-tailed Student's *t* test. No statistical differences between intact controls and sham operated (Sham + PBS) animals were observed, and therefore all analyses other than weight gain (shown in Figure 2) compared Sham + PBS, OVX + PBS, and OVX + HA animals. Differences among these multiple groups were measured using a one-way ANOVA and Tukey's comparison post-test. Statistical differences between two groups were then confirmed using a two-tailed Student's *t* test. p values of <0.05 were considered to be statistically significant.

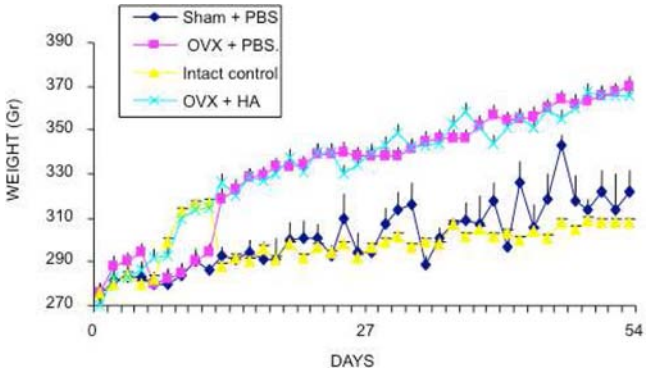

Figure 2. Weight gain. Animals were weighed on weekdays, D1–D54. The weight gain of the two ovariectomized animal groups (OVX + PBS and OVX + HA) is significantly greater than that of the controls (intact control and Sham + PBS) from D13 to D54 ($p < 0.01$). Values represent the mean ± SEM.

RESULTS

Oral HA Does Not Affect Weight Gain of Ovariectomized Rats. No animals died during the study, and no clinical signs of morbidity were observed in any of the groups given HA or PBS gavage. Weight gain was similar in all groups for the first 8 days (Figure 2). However, as expected, OVX animals (+ PBS or + HA, groups 3 and 4) gained weight at significantly greater rates ($p < 0.001$) between days 10 and 56 of the study than non-OVX animals (Sham + PBS and intact controls, groups 1 and 2). No detectable difference in the weight gain of PBS-treated versus HA-treated animals was observed (Figure 2), confirming a previous study.[16]

Oral HA Results in Detectable Increases in Intact Serum HA. In a separate experimental series, five female rats received HA gavage, and then serum levels were sampled from 0 to 48 h (Figure 3). Small but significant ($p < 0.05$) increases in serum HA levels were detected above baseline at all times between 2 and 48 h following the gavage as previously reported.[15,22] To determine if daily HA gavage resulted in a sustained increase in serum HA levels, blood was sampled on days 26 and 54 from OVX + PBS and OVX + HA animals (Figures 1 and 4) just prior to the daily gavage. By day 54, serum HA levels were sustained at a significantly higher level ($p < 0.05$) in the animals receiving HA gavage compared to those receiving PBS only (Figure 4). Serum HA levels were measured using a biotinylated protein probe, which binds to a minimum of 10 disaccharides, thus not permitting us to assess the size variation of unfractionated serum HA. To detect and quantify MW variations, serum HA was concentrated by precipitation and then chromatographed on gel filtration columns. The size profile was similar in each group, which ranged from 100 to 300 kDa and which were not statistically different from one another ($p > 0.05$; Figure 5). These results indicated that there were no detectable/major differences in the molecular weights of serum HA in the experimental and control animal groups and that serum HA was largely in intermediate to high MW forms.

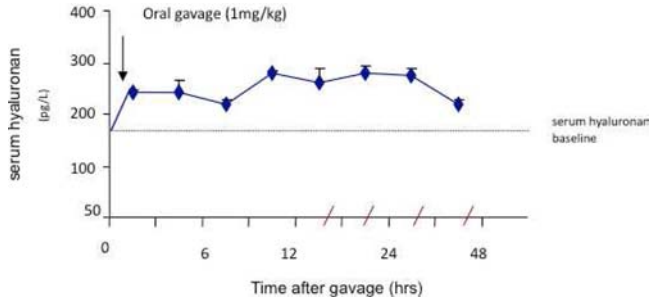

Figure 3. Serum HA levels 2–36 h after gavage. Serum HA levels were quantified using ELISA from a separate experimental series ($n = 5$ rats) to confirm that gavage resulted in acute detectable increases in serum HA. Blood was sampled every 2 h from 0 to 48 h after a single HA gavage. Pregavage samples were used to determine baseline levels. Serum HA levels are slightly but significantly ($p < 0.05$) increased above basal levels between 2 and 36 h following HA gavage. Hatch marks on the *X*-axis mark nonlinear time breaks. Values represent the mean ± SEM.

