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Alpha-1 antitrypsin reduces ovariectomy-induced bone loss in mice

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Proinflammatory cytokines are primary mediators of bone loss in estrogen deficiency. This study determined whether alpha-1 antitrypsin (AAT), a multifunctional protein with proteinase inhibitor and anti-inflammatory activities, mitigates bone loss induced by estrogen deficiency. Mice were either sham-operated or ovariectomized and injected with either AAT or phosphate buffered saline (PBS). Ovariectomy resulted in decreased wet uterus weight, significant bone loss, increased serum leptin concentrations, and higher body weight compared to sham. AAT injection increased tibial trabecular bone volume/total volume and trabecular thickness compared to PBS injection in ovariectomized mice. Ovariectomized mice with AAT treatment had higher uterus weight, lower serum osteocalcin levels, fewer bone marrow tartrate-resistant acid phosphatase–positive osteoclasts, and less expression of calcitonin receptor in bone than that in PBS-injected mice. These data demonstrate that AAT mitigates ovariectomy-induced bone loss in mice possibly through inhibiting osteoclast activity and bone resorption.

Keywords: alpha 1 antitrypsin; ovariectomy; bone loss; osteoporosis

Introduction

Osteoporosis is one of the most common disorders in postmenopausal women due to the decreased production of estrogen. Both *in vivo* and *in vitro* studies show that proinflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1, macrophage colony stimulating factor (M-CSF), and IL-6, are primary mediators of postmenopausal osteoporosis.[1] These proinflammatory cytokines have been shown capable of stimulating osteoclast activity through regulating receptor activator of NF-κB ligand (RANKL)/RANK/osteoprotegerin (OPG) pathway.[2,3] Therefore, identifying strategies that reduce inflammation may help prevent osteoporosis.

Alpha-1 antitrypsin (AAT) is a multifunctional protein with proteinase inhibitory, cytoprotective, and anti-inflammatory activities.[4] It has been shown that AAT inhibits acute inflammatory infiltration, affects neutrophil migration, and regulates neutrophil chemotaxis.[5,6] Importantly, AAT inhibits lipopolysaccharide-stimulated release of TNF-α and IL-1β in human monocytes and enhances the production of the anti-inflammatory cytokine IL-10.[7,8] In this study, we tested the possible protective and therapeutic effects of AAT on bone loss in ovariectomized mice.

Materials and methods

Animals and treatments

Thirty-eight female C57BL/6 mice (seven-week-old; Charles River Laboratories, Wilmington, MA) were either bilaterally ovariectomized (OVX) or exposed to a sham-operated procedure at Charles River Laboratories, and shipped one week after the operation. Access to food (Purina Rat Chow #5012; Ralston-Purina, St. Louis, MO) and deionized water was *ad libitum* throughout the study. The animal protocol for the study was approved by the USDA-ARS Grand Forks Human Nutrition Research Center Animal Care and Use

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Committee. Animals were maintained and processed in accordance with the NIH Guide for the Care and Use of Laboratory Animals. After a one-week acclimation period, the ovariectomized mice were randomly assigned to an AAT-injected group ($n = 13$) or phosphate buffered saline (PBS)–injected group ($n = 12$), whereas sham-operated mice ($n = 13$) were injected with PBS and used as controls. Clinical grade of human AAT (Prolastin C®, Research Triangle Park, NC, Talecris, 2 mg/mouse, every three days for 30 days) or PBS was administered by intraperioneal injection. The initial and final body weights of mice were recorded.

Sample preparation

Mice were euthanized with a ketamine cocktail (1.37:1 mixture of ketamine [Animal Health Co., St. Joseph, MO]: xylazine [Phoenix Scientific, St. Joseph, MO]) at the end of the study. Blood samples were collected and allowed to clot at room temperature for three hours, and serum was obtained following centrifugation for 20 min, 1,500 *g* at 4° C. All serum samples were stored at –80 °C until analyzed.

