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The Effects of the Receptor for Advanced Glycation End Products (RAGE) on Bone Metabolism under Physiological and Diabetic Conditions

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Running title: The Effects of RAGE on Bone Metabolism

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Abstract

It has been reported that AGEs and the receptor for AGEs (RAGE) have been linked to the pathogenesis of diabetic microangiopathy. However, the relationship between RAGE and alteration in bone metabolism is unclear. Therefore, in order to determine the role of RAGE in bone metabolism, we investigated the effects of RAGE deletion on bone metabolism under physiological and diabetic conditions using RAGE knockout mice (RAGE-KO). Eight-week-old male RAGE-KO and wild-type littermates (WT) were intraperitoneally injected with either streptozotocin or vehicle. Mice were classified into four groups: 1) nondiabetic WT; 2) nondiabetic RAGE-KO; 3) diabetic WT; and 4) diabetic RAGE-KO. After 12 weeks of streptozotocin or vehicle treatment, the physical properties of femora and the static and dynamic parameters of bone histomorphometry of tibiae were assessed. The deletion of RAGE affected neither body weights nor hemoglobin A1c levels. RAGE deletion resulted in increased bone mineral density due to decreased osteoclast function under physiological conditions that is no accumulation of AGEs. In contrast, lacking RAGE did not affect the alteration in bone metabolism under diabetic conditions, suggesting that AGEs-RAGE interaction may not be involved in the pathogenesis of diabetic osteopenia, although RAGE plays a crucial role in bone metabolism.

Key Words

advanced glycation end products (AGEs); diabetes mellitus; diabetic osteopenia;
diabetic bone disorder; streptozotocin

Introduction

Diabetes mellitus causes alterations in bone metabolism, such as reductions in bone mass [1], increased fracture rates [2], and delayed fracture healing [3]. However, there are few effective therapies for diabetic osteopenia, and the mechanisms leading to diabetic bone disorder have not been clearly identified, although several theories based on animal models exist [4-7].

In diabetes mellitus, advanced glycation end products (AGEs) form and accumulate progressively. AGEs result from nonenzymatic reactions of carbohydrates and oxidized lipids with proteins [8]. AGEs induce alterations in the structure and function of different proteins, which have been proposed as the pathological mechanisms underlying chronic complications in diabetes, such as nephropathy, retinopathy, neuropathy, and atherosclerosis. Regarding bone metabolism, it has been reported that AGEs enhance osteoclast-induced bone resorption in cultured mouse unfractionated bone cells [9], and that N-carboxymethyl lysine(CML)-modified collagen injected in vivo stimulated apoptosis in calvarial periosteal cells compared to injection with unmodified collagen [10]. In addition, AGE levels are elevated in the serum and urine samples of patients with osteoporosis [11,12]. Considering these findings, these products may also play an important role in the pathogenesis of diabetic osteopenia, although their mechanisms of action remain unclear.

Several receptors that bind AGEs have been identified to date. Among them, the Receptor for AGEs (RAGE) is one of the best characterized AGE receptors. RAGE is a cell-surface protein isolated from bovine lung, and it belongs to immunoglobulin superfamily.

In addition, RAGE has an extracellular region containing one V-type immunoglobulin domain and two C-type immunoglobulin domains [13,14]. A growing body of evidence shows that AGEs-RAGE interactions are involved in the development of atherosclerosis and diabetic microangiopathy. Since RAGE is expressed on various cells, such as endothelial cells, smooth muscle cells, fibroblasts, osteoblasts, and osteoclasts, and at lower levels in healthy animals but at significantly higher levels in diabetes [15-18], we hypothesized that AGEs-RAGE interactions may play an important role in the development of diabetic bone disorder. In the present study, we investigated the effects of RAGE deletion on bone metabolism under physiological and diabetic conditions using RAGE knockout mice (RAGE-KO) [19,20] and a streptozotocin-induced diabetic mice model [4].

