Bisphosphonates such as alendronate (ALD), although controversial, are worthy of investigation for the enhancement of implant osseointegration in patients with low bone mass who are already taking bisphosphonates for osteoporosis. These patients may receive additional benefits and be acceptable candidates for dental implants without needing to change their medication regimen and possibly as a result of their medication regimen. The purpose of this study was to compare implant osseointegration in maxillary bone of normal rats with a rat model of postmenopausal estrogen deficiency (ovariectomized [OVX]), with and without ALD. An experimental group of 32 rats was divided in 4 groups: ALD-OVX (n = 8 OVX with ALD), OVX (n = 8 OVX without ALD), ALD (n = 8 normal rats with ALD), and control (n = 8 normal rats). All rats received one titanium microscrew implant in the left edentulous region of the maxillary arch. The ALD-OVX and ALD groups received subcutaneous injections of ALD 3 times a week. On the fourth week after ALD administration, an implant was placed in all 32 rats. The maxilla of each rat was radiographed 4 times: at 0, 7, 14, and 28 days. On day 28 after implant placement, all rats were killed, and the peri-implant tissue was embedded in plastic or paraffin for histological examination. The X rays were used for a chronologic calculation of the contact ratio between implant and bone surfaces. Radiographic bone density was determined at 3 points: mesial, apical, and distal. The results show that osseointegration of the implants was impaired in the estrogen-deficient OVX rats compared with the ALD-OVX rats. Fifty percent of the implants were lost at 2 weeks in the OVX group. Radiographic evidence suggested that none of the implants in the OVX group osseointegrated. In the histologic examination more bone was observed around implants from the ALD-OVX and ALD groups than around implants from the OVX group. The OVX group presented a dramatic reduction in implant bone contact at 2 weeks and a significant 13% reduction at 4 weeks vs day of implant (P = .006). The ALD-OVX group presented 50% more bone density than the OVX group (P = .0003). Both ALD groups (ALD and ALD-OVX) had significantly higher radiographic bone density than the other groups (P < .01 for each comparison). In conclusion, osseointegration of implants was enhanced by ALD. Radiographic bone density and contact ratio improved with ALD administration. Implant osseointegration was impaired by estrogen deficiency in the OVX group.

Key Words: implant, osteoporosis, bisphosphonates, alendronate, osseointegration, rat, ovariectomy
Osteoporosis is a systemic skeletal disease characterized by low bone mass and microarchitectural deterioration of bone tissue, with a consequent increase in bone fragility and susceptibility to fracture. Typical characteristics of osteoporosis are reduced bone mineral density and decreased trabecular bone volume. Osteoporosis is also characterized by an imbalance between the formation and resorption of bone, where osteoclastic activity remains relatively normal but osteoblastic activity is diminished.

Bisphosphonates, potent bone antiresorptive agents, are known to be some of the most reliable and prevalent drugs in osteoporosis treatment. Alendronate (ALD; sodium 4-amino-1-hydroxybutylidene-1, 1-bisphosphonate trihydrate), an aminobisphosphonate, is well known as a potent inhibitor of osteoclastic bone resorption. The efficacy of bisphosphonates has been demonstrated in osteoporosis as well as in various other metabolic bone disorders such as Paget’s disease and hypercalcemia of malignancy. Bisphosphonates are synthetic compounds extensively used for the treatment of systemic bone loss due to estrogen depletion. It has been shown that ALD is a potent inhibitor of bone resorption without significantly affecting bone formation. Alendronate provides robust clinical efficacy in the management of osteopenia and osteoporosis by normalizing increased and negatively balanced bone turnover. The mode of action of ALD includes suppression of the differentiation of osteoclast precursors and the maturation of mature osteoclast function, resulting in decreased bone turnover. It also causes apoptosis of osteoclasts.

Long-term clinical success of endosseous dental implants is critically related to direct contact of bone and implant. This condition is called osseointegration and ensures primary mechanical stability to the implant after implantation. Osseointegration is defined as a “direct structural and functional connection between ordered living bone and the surface of a load-carrying implant without intervening fibrous tissue.” Osseointegration is achieved by the ingrowth of bone into the surface of the implant. Alendronate may have the potential to improve osseointegration of implants. This drug may potently inhibit osteoclast-mediated bone resorption while minimally inhibiting osteoblast activity. Bisphosphonates may enhance the ability of periprosthetic bone to integrate with the implant, leading to more rapid and improved osseointegration. Bisphosphonates may be able to reverse the negative effect of postmenopausal osteopenia on the osseointegration of implants. Alendronate also increases the bone mass and strength in ovariectomized (OVX) rats. Treatment with ALD prevents a decrease in bone mineral and helps maintain the mechanical properties of bone after ovariectomy without impairing bone mineralization. As preclinical and clinical evidence suggests, osteoporosis may impair osseointegration. Patients with low bone mass who are already taking bisphosphonates for osteoporosis or metabolic bone disorders may receive additional benefits without needing to change their medication regimen. These benefits may effectively prevent osteoporotic-related fractures and enhance osseointegration of the implant.