The uterus of each mouse was removed through a midline incision, and a wet uterine weight was measured. The left tibia of each mouse was removed and cleaned of adherent tissue and stored in –20 °C before being scanned by microcomputerized tomography (μCT). The right femur from each mouse was quickly excised and soft tissue was removed. Then, bones were flash-frozen in liquid nitrogen and stored at –80 °C until being pulverized with a liquid nitrogen-cooled steel mortar and pestle for total RNA purification (see later).

Biochemical measurements

Serum concentrations of leptin and osteocalcin were measured by commercial enzyme-linked immunosorbent assay kits from ALPCO Diagnostics (Windham, NH) and Biomedical Technologies Inc. (Stoughton, MA), respectively, according to the manufacturer's instructions.

Bone marrow cells harvest and osteoclast formation

Murine bone marrow cells were harvested from long bones, washed, and cultured for osteoclast formation, as described previously.[9]

Measurement of mRNA levels in bone

Total RNA was purified from whole bone samples by using trizol (Carlsbad, CA) reagent as detailed previously,[10] and real-time PCR was performed as described.[11]

The sense and antisense primer sequences were as follows: for RANKL 5′-CCT GAG GCC CAG CCA TTT-3′, and 5′-CTT GGC CCA GCC TCG AT-3′; for calcitonin receptor (CTR) 5′-ACA TGA TCC AGT TCA CCA GGC AGA -3′, and 5′-AGG TTC TTG GTG ACC TCC CAA CTT-3′; and for GAPDH 5′-TGC ACC ACC AAC TGC TTA G -3′, and 5′- GGA TGC AGG GAT GAT GTT C -3′.

Bone structure determined by μCT

The left tibia from each mouse was cleaned of adherent tissue and placed in a holder of 10.2 mm in diameter and scanned by a Scanco μCT scanner (μCT-40; Scanco Medical AG, Bassersdorf, Switzerland) using a 12 μm isotropic voxel size with X-ray source power of 55 kV and 145 μA and integration time of 300 milliseconds. A detailed method has been described.[11]

Data analysis

Data are expressed as group means ± SD and were analyzed by one-way analysis of variance (ANOVA) followed by Tukey–Kramer's multiple comparison procedure (JMP, version 9.0.0, SAS Institute, Inc., Cary, NC). In all of the analyses, $P < 0.05$ was considered to be statistically significant.

Results and discussion

AAT treatment increases uterine weight in ovariectomized mice

There were no differences in initial body weights among the three treatment groups. All animals gained weight regardless of treatments. Compared to Sham, ovariectomy resulted in 16–20% higher body weight ($P < 0.01$), a finding that is consistent with other reports that estrogen deficiency is associated with weight gain, primarily fat mass in humans and animals.[12–14] The difference in body weight between OVX-PBS and OVX-AAT treatment mice was not statistically significant (Fig. 1). Ovariectomized mice had lower mean uterine wet weight than sham mice (Fig. 2), confirming an interruption in gonadal estrogen production due to a successful ovariectomy. It is obvious that estrogen deficiency affects uterus growth or leads to regression of uterus. Interestingly, the uterus weights of mice receiving AAT injections were 66% higher than PBS-injected animals ($P < 0.01$; Fig. 2). However, the effect of AAT on the uterus has never been reported. The mechanisms

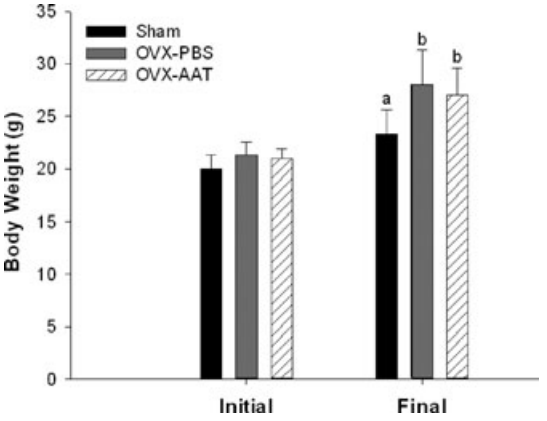

Figure 1. Body weight of sham-operated (Sham) or ovariectomized mice injected with either phosphate buffered saline (OVX-PBS) or alpha-1 antitrypsin (OVX-AAT) for 30 days. Values are means ± SD ($n = 13$, 12, and 13 for the Sham, OVX-PBS, and OVX-AAT groups, respectively). Data without a common superscript letter are different ($P < 0.05$) among treatment groups.