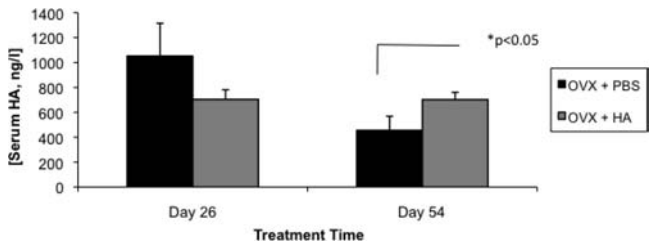

Figure 4. Serum HA levels at days 26 and 54 of the study. Serum HA levels at days 26 and 54 were determined using blood sampled prior to daily HA gavage using ELISA as described under Materials and Methods. These experiments permitted quantification of serum HA that was chronically elevated as a result of the history of previous, daily HA gavages. Results show that by day 54, HA gavage has resulted in a sustained increase in serum HA (asterisk, $p < 0.05$) when compared to OVX + PBS. Analyses were performed as triplicate samples, $n = 4$ animals from each group were sampled. Values are the mean ± SEM.

Oral HA Reduces the Levels of Serum Collagen Telopeptides. Bone density/mineral content measurements are relatively insensitive as methods for detecting acute and small changes in bone density following treatment.[15] Instead, serum biochemical markers associated with bone resorption are now commonly used to detect early and small changes in bone metabolism in response to treatment.[20] Serum collagen I

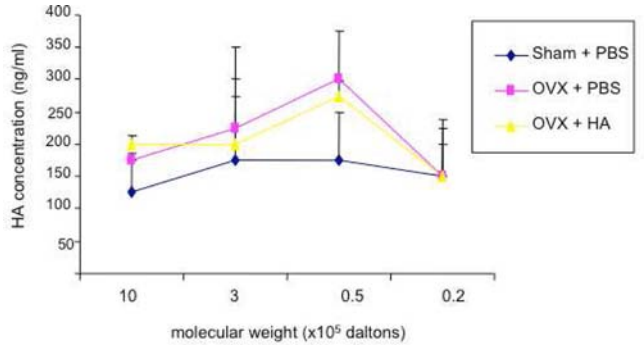

Figure 5. Molecular weight profiles of serum HA following PBS or HA gavage. Serum HA from Sham + PBS, OVX + PBS, and OVX + HA animals was isolated by precipitation and then fractionated on gel filtration columns. HA was quantified from each fraction using ELISA, and molecular weights were determined relative to sized dextran blue polymers. The molecular weight profiles indicate serum HA is high molecular weight and ranges between 20 and 1000 kDa. Values for each group are not significantly different from one another ($p > 0.05$).

telopeptides are used to detect early changes in bone mineral turnover, and nitrate/nitrite levels are markers for oxidative stress associated with osteopenia/osteoporosis.[23] Day 54 serum levels of collagen N-telopeptides were significantly increased in OVX + PBS animals compared to Sham + PBS ($p < 0.001$; Figure 6), indicating osteopenia-related metabolic/turnover

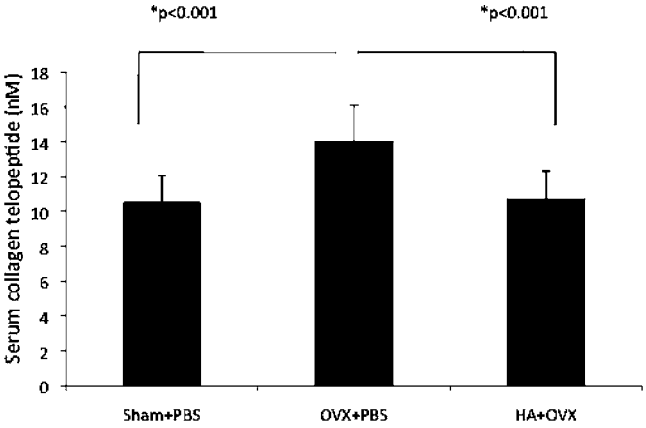

Figure 6. Serum collagen N-telopeptide levels. The levels of serum collagen N-telopeptides in OVX + PBS are significantly elevated at day 54 compared to Sham + PBS and OVX + HA. Values are the mean ± SEM.

changes had occurred within this time frame. In contrast, levels in OVX + HA animals were not significantly different from Sham + PBS levels at either time point, indicating that HA gavage had suppressed this early bone turnover associated with osteopenia.