MATERIALS AND METHODS

Animals and experimental design

The generation and maintenance of RAGE-KO mice, backcrossed onto the C57BL/6 strain, have been described previously [19,20]. The genotype of each animal was confirmed by polymerase chain reaction analysis on purified tail DNA. Eight-week-old male RAGE-KO and wild-type littermates (WT) were intraperitoneally injected with either streptozotocin (STZ; Sigma Chemical Co., St Louis, MO, USA; 100 mg/kg body weight in 100 μ L of sterile citrate buffer, pH 4.5) or a citrate vehicle on two consecutive days. Mice with venous blood glucose levels >17 mmol/L obtained from the tail, which was measured using Glutest-Ace (Sanwa Kagaku Kenkyusho, Nagoya, Japan), were included in the study as diabetic mice models. The mice were classified into four groups ($n = 8$ in each group): 1) nondiabetic WT; 2) nondiabetic RAGE-KO; 3) diabetic WT; and 4) diabetic RAGE-KO. They had free access to standard chow and tap water throughout the experiment. In previous experiments, no significant difference in bone mineral density (BMD) was evident between nondiabetic and diabetic WT 4 weeks after STZ injection [4]. In the present experiment therefore, the mice were followed for 12 weeks after STZ injection. Body weights and hemoglobin A1c (HbA1c) levels were measured at 0 and 12 weeks after STZ treatment. HbA1c levels were determined with DCA2000 analyzer (Bayer Medical, Tokyo, Japan) using venous blood from the tail. The procedures were approved by the Institutional Animal Care and Use Committee guidelines of Kobe University Graduate School of Medicine.

Serum measurements

The mice were killed under ether anesthesia to obtain blood via cardiac puncture. Blood serum was prepared from each sample by centrifugation for 5 min at 3000 rpm. Serum samples were stored at -80°C until analysis. Serum creatinine (Cr) levels and serum concentrations of Ca and P were measured using Fuji DRI-CHEM 3500 (Fuji Film, Tokyo, Japan) according to the instructions of the manufacturer. Serum concentrations of parathyroid hormone (PTH) were determined using a mouse PTH ELISA kit (Immutopics Inc., San Clemente, CA, USA).

Sample preparation and skeletal morphology

Bone radiographs of excised femora were taken with a Type SRO-M50 soft X-ray apparatus (Sofron, Tokyo, Japan). BMDs of the left femora were measured by single energy X-ray absorptiometry using a bone mineral analyzer (DCS-600R; Aloka Co. Ltd., Tokyo, Japan). All histological analyses were performed on 12 weeks of STZ treated mice. For Villanueva-Goldner staining, the right tibiae were excised, fixed with 70% ethanol, embedded in methyl methacrylate, and sectioned into 6- μm thick slices. For double-labeling, mice were injected subcutaneously with calcein (8 mg/kg body weight) at 3 d and 10 d before killing. The specimens were subjected to histomorphometric analysis using a light microscope equipped with a micrometer and an image analyzer (Osteoplan II; Carl Zeiss, Thornwood, NY, USA). Parameters for the trabecular bone were measured in an area of 1.2 mm in length from 0.6 mm below the growth plate at the proximal metaphysis of the tibiae. All parameters complied with the guidelines of the nomenclature committee of the American Society of Bone and Mineral Research [21].

Statistical analyses

Results were expressed as mean $\pm$ SD. Data were examined by one-way ANOVA, followed by Tukey's HSD test. All statistical analyses were performed using SPSS for Windows ver. 12.0 (SPSS Inc., Chicago, IL, USA). $p < 0.05$ was considered statistically significant.

RESULTS

Animal profiles

There were no differences observed in body weights and HbA1c levels between WT and RAGE-KO at the time of STZ or buffer treatment (data not shown). The body weights of the diabetic mice were significantly ($p < 0.01$) reduced compared with the nondiabetic mice at the end of the experiment. HbA1c levels were significantly ($p < 0.01$) higher in the diabetic than in the nondiabetic mice during the experiment. However, RAGE deletion affected neither body weights nor HbA1c levels in both nondiabetic and diabetic groups (Table 1). Metabolic measurements at the end of the 12-week experimental period demonstrated that renal function was comparable in all four groups. Furthermore, no significant differences in serum levels of Ca and P were found among the groups (Table 1). Though there is no significant difference, serum PTH levels tended to be lower in RAGE-KO than in WT. On the other hand, diabetic induction did not affect serum concentration of PTH in WT or RAGE-KO.