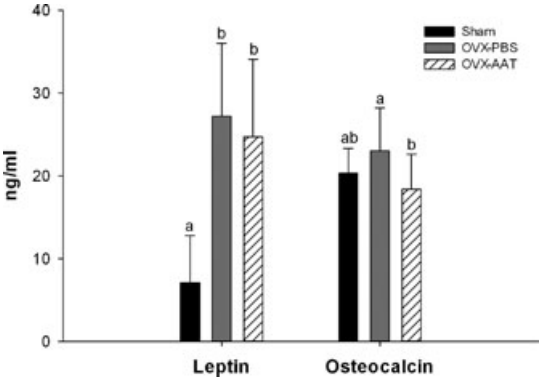

Figure 3. Serum leptin and osteocalcin concentrations in sham-operated (Sham) or ovariectomized mice injected with either phosphate buffered saline (OVX-PBS) or alpha-1 antitrypsin (OVX-AAT) for 30 days. Values are means ± SD ($n = 13$, 12, and 13 for the Sham, OVX-PBS, and OVX-AAT groups, respectively). Data without a common superscript letter are different ($P < 0.05$) among treatment groups.

underlying this observation remain elusive. It is possible that AAT inhibited OVX-induced apoptosis of uterine cells by inhibiting caspases.[15,16]

Concentrations of serum leptin, a small protein secreted primarily by the adipocytes, in OVX mice were higher than in sham-operated mice ($P < 0.01$), an observation in agreement with the increased fat mass in OVX animals (Fig. 3). AAT treatment did not significantly reduce serum leptin levels in OVX animals. The action of leptin on bone remains controversial, in that both stimulatory and inhibitory effects on bone formation have been reported.[17,18] The ultimate outcome of leptin on bone metabolism may be determined by its levels or the mode of action (either peripheral or central). We observed that AAT treatment completely prevented OVX-induced increases of serum osteocalcin levels (Fig. 3).

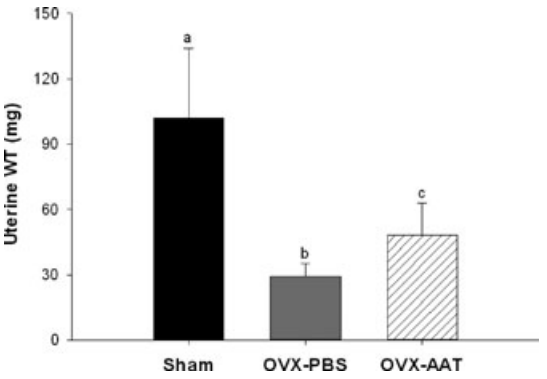

Figure 2. Uterine weights of sham-operated (Sham) or ovariectomized mice injected with either phosphate buffered saline (OVX-PBS) or alpha-1 antitrypsin (OVX-AAT) for 30 days. Values are means ± SD ($n = 13$, 12, and 13 for the Sham, OVX-PBS, and OVX-AAT groups, respectively). Data without a common superscript letter are different ($P < 0.05$) among treatment groups.