Although a causal relationship between oxidative stress and osteoporosis is controversial and not specific to osteoporosis,[2,24] surrogate markers of serum antioxidant enzyme deficiency, measured as the serum nitrate and nitrite levels (metabolites of nitrous oxide), have been linked to bone catabolism.[16] Thus, drops in serum nitrate/nitrite levels correlate with the development of osteoporosis in menopausal women and in ovariectomized rats.[16,24] Serum nitrate/nitrate levels were sampled at day 54. Serum nitrate/nitrite levels were indeed significantly lower in OVX + PBS animals than in weight or Sham + PBS controls (Figure 7). However, levels in OVX + HA were similar to control groups, indicating HA did not influence this marker for osteopenia.

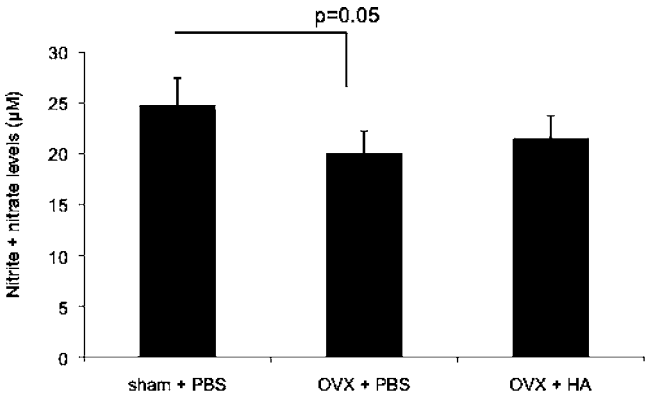

Figure 7. Serum nitrate and nitrite levels. OVX + PBS animals exhibit a decreased level of serum nitrate and nitrite ($p = 0.05$) compared to controls animals. Levels in OVX + HA animals were not significantly different from the control ($p > 0.05$).

Oral Administration of HA Results in Retention of Femur Bone Mineral Content. Bone mineral content (BMC) and density (BMD) are currently the best predictors for fracture resulting from osteoporosis and therefore are used clinically as a surrogate phenotype for frank osteoporosis.[25] However, the sensitivity and specificity of dual-energy X-ray absorptiometry (DXA) for measuring characteristics that are relevant to fracture prediction are relatively low.[25] Here, we used the more sensitive tomography-assisted analysis of MRI/micro-CT images to detect changes in bone mineral density and content (Figure 8).[26] We had previously shown that this

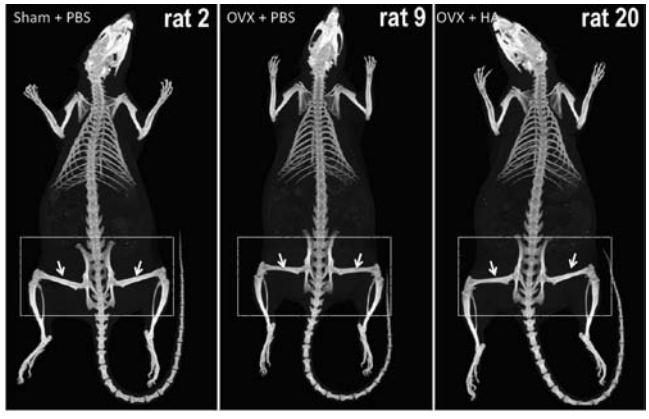

Figure 8. MRI/micro-CT images of representative rats from different groups. Images were obtained with MRI of rats euthanized at day 54. Individual rats from each treatment group are shown. Images of all rats for each group (Figure 1) were taken, and these were analyzed by tomography to obtain bone mineral density and content measurements.

method is a highly precise measure for detecting osteopenia in cadaver rats.[19] Tomography analyses of MRI/micro-CT images showed that at day 54, OVX + PBS and OVX + HA animals exhibited a significant decrease in whole-body BMD relative to the Sham + PBS control (Tables 1 and 2). OVX + PBS animals also exhibited a significant decrease in whole body BMC relative to Sham + PBS controls. HA-treated animals were not