Bone X-ray and bone mineral density

No significant differences in bone morphology shape could be detected between all four groups. However, RAGE deletion induced higher BMD compared with WT under physiological conditions. On the other hand, diabetic mice showed severe osteopenia on X-ray of the femora of both WT and RAGE-KO (Fig. 1A). To assess the distribution of BMD in the femora, the bones were divided longitudinally into three parts, and the BMD of each part was measured (Fig. 1B). Under physiological conditions, the BMD of each part was increased in RAGE-KO compared with WT. Under diabetic condition, the BMD of each part decreased to a

similar extent in diabetic WT as well as diabetic RAGE-KO, suggesting that trabecular bone and cortex bone were equally affected by diabetic conditions and that RAGE deletion did not affect bone metabolism in diabetes (Fig. 1B).

Bone histomorphometry

Villanueva-Goldner staining indicated decreases of trabecular bone, stained green, and osteoid volume, stained red, in diabetic WT and RAGE-KO (Figs. 2C, 2D) compared with nondiabetic mice (Figs. 2A, 2B). There was no significant difference between WT and RAGE-KO in both nondiabetic and diabetic groups. Calcein double-labeling showed that the double-label was clearly recognized in nondiabetic WT and RAGE-KO (Figs. 2E, 2F), while the double-label almost diminished in diabetic WT and RAGE-KO (Figs. 2G, 2H). However, no significant difference was observed between WT and RAGE-KO.

To determine whether the increased BMD of nondiabetic RAGE-KO was due to increased bone formation or decreased bone resorption, histomorphometric analyses of the proximal tibiae were performed. Histomorphometric measurements revealed no significant differences in the bone formation parameters of osteoid volume/bone volume (OV/BV), osteoid surface/bone surface (OS/BS), and osteoblast surface/bone surface (Ob.S/BS) between nondiabetic WT and nondiabetic RAGE-KO (Figs. 3A–C). In addition, there were no significant differences in the mineral apposition rate (MAR), reflecting the ability of individual osteoblasts to form bone, and the bone formation rate (BFR/BS), determined by the number and function of osteoblasts, between nondiabetic WT and nondiabetic RAGE-KO (Figs. 3D, 3F). In contrast, bone resorption parameters [osteoclast number/bone perimeter (N.Oc/B.Pm) and osteoclast

surface/bone surface (Oc.S/BS)] were significantly lower in nondiabetic RAGE-KO compared with nondiabetic WT (Figs. 3H, 3I). These results suggest that increased BMD by RAGE deletion is due to suppressed osteoclast formation.

On the other hand, not only bone formation parameters, but also bone resorption parameters, were significantly decreased in the diabetic group compared with nondiabetic group. However, no significant differences were detected between diabetic WT and RAGE-KO.

DISCUSSION

In the current study, RAGE-KO mice showed significantly higher BMD compared with WT under physiological conditions, although AGEs accumulation was not observed in physiological conditions. Histomorphometric analysis revealed that increased BMD observed in RAGE-KO was due to suppressed osteoclast formation. On the other hand, RAGE deletion did not affect bone metabolism in diabetic mice, but did induce increased formation and accumulation of AGEs. Taken together, contrary to expectations, the effects of RAGE deletion on bone metabolism were confirmed, not under diabetic conditions but physiological conditions.

The skeletal phenotypes of the RAGE-KO mice under physiological conditions have been described by some investigators. Consistent with our findings, Zhou et al. reported that mice lacking RAGE had increased bone mass and bone mineral density and decreased bone resorptive activity [22]. On the other hand, Philip et al. reported that RAGE-KO female mice showed lack of difference in BMD compared with WT, but a significant decrease in osteoblast differentiation markers and an apparent increase in body weight and percentage of fat [23]. Although the reason why these different findings were observed is unclear, it may be due to sex differences. These findings including our results suggest that RAGE plays a crucial role in bone metabolism at least under physiological conditions.

Possible mechanisms by which suppressed osteoclast formation due to RAGE deletion is observed only under physiological condition is because RAGE has multiple ligands. RAGE was initially identified as a receptor for CML-modified proteins, one of the most

popularly studied AGEs [24]. To date, it has also been reported that RAGE interacts not only with AGEs, but also with nonglycated ligands, such as amphotericin/ high-mobility group box 1 protein (HMGB1) [25,26], S100/calgranulin [27], amyloid fibrils [28], and a leukocyte integrin, Mac-1 [29]. Furthermore, binding of ligands to RAGE on different cells such as endothelial cells and smooth muscle cells, has been shown to activate various signaling pathways including Erk1/2 (p44/p42), p38 and JNK MAP kinases, Rho GTPases, phosphoinositol 3-kinase, and the JAK/STAT pathway, as well as NF- κ B signaling [30]. In particular, Zhou et al. have recently reported that RAGE plays an important role in osteoclast function, and that RAGE deletion impairs signaling downstream of $\alpha_v\beta_3$ integrin [22]. Although the role of RAGE under physiological conditions remain unclear, RAGE ligands, except for AGEs, may be involved in osteoclast regulation.