AAT treatment mitigates ovariectomy-induced bone loss

As expected, ovariectomy induced significant bone loss. OVX mice had lower tibial trabecular BV (bone volume), BV/TV (total volume), Tb.N (trabecular number), Conn.Dn (connectivity density), and higher Tb.Sp (trabecular separation) and SMI (structure model index) than sham mice ($P < 0.01$; Table 1). These results are consistent with previous observations that estrogen deficiency is detrimental to bone metabolism. Our results also demonstrate that the potential increase in mechanical loading due to increased body weight in OVX mice did not confer enough protection against bone deterioration due to estrogen deficiency. Intriguingly, ovariectomized mice with AAT injection had 32% and 10% higher BV/TV and Tb.Th (trabecular thickness; $P < 0.05$), respectively, than those receiving PBS injection, indicating that AAT treatment significantly improved bone microarchitecture. Although BV/TV in OVX-AAT mice was still lower than in sham, there were no differences in tibial Tb.Th and SMI between sham and OVX-AAT groups ($P > 0.05$), indicating that

Table 1. Trabecular structural indices of proximal tibia in sham-operated (Sham) or ovariectomized mice injected with either phosphate buffered saline (OVX-PBS) or alpha-1 antitrypsin (OVX-AAT) for 30 days

Indices	Sham	OVX-PBS	OVX-AAT	ANOVA (P)
BV (mm^3)	0.15 ± 0.02^a	0.09 ± 0.03^b	0.10 ± 0.02^b	< 0.01
TV (mm^3)	1.84 ± 0.18	1.86 ± 0.32	1.81 ± 0.17	0.81
BV/TV (%)	8.2 ± 1.0^a	4.4 ± 1.3^b	5.8 ± 1.0^c	< 0.01
Tb.N (per mm)	3.16 ± 0.35^a	2.35 ± 0.21^b	2.42 ± 0.25^b	< 0.01
Tb.Th (mm)	$0.051 \pm 0.005^{a,b}$	0.048 ± 0.003^a	0.053 ± 0.004^b	0.03
Tb.Sp (mm)	0.32 ± 0.04^a	0.41 ± 0.05^b	0.42 ± 0.05^b	< 0.01
Conn.Dn (per mm^3)	54 ± 11^a	18 ± 8^b	26 ± 15^b	< 0.01
SMI	2.4 ± 0.2^a	2.8 ± 0.4^b	$2.6 \pm 0.2^{a,b}$	< 0.01

NOTE: Values are means ± SD ($n = 13$, 12, and 13 for the Sham, OVX-PBS, and OVX-AAT groups, respectively). Data were analyzed with one-way analysis of variance (ANOVA) followed by Tukey–Kramer's multiple comparison procedure. Data without a common superscript letter are different ($P < 0.05$) among treatment groups. BV, bone volume; TV, total volume; Tb.N, trabecular number; Tb.Th, trabecular thickness; Tb.Sp, trabecular separation; Conn.Dn, connectivity density; SMI, structure model index.

AAT reversed the detrimental effects of OVX on these parameters.

To understand the mechanism underlying the protective effect of AAT on bone loss, expression of bone resorption markers were evaluated in all animals. Results from these studies showed that AAT treatment completely abolished OVX-induced increase in bone marrow tartrate-resistant acid phosphatase–positive multinucleated osteoclasts ($P < 0.01$; Fig. 4) as well as the expression of RANKL ($P < 0.05$) and CTR ($P < 0.01$; Fig. 5). Importantly, neither osteoclast number nor the expression of RANKL and CTR was different between OVX-AAT and sham groups ($P > 0.05$).

Bone mass reflects the balance of bone resorption and formation, which involves bone resorption by osteoclasts followed by bone formation by osteoblasts.[19] At the cellular level, bone metabolism is regulated through the RANKL/RANK/OPG signaling pathway, and studies have shown bone loss to