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Table 1. Femur and Whole Body Bone Mineral Density[a]

animal group	no. of animals	femur (cm^3)	SD	whole body (cm^3)	SD
Sham + PBS	3	1.071	0.004	0.545	0.016
OVX + PBS	5	1.062*	0.004	0.513*	0.021
OVX + HA	12	1.063*	0.003	0.511*	0.014

[a]Bone mineral density (mean and SD) values are shown for each rat treatment group. The asterisks mark significance differences from the Sham + PBS control (*, $p < 0.05$). Results show that the femur and whole body bone mineral density of both OVX + PBS and OVX + HA are significantly decreased relative to the control. However, OVX + PBS and OVX + HA were not significantly different from one another.

Table 2. Femur and Whole-Body Mineral Content[a]

animal group	no. of animals	femur (cm^3)	SD	whole body (cm^3)	SD
Sham + PBS	3	0.461	0.051	10.14	0.583
OVX + PBS	5	0.386*	0.031	9.571*	0.367
OVX + HA	12	0.414*	0.031	9.763*	0.538

[a]Bone mineral content (mean and SD) values are shown for each rat treatment group. The asterisks mark significant differences from the mean of the Sham + PBS control (*, $p < 0.05$). Results show that femur and whole body bone mineral content of OVX + PBS is significantly decreased relative to the control. However, the OVX + HA group means are not different from either the control or OVX + PBS.

significantly different from controls in this particular property (Table 2).

Femurs are particularly susceptible to bone loss in the OVX rat model,[27,28] and therefore femur bone mineral density and content were next compared in OVX + HA, OVX + PBS, and Sham + PBS animal groups from the same MRI/micro-CT images (e.g., Figure 8). Femur bone mineral density was significantly decreased in both the OVX + HA and OVX+ PBS animals compared to the control, Sham + PBS animals (Table 1). However, only OVX + PBS animals exhibited a significant drop in femur bone mineral content relative to the controls (Table 2). Collectively, these results suggest that HA gavage may have had a slight effect on preventing bone mineral content loss, but this difference was not statistically significant when compared to OVX + PBS animals.

DISCUSSION

Our results show that gavage with high molecular weight HA reduced early stages of bone resorption resulting from ovariectomy in young female rats as detected by a specific serum marker for collagen catabolism. These results suggest that oral HA significantly reduces very early changes in bone resorption associated with mild osteopenia and indicate a potential use for oral HA as a nutritional supplement to support bone health following estrogen depletion. Our results also provide a foundation for further studies to determine the consequences of oral HA over longer treatment periods and in older animals.

A number of animal models are currently used to investigate the mechanisms of and to identify treatments for osteoporosis.[27,28] None of the currently used animal models replicate a key feature of human osteoporosis, which is susceptibility of osteoporotic bones to fracture. Thus, animal models more accurately mirror the process of osteopenia in humans. However, because early osteopenia is a predictor of susceptibility to osteoporosis, the ovariectomized rat model replicates many of the features of osteopenia following menopause, in particular, accelerated bone loss due to increased resorption as a result of estrogen loss, decreased gut calcium absorption, and response to hormonal therapy.[27] In this model, the bone density of the proximal tibia metaphysis is significantly reduced 14 days after ovariectomy, whereas that of the femoral neck and lumbar vertebrae is significantly reduced at 30 and 60 days, respectively. Our measurement of the femur bone mineral content and density is therefore appropriate to our experimental design of treating 3-month-old animals for 54 days with HA. Measurement of the cortical bone width and marrow cavity of the femur and tibia provide more sensitive quantification of total femur bone or femur neck, but this analysis requires treatment of older rats, usually 90−120 days after ovariectomy. The trend toward an ability of HA to reduce loss of bone mineral content, judged from the lack of difference between sham-operated controls and OVX + HA, may warrant more detailed analysis of the femur (e.g., femur neck vs shaft), longer treatment, and examination of different specific sites (e.g., lumbar vertebra vs tibia).