In the current study, under diabetic conditions, RAGE deletion did not affect diabetic osteopenia, although the formation and accumulation of AGEs were augmented in diabetes. RAGE is expressed on different cells, such as endothelial cells, smooth muscle cells, fibroblasts, osteoblasts, and osteoclasts; at lower levels in healthy animals but at significantly higher levels in diabetic animals [15-18]. Furthermore, multiple studies have demonstrated that the binding of AGEs to RAGE on cells critically involved in inflammatory responses results in cellular perturbation [31]. However, though there is a growing body of evidence to show that AGEs-RAGE interactions are involved in the development of various diabetic complications, the results of the present study suggest that AGEs-RAGE interactions may not contribute to the pathogenesis of diabetic osteopenia.

Other possible reason why RAGE deletion did not affect bone metabolism under diabetic conditions is that soluble RAGE as well as cell-surface RAGE are eliminated in RAGE-KO. Recently, numerous truncated forms of RAGE have been reported [32-34]. Two major spliced variants of RAGE mRNA, N-terminal and C-terminal truncated forms, have been well characterized [32]. The N-truncated isoform of RAGE mRNA codes for a 303-amino-acid protein lacking the N-terminal signal sequence and the first V-like extracellular domain. The N-truncated form is incapable of binding with AGEs because the V-domain is essential for the binding of ligands [13]. This form of RAGE has been suggested to be involved in angiogenic regulation independent of the classical RAGE signaling pathway [35].

The C-terminal truncated form of RAGE lacks the exon 10 sequences encoding the transmembrane and intracytoplasmic domains [32]. This spliced-variant RAGE mRNA encodes a protein consisting of 347 amino acids with a 22-amino-acid signal sequence, and is released from cells. This form of RAGE is present in circulation and is called soluble RAGE (sRAGE). Lalla et al. reported that blockade of AGEs suppressed diabetic alveolar bone loss in periodontal disease in mice using sRAGE as a decoy by binding RAGE ligands and preventing their interaction with cell-surface RAGE [36]. Moreover, in vivo studies revealed that administration of sRAGE to diabetic animals resulted in diminished vascular hyperpermeability and suppression of accelerated atherosclerosis [37,38]. In other words, sRAGE might play an important role in bone metabolism in diabetes, a role which enhances the formation and accumulation of AGEs. If there were conditional knockout mice such as mice lacking only cell-surface RAGE and mice lacking only RAGE of osteoblast or osteoclast, the

role of RAGE on bone metabolism could be well evaluated. In addition, the diabetic mice did not grow as well as the non-diabetic mice. Since body mass affects bone metabolism, the reduced body weight might be the reason why AGE-RAGE interaction has little to do with diabetic osteopenia.

In conclusion, our present study demonstrates that RAGE deletion results in increased bone mineral density due to decreased osteoclast function under physiological conditions. On the other hand, lacking RAGE did not affect alteration in bone metabolism in diabetes, suggesting that AGEs-RAGE interactions may not be involved in the pathogenesis of diabetic osteopenia, although RAGE plays a crucial role in bone metabolism.

Acknowledgments

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Table 1. Metabolic measurements

	Nondiabetic Mice		Diabetic Mice	
	N-WT	N-RAGE	D-WT	D-RAGE
Body Weight (g)	28.8 ± 1.3	27.8 ± 1.9	22.1 ± 3.0 ^a	21.4 ± 2.4 ^a
HbA1c (%)	3.8 ± 0.2	3.7 ± 0.4	12.5 ± 1.8 ^a	11.4 ± 1.4 ^a
Serum Cr (mg/dL)	0.38 ± 0.07	0.39 ± 0.06	0.36 ± 0.07	0.37 ± 0.11
PTH (pg/mL)	38.6 ± 12.5	28.1 ± 10.0	46.3 ± 25.2	31.3 ± 15.7
Serum Ca (mg/dL)	9.3 ± 0.9	9.8 ± 1.2	8.7 ± 2.0	8.9 ± 1.9
Serum P (mg/dL)	9.6 ± 0.8	9.9 ± 1.4	10.0 ± 1.5	10.1 ± 2.4

Data were obtained 12 weeks after STZ or citrate vehicle injection.

