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 UA. 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 most 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, 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, thiazolidinediones, anticonvulsants, and others1–4 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.3 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.3 Coupling of osteoblast and osteoclast activity is regulated by parathyroid hormone, vitamins C/D, and estrogen/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 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 vitro10 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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intermediate HA fragments (e.g., 20–100 kDa) promote osteoclast functions. 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. High molecular weight HA therefore 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. One paper documented reduced osteopenia following oral administration of HA in ovariectomized rats. An additional study showed that oral administration of high molecular weight HA reduced inflammatory cytokines 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. IL10 production results from an interaction between HA and TLR2,4, and mutations in these receptors are associated with IL10 production. 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 bone health. HA has an impressive safety record, can be taken as oral supplements like vitamins, and should therefore improve bone health. HA has an impressive safety record, can be taken as oral supplements like vitamins, and should therefore improve bone health.

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. The molecular weight of the HA used was documented to range from 500 kDa to 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.

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, Harlan Teklad Laboratories) was given ad libitum and tap water ad libitum. Animals were euthanized by CO2 asphyxiation after sacrifice at the end of the experiment. Blood was harvested from tail veins (0.3 mL) at the same time as at the end of the experiment. Blood was harvested from tail veins (0.3 mL) at the same time as at the end of the experiment. Blood was harvested from tail veins (0.3 mL) at the same time as at the end of the experiment. Blood was harvested from tail veins (0.3 mL) at the same time as at the end of the experiment.
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. 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. 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. Values of <0.05 were considered to be statistically significant.

**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.

**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. 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.

**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. 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. Serum collagen I...
Telopeptides are used to detect early changes in bone mineral turnover, and nitrate/nitrite levels are markers for oxidative stress associated with osteopenia/osteoporosis. 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 changes had occurred within this time frame. In contrast, levels in OVX + HA animals were not significantly different from control groups, indicating HA did not influence this marker for osteopenia.

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. However, the sensitivity and specificity of dual-energy X-ray absorptiometry (DXA) for measuring characteristics that are relevant to fracture prediction are relatively low. 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). We had previously shown that this method is a highly precise measure for detecting osteopenia in cadaver rats. 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 significantly different from control groups.
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. 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. 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 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 MWav HA prevented bone loss but not the 750 kDa MWav HA fraction. The HA solution used in our study was polydispersed and ranged between 500 kDa and 1.6 kDa with a MWav of $\approx$900 kDa. Thus, although we administered the same concentration of HA (1 mg/kg), it is possible that 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 function as well as to promote osteoblast differentiation, 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. 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. 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. IL-10 and other anti-inflammatory cytokines are implicated in protecting against bone loss induced by ovariectomy. 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 including calcium, 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. 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. 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

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REFERENCES