Although our results generally support the conclusion of a previous study[16] that oral HA may be protective against bone loss due to ovariectomy, we were unable to confirm that HA significantly prevented the decreased femur mineral content and density observed by 30 days after gavage. This discrepancy is not likely to result from a difference in methods for measuring bone mineral content/density but may indicate a critical importance for the amount of high molecular weight HA needed to suppress bone loss. For example, the previous study showed that the 1.6 MDa MW_{av} HA prevented bone loss but not the 750 kDa MW_{av} HA fraction. The HA solution used in our study was polydisperse and ranged between 500 kDa and 1.6 kDa with a MW_{av} of ≈900 kDa. Thus, although we administered the same concentration of HA (1 mg/kg), it is unlikely our HA solution contained as much 1.6 MDa polymer as the previous study. Future studies are required in different animal models of estrogen depletion to provide an estimate of the dose and length of treatment time that may be useful for reducing the risk of osteoporosis and osteopenia in humans. Future studies are also required to determine the long-term effects of HA supplements on bone resorption and to assess if HA affects bone resorption in other models of bone loss, for example, immobilization and dietary restrictions.

The mechanisms by which oral HA exerts its effects on bone metabolism were not addressed in this study, but the evidence that HA gavage results in very minor increases in serum HA predict it is likely indirect. For example, although previous studies have reported the ability of high molecular weight HA to directly suppress osteoclast functions[16] as well as to promote osteoblast differentiation,[10,29] these effects occur in the presence of micrograms per milliliter levels of HA, which are higher than the picograms per liter increases observed in serum after oral administration of HA.[15,30] HA can potentially influence bone resorption by several indirect mechanisms. A major function of HA, which might also contribute to bone density, is the regulation of the pro-inflammatory arm of the immune system during response to injury and disease progression.[31,32] For example, HA gavage has recently been shown to block production of systemic pro-inflammatory cytokines and increase systemic production of anti-inflammatory cytokines (e.g., IL-10) by acting on the gut luminal

epithelial cells and trafficking immune cells.[14] IL-10 and other anti-inflammatory cytokines are implicated in protecting against bone loss induced by ovariectomy.[33] Collectively, these studies raise the possibility that oral HA could indirectly affect osteopenia by binding to HA receptors such as TLR-4 on immune cells within the gut epithelium, resulting in altered systemic inflammatory cytokine production, which attenuates osteoclastogenesis to reduce susceptibility to osteopenia. One alternative mechanisms is that HA, which efficiently binds to cations[34] including calcium,[35,36] may promote gut uptake and increase serum levels of this cation, which has been related to bone density. Analysis of rat models of calcium insufficiency might be useful in assessing this last possibility.

The mechanism by which small amounts of HA are taken up by the gut has, to our knowledge, not been established but HA shares this property with other large polysaccharides including chondroitin and dermatan sulfates.[37] The association of HA with chylomicrons may spare small amounts of this polymer from digestion with hyaluronidases, and this association may also facilitate its uptake from the gut.[38] Alternatively, gut HA may indirectly contribute to increased serum levels by secondarily stimulating endogenous production of this glycosaminoglycan by, for example, trafficking cells. Regardless of mechanism, our results confirm that oral intake of high molecular weight HA increases the amount of large HA in blood, providing a rationale for using oral HA as a nutrient supplement. In summary, the major conclusion from this study is that HA administered 5 days/week by oral gavage to rats significantly reduces their bone turnover associated with estrogen depletion.

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Notes

The authors declare no competing financial interest.

REFERENCES

(1) Choong, K.; Basaria, S. Emerging cardiometabolic complications of androgen deprivation therapy. *Aging Male* **2010**, *13* (1), 1−9.

(2) Li, W. F.; Hou, S. X.; Yu, B.; Li, M. M.; Ferec, C.; Chen, J. M. Genetics of osteoporosis: accelerating pace in gene identification and validation. *Hum. Genet.* **2010**, *127* (3), 249−285.

(3) Mangione, R. A. Celiac disease and osteoporosis. *Am. J. Health Syst. Pharm.* **2008**, *65* (17), 1601; author reply 1601−1602.

(4) Post, T. M.; Cremers, S. C.; Kerbusch, T.; Danhof, M. Bone physiology, disease and treatment: towards disease system analysis in osteoporosis. *Clin. Pharmacokinet.* **2010**, *49* (2), 89−118.

(5) Sabo, D.; Bernd, L.; Pfeil, J.; Reiter, A. Bone quality in the lumbar spine in high-performance athletes. *Eur. Spine J.* **1996**, *5* (4), 258−263.