N-WT = nondiabetic wild-type mice; N-RAGE = nondiabetic RAGE-KO mice; D-WT = diabetic wild-type mice; D-RAGE = diabetic RAGE-KO mice.

Data are presented as mean ± SD. ^a*p* < 0.01 vs. nondiabetic wild-type mice.

Figure Legends

Fig. 1. Bone X-ray and BMD

No significant differences in bone shape could be detected between all four groups (A). Three regions (distal, middle, and proximal) of femora were analyzed, and BMD of each fraction was measured (B). Data are presented as mean $\pm$ SD. N-WT = nondiabetic WT mice; N-RAGE = nondiabetic RAGE-KO mice; D-WT = diabetic WT mice; D-RAGE = diabetic RAGE-KO mice. ^a $p < 0.05$ vs. nondiabetic wild-type mice.

Fig. 2. Villanueva-Goldner stain and calcein double-labeling

Compared with non-diabetic WT (A) and RAGE-KO (B) mice, decreases of trabecular bones stained green and osteoid volume stained red in D-WT (C) mice as well as D-RAGE-KO (D) mice were confirmed. In addition, compared with N-WT (E) and N-RAGE-KO (F) mice, decreases of calcein double-labeling in both D-WT (G) and D-RAGE-KO (H) mice were confirmed. There were no significant differences between WT and RAGE-KO under diabetic conditions as well as physiological conditions (magnification, 200 $\times$).

Fig. 3. Histomorphometric analysis

Data are presented as mean $\pm$ SD. N-WT, N-RAGE, D-WT, D-RAGE. ^a $p < 0.05$ vs.

non-diabetic wild-type mice. OV/BV = osteoid volume/bone volume; OS/BS = osteoid

surface/bone surface; Ob.S/BS = osteoblast surface/bone surface; MAR = mineral apposition

rate ; dLS/BS = double-labeled surface/bone surface; BFR/BS = bone formation rate/ bone
surface; ES/BS = eroded surface/bone surface; $N.Oc/B.Pm$ = osteoclast number/bone
perimeter; $Oc.S/BS$ = osteoclast surface/bone surface.