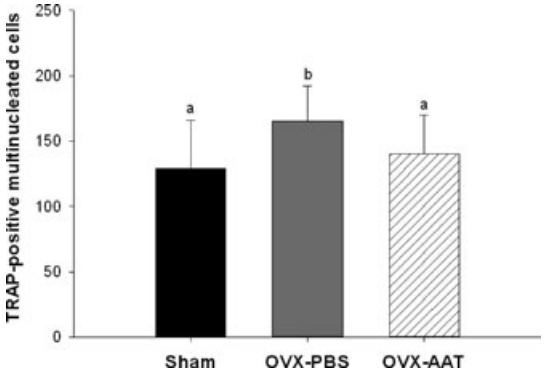

Figure 4. Formation of tartrate-resistant acid phosphatase (TRAP)–positive multinucleated osteoclasts by bone marrow cells from sham-operated (Sham) or ovariectomized mice injected with either phosphate buffered saline (OVX-PBS) or alpha-1 antitrypsin (OVX-AAT) for 30 days. Values are means ± SD ($n = 13$, 12, and 13 for the Sham, OVX-PBS, and OVX-AAT groups, respectively). Data without a common superscript letter are different ($P < 0.05$) among treatment groups.

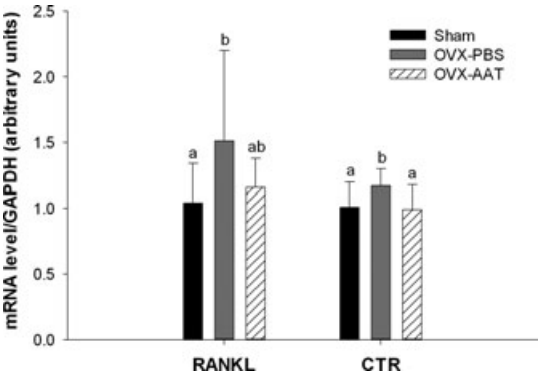

Figure 5. Gene expression levels in whole bone from sham-operated (Sham) or ovariectomized mice injected with either phosphate buffered saline (OVX-PBS) or alpha-1 antitrypsin (OVX-AAT) for 30 days as measured by quantitative real-time PCR. Values are means ± SD ($n = 13$, 12, and 13 for the Sham, OVX-PBS, and OVX-AAT groups, respectively). Data without a common superscript letter are different ($P < 0.05$) among treatment groups.

be correlated with RANKL expression.[10,20,21] The finding that OVX-AAT mice had higher BV/TV and Tb.Th than OVX-PBS animals clearly demonstrates that AAT is beneficial to tibial trabecular bone microstructure. These structural changes are associated with a decrease in bone resorption (decline in osteoclast number and CTR expression). Interestingly, we found that mice in the OVX-AAT group had lower serum osteocalcin concentration than OVX-PBS ($P < 0.05$) but not sham ($P > 0.05$) groups (Fig. 3), indicating AAT treatment decreased bone formation. The change is likely due to the decrease in bone turnover in AAT-treated animals.

This study is the first to demonstrate that AAT mitigates OVX-induced bone loss in mice. Although the mechanisms remain unclear, these findings suggest that AAT improves bone microstructure in OVX mice by serving as an anti-inflammatory agent. It is well accepted that increased bone resorption relative to bone formation is the main cause of bone loss in estrogen deficiency.[22] The change is accompanied by an increase in proinflammatory cytokines, such as TNF-α, IL-1β, M-CSF, GM-CSF, and IL-6.[1] These cytokines have been shown to be capable of stimulating osteoclast activity through regulating the RANKL/RANK/OPG pathway.[3,22] Unfortunately, the serum concentrations of TNF-α and IL-1β were too low, and changes were undetectable with the kits used in the study.

Acknowledgments

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Conflicts of interest

The authors declare no conflicts of interest.