(6) Tucker, K. L. Osteoporosis prevention and nutrition. *Curr. Osteoporosis Rep.* **2009**, *7* (4), 111−117.

(7) Cao, J. J.; Singleton, P. A.; Majumdar, S.; Boudignon, B.; Burghardt, A.; Kurimoto, P.; Wronski, T. J.; Bourguignon, L. Y.; Halloran, B. P. Hyaluronan increases RANKL expression in bone marrow stromal cells through CD44. *J. Bone Miner. Res.* **2005**, *20* (1), 30−40.

(8) Prince, C. W. Roles of hyaluronan in bone resorption. *BMC Musculoskeletal Disord.* **2004**, *5*, 12−15.

(9) Bastow, E. R.; Byers, S.; Golub, S. B.; Clarkin, C. E.; Pitsillides, A. A.; Fosang, A. J. Hyaluronan synthesis and degradation in cartilage and bone. *Cell. Mol. Life Sci.* **2008**, *65* (3), 395−413.

(10) Pilloni, A.; Bernard, G. W. The effect of hyaluronan on mouse intramembranous osteogenesis in vitro. *Cell Tissue Res.* **1998**, *294* (2), 323−333.

(11) Aslan, M.; Simsek, G.; Dayi, E. The effect of hyaluronic acid-supplemented bone graft in bone healing: experimental study in rabbits. *J. Biomater. Appl.* **2006**, *20* (3), 209−220.

(12) Chazono, M.; Tanaka, T.; Komaki, H.; Fujii, K. Bone formation and bioresorption after implantation of injectable β-tricalcium phosphate granules-hyaluronate complex in rabbit bone defects. *J. Biomed. Mater. Res. A* **2004**, *70* (4), 542−549.

(13) Moffatt, P.; Lee, E. R.; St-Jacques, B.; Matsumoto, K.; Yamaguchi, Y.; Roughley, P. J. Hyaluronan production by means of Has2 gene expression in chondrocytes is essential for long bone development. *Dev. Dyn.* **2011**, *240* (2), 404−412.

(14) Asari, A.; Kanemitsu, T.; Kurihara, H. Oral administration of high molecular weight hyaluronan (900 kDa) controls immune system via Toll-like receptor 4 in the intestinal epithelium. *J. Biol. Chem.* **2010**, *285* (32), 24751−24758.

(15) Balogh, L.; Polyak, A.; Mathe, D.; Kiraly, R.; Thuroczy, J.; Terez, M.; Janoki, G.; Ting, Y.; Bucci, L. R.; Schauss, A. G. Absorption, uptake and tissue affinity of high-molecular-weight hyaluronan after oral administration in rats and dogs. *J. Agric. Food Chem.* **2008**, *56* (22), 10582−10593.

(16) Stancikova, M.; Svik, K.; Istok, R.; Rovensky, J.; Velebny, V. The effects of hyaluronan on bone resorption and bone mineral density in a rat model of estrogen deficiency-induced osteopenia. *Int. J. Tissue React.* **2004**, *26* (1−2), 9−16.

(17) Jiang, D.; Liang, J.; Noble, P. W. Hyaluronan in tissue injury and repair. *Annu. Rev. Cell Dev. Biol.* **2007**, *23*, 435−461.

(18) Kim, Y. S.; Koh, J. M.; Lee, Y. S.; Kim, B. J.; Lee, S. H.; Lee, K. U.; Kim, G. S. Increased circulating heat shock protein 60 induced by menopause, stimulates apoptosis of osteoblast-lineage cells via up-regulation of toll-like receptors. *Bone* **2009**, *45* (1), 68−76.

(19) Granton, P. V.; Norley, C. J.; Umoh, J.; Turley, E. A.; Frier, B. C.; Noble, E. G.; Holdsworth, D. W. Rapid in vivo whole body composition of rats using cone beam muCT. *J. Appl. Physiol.* **2010**, *109* (4), 1162−1169.

(20) Lewiecki, E. M. Benefits and limitations of bone mineral density and bone turnover markers to monitor patients treated for osteoporosis. *Curr. Osteoporosis Rep.* **2010**, *8* (1), 15−22.

(21) Haserodt, S.; Aytekin, M.; Dweik, R. A. A comparison of the sensitivity, specificity, and molecular weight accuracy of three different commercially available hyaluronan ELISA-like assays. *Glycobiology* **2011**, *21* (2), 175−183.