Figure 1

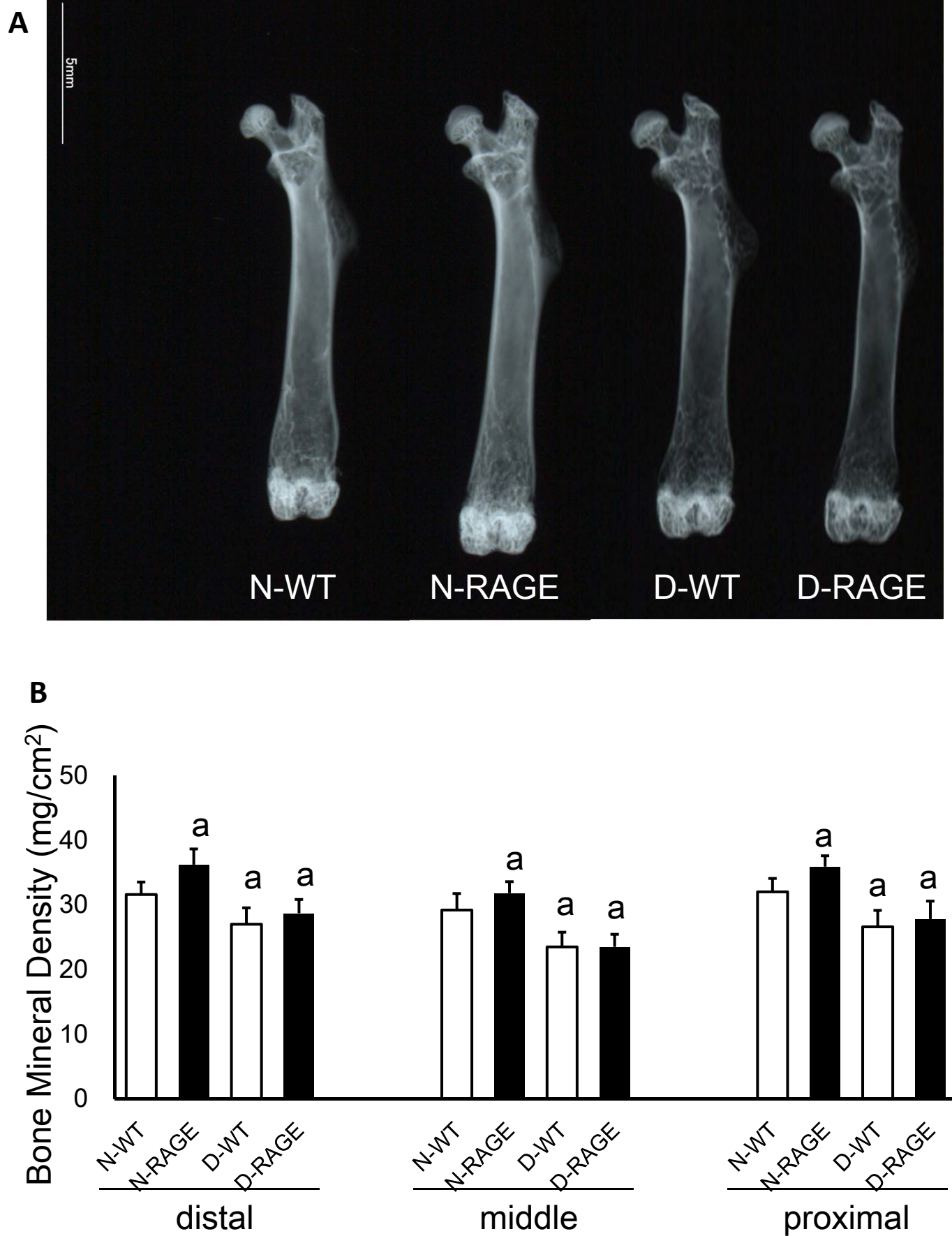


Figure 2

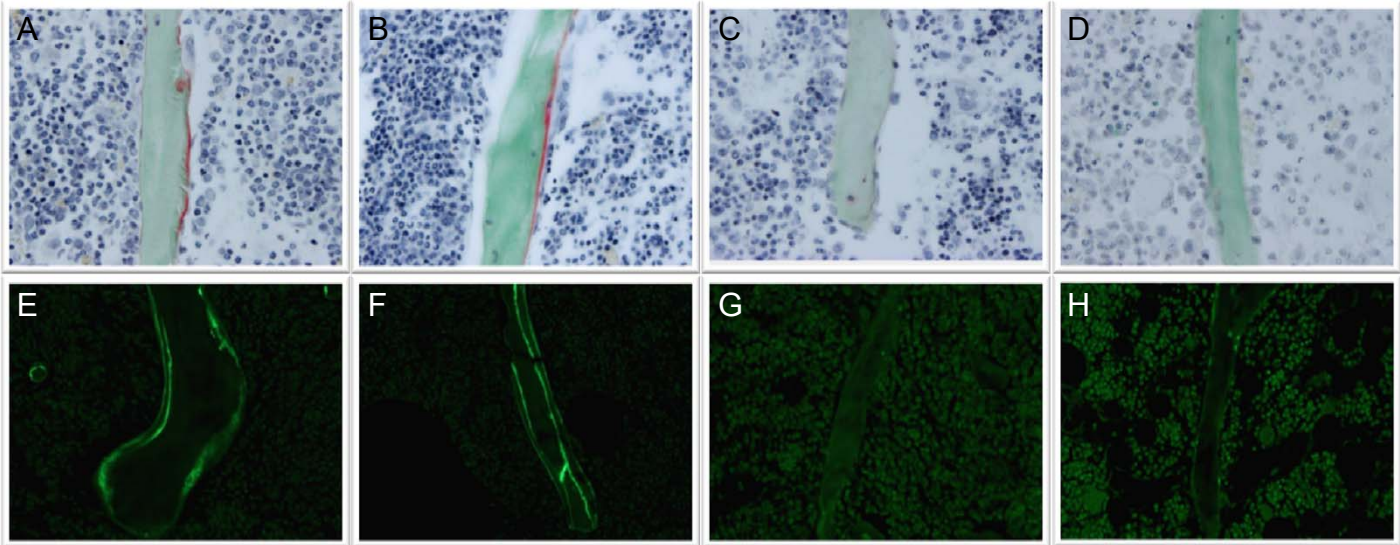


Figure 3