References

1. Mundy, G.R. 2007. Osteoporosis and inflammation. *Nutr. Rev.* **65**(Pt 2): S147–S151.
2. Pfeilschifter, J., R. Koditz, M. Pfohl & H. Schatz. 2002. Changes in proinflammatory cytokine activity after menopause. *Endocr. Rev.* **23:** 90–119.
3. Khosla, S. 2001. Minireview: the OPG/RANKL/RANK system. *Endocrinology* **142:** 5050–5055.
4. Janciauskiene, S.M., R. Bals, R. Koczulla, *et al*. 2011. The discovery of alpha1-antitrypsin and its role in health and disease. *Respir. Med.* **105:** 1129–1139.
5. Dhami, R., B. Gilks, C. Xie, *et al*. 2000. Acute cigarette smoke-induced connective tissue breakdown is mediated by neutrophils and prevented by alpha1-antitrypsin. *Am. J. Respir. Cell. Mol. Biol.* **22:** 244–252.
6. Al-Omari, M., E. Korenbaum, M. Ballmaier, *et al*. 2011. Acute-Phase Protein alpha1-Antitrypsin Inhibits Neutrophil Calpain I and Induces Random Migration. *Mol. Med.* **17:** 865–874.
7. Janciauskiene S, S. Larsson, P. Larsson, *et al*. 2004. Inhibition of lipopolysaccharide-mediated human monocyte activation, in vitro, by alpha1-antitrypsin. *Biochem. Biophys. Res. Commun.* **321:** 592–600.
8. Janciauskiene, S.M., I.M. Nita & T. Stevens. 2007. Alpha1-antitrypsin, old dog, new tricks. Alpha1-antitrypsin exerts in vitro anti-inflammatory activity in human monocytes by elevating cAMP. *J. Biol. Chem.* **282:** 8573–8582.
9. Cao, J.J., L. Sun & H. Gao. 2010. Diet-induced obesity alters bone remodeling leading to decreased femoral trabecular bone mass in mice. *Ann. N Y Acad. Sci.* **1192:** 292–297.
10. Cao, J., L. Venton, T. Sakata & B.P. Halloran. 2003. Expression of RANKL and OPG correlates with age-related bone loss in male C57BL/6 mice. *J. Bone Miner. Res.* **18:** 270–277.
11. Cao, J.J., B.R. Gregoire & H. Gao. 2009. High-fat diet decreases cancellous bone mass but has no effect on cortical bone mass in the tibia in mice. *Bone.* **44:** 1097–1104.
12. Ley, C.J., B. Lees & J.C. Stevenson. 1992. Sex- and menopause-associated changes in body-fat distribution. *Am. J. Clin. Nutr.* **55:** 950–954.
13. Chen, Y. & M.L. Heiman. 2001. Increased weight gain after ovariectomy is not a consequence of leptin resistance. *Am. J. Physiol. Endocrinol. Metab.* **280:** E315–E322.
14. McElroy, J.F. & G.N. Wade. 1987. Short- and long-term effects of ovariectomy on food intake, body weight, carcass composition, and brown adipose tissue in rats. *Physiol. Behav.* **39:** 361–365.
15. Zhang, B., Y. Lu, M. Campbell-Thompson, *et al*. 2007. Alpha1-antitrypsin protects beta-cells from apoptosis. *Diabetes* **56:** 1316–1323.
16. Petrache, I., I. Fijalkowska, T.R. Medler, *et al*. 2006. alpha-1 antitrypsin inhibits caspase-3 activity, preventing lung endothelial cell apoptosis. *Am. J. Pathol.* **169:** 1155–1166.
17. Iwaniec, U.T., M.G. Dube, S. Boghossian, *et al*. 2009. Body mass influences cortical bone mass independent of leptin signaling. *Bone* **44:** 404–412.
18. Elefteriou, F., S. Takeda, K. Ebihara, *et al*. 2004. Serum leptin level is a regulator of bone mass. *Proc. Natl. Acad. Sci. U. S. A.* **101:** 3258–3263.
19. Novack, D.V. & S.L. Teitelbaum. 2008. The osteoclast: friend or foe? *Annu. Rev. Pathol.* **3:** 457–484.
20. Lacey, D.L., E. Timms, H.L. Tan, *et al*. 1998. Osteoprotegerin ligand is a cytokine that regulates osteoclast differentiation and activation. *Cell* **93:** 165–176.
21. Simonet, W.S., D.L. Lacey, C.R. Dunstan, *et al*. 1997. Osteoprotegerin: a novel secreted protein involved in the regulation of bone density. *Cell* **89:** 309–319.
22. Boyle, W.J., W.S. Simonet & D.L. Lacey. 2003. Osteoclast differentiation and activation. *Nature* **423:** 337–342.