(22) Simarro, M.; Mauger, D.; Rhee, K.; Pujana, M. A.; Kedersha, N. L.; Yamasaki, S.; Cusick, M. E.; Vidal, M.; Garcia-Blanco, M. A.; Anderson, P. Fas-activated serine/threonine phosphoprotein (FAST) is a regulator of alternative splicing. *Proc. Natl. Acad. Sci. U.S.A.* **2007**, *104* (27), 11370−11375.

(23) Cox, G.; Einhorn, T. A.; Tzioupis, C.; Giannoudis, P. V. Bone-turnover markers in fracture healing. *J. Bone Jt. Surg., Br. Vol.* **2010**, *92* (3), 329−334.

(24) Sendur, O. F.; Turan, Y.; Tastaban, E.; Serter, M. Antioxidant status in patients with osteoporosis: a controlled study. *Joint, Bone, Spine* **2009**, *76* (5), 514−518.

(25) Compston, J. Monitoring bone mineral density during antiresorptive treatment for osteoporosis. *BMJ* **2009**, *338*, b1276.

(26) Muller, R. Hierarchical microimaging of bone structure and function. *Nat. Rev. Rheumatol.* **2009**, *5* (7), 373−381.

(27) Kalu, D. N. The ovariectomized rat model of postmenopausal bone loss. *Bone Miner.* **1991**, *15* (3), 175−191.

(28) Lelovas, P. P.; Xanthos, T. T.; Thoma, S. E.; Lyritis, G. P.; Dontas, I. A. The laboratory rat as an animal model for osteoporosis research. *Comp. Med.* **2008**, *58* (5), 424−430.
(29) Huang, L.; Cheng, Y. Y.; Koo, P. L.; Lee, K. M.; Qin, L.; Cheng, J. C.; Kumta, S. M. The effect of hyaluronan on osteoblast proliferation and differentiation in rat calvarial-derived cell cultures. *J. Biomed. Mater. Res. A* **2003**, *66* (4), 880−884.
(30) Banzato, A.; Rondina, M.; Melendez-Alafort, L.; Zangoni, E.; Nadali, A.; Renier, D.; Moschini, G.; Mazzi, U.; Zanovello, P.; Rosato, A. Biodistribution imaging of a paclitaxel-hyaluronan bioconjugate. *Nucl. Med. Biol.* **2009**, *36* (5), 525−533.
(31) Jiang, D.; Liang, J.; Noble, P. W. Hyaluronan as an immune regulator in human diseases. *Physiol. Rev.* **2011**, *91* (1), 221−264.
(32) Shirali, A. C.; Goldstein, D. R. Activation of the innate immune system by the endogenous ligand hyaluronan. *Curr. Opin. Organ Transplant.* **2008**, *13* (1), 20−25.
(33) Rahman, M. M.; Bhattacharya, A.; Banu, J.; Kang, J. X.; Fernandes, G. Endogenous n-3 fatty acids protect ovariectomy induced bone loss by attenuating osteoclastogenesis. *J. Cell. Mol. Med.* **2009**, *13* (8B), 1833−1844.
(34) Villegas-Navarro, A.; Dominguez, R.; Reyes, J. L.; Dieck, T. A. In vitro lead cation-hyaluronic acid interaction. *Exp. Toxicol. Pathol.* **1995**, *47* (5), 409−411.
(35) Blokhuis, T. J. Formulations and delivery vehicles for bone morphogenetic proteins: latest advances and future directions. *Injury* **2009**, *40* (Suppl. 3), S8−11.
(36) Lewis, K. N.; Thomas, M. V.; Puleo, D. A. Mechanical and degradation behavior of polymer-calcium sulfate composites. *J. Mater. Sci. Mater. Med.* **2006**, *17* (6), 531−537.
(37) Dawes, J.; Hodson, B. A.; Pepper, D. S. The absorption, clearance and metabolic fate of dermatan sulphate administered to man − studies using a radioiodinated derivative. *Thromb. Haemost.* **1989**, *62* (3), 945−949.
(38) Reed, R. K.; Townsley, M. I.; Pitts, V. H.; Laurent, T. C.; Taylor, A. E. Increased lymphatic flux of hyaluronan from cat intestine during fat absorption. *Am. J. Physiol.* **1992**, *263* (1 Part 1), G6−11.