The rat model has been used previously by various researchers. Duarte et al studied implant osseointegration, estrogen supplementation and ALD on rat tibia. This group concluded that estrogen deficiency has a negative impact on implant osseointegration. The protocol used in our experiment was similar to the one used by Duarte et al. In this study the ALD dosage established was 5 mg/kg subcutaneous, 3 times a week. Implants were placed 21 days after ovariectomy and ALD administration. In other studies where implants were placed in rats, new bone formation appeared as soon as 5 days after implantation and at 28 days the implants in rats osseointegrated. Other researchers studied the induction of an osteoporosis-like condition on rats after ovariectomy. The histology and microradiograph evaluation of changes showed many of the characteristics associated with osteoporotic bone in humans. These included porosity in the cortical bone, disruption and thinning of the trabecular bone, accumulation of cement lines, and presence of undermineralized matrix. Ovariectomy treatment led to a significant reduction in bone at the periphery of the implant/tissue interface.

Previous studies have examined implant osseointegration in estrogen deficiency on rat tibia, but few studies have examined the effect of estrogen deficiency and ALD administration on the maxilla and mandible. The purpose of this study was to compare implant osseointegration in the maxillary bone of normal rats with osseointegration in a rat model of postmenopausal estrogen deficiency (OVX) with and without ALD treatment.

Materials and Methods

All reagents in this study were obtained from Sigma (St Louis, Mo), except where noted, and were of the highest quality available.
Experimental protocol

Thirty-two Sprague-Dawley retired breeder female rats (Harlan, Indianapolis, Ind) were used in this study. Each rat was between 6 and 9 months old and had an average body weight of 300 to 350 g. The rats were provided with food and water ad libitum, and no special diet was provided. Each rat was weighed and examined once a week to ensure that they were eating and growing normally. No significant differences between groups were detected. An animal protocol was reviewed and approved by the Committee for Care and Use of Laboratory Animals at the Medical College of Georgia. One control group and 3 experimental groups were divided as follows: ALD-OVX (n = 8 OVX with ALD), OVX (n = 8 OVX without ALD), ALD (n = 8 normal rats with ALD), and control (n = 8 normal rats). Two experimental groups (OVX and ALD-OVX) were purchased from Harlan with bilateral ovariectomy from dorsal approach. Two experimental groups (ALD-OVX and ALD) received a bisphosphonate, 4-amino-1-hydroxy-1-phosphonobutyl phosphonic acid (alendronate sodium trihydrate). They were treated with subcutaneous injections of 5 mg/kg ALD 3 times a week. The bisphosphonate was diluted with physiologic phosphate-buffered saline. Administration of ALD began 4 days after the ovariectomy surgery because of time required for transportation. Four weeks later, the implant was placed in the maxillary arch mesial to the left first molar. The ALD groups continued to receive the drug for the duration of the experiment.

A titanium microscrew implant (3 mm in length, 1 mm in diameter) (Stryker Leibinger, Kalamazoo, Mich) was placed in the maxillary arch mesial to the left first molar for each animal. This region is commonly referred to as an edentulous space without opposing teeth.

Implant surgery

The rats received an anesthetic cocktail intramuscularly composed of ketamine (100 mg/mL), xylazine (20 mg/mL), and acepromazine (10 mg/mL) at a dosage of 0.5 to 0.7 mL/kg. Initial crestal incision was done, and a full mucoperiosteal flap was elevated on the left side, mesial to the first molar, on top of the ridge to expose the bone. Using a low-speed handpiece (Lone Star Dental, Arlington, Tex) and a pilot drill, an osteotomy site was drilled directly on top of the ridge parallel to the first molar. A mark was made on the drill to ensure that each site was prepared to the same depth. After confirming that the floor of the nose was not penetrated, the implant was placed and positioned using a 1.5-mm screwdriver (Small Parts, Miami Lakes, Fla) to reach the most apical position of the osteotomy site (Figure 1). After assessing the implant, the flap was closed with sutures when needed. Sutures were removed 1 week after surgery.

Bone contact measurements

Soft no. 2 radiographs (Kodak, Rochester, NY) were taken of the maxilla at 4 different time points with the Faxitron Series 43807N X ray system (Hewlett-Packard, Palo Alto, Calif) on anesthetized rats. Soft X rays were taken for 10 seconds at 2.5 mA and 50 Kvp. The radiographs were taken the day of implant surgery, 1 week after surgery, 2 weeks after surgery, and 4 weeks after surgery (Figure 2).

Scion Imaging software (Frederick, Md) was used to estimate bone contact ratio on scanned X rays at days 0, 7, 14, and 28. The ratio was determined by measuring the implant length surrounded by bone divided by total implant length. Investigators were blinded for all measurements. Each measurement was repeated 3 times and then averaged. The contact ratio was compared for the 4 groups chronologically at 4 time points. In the area studied, the existing bone does not cover the implant completely, and the implant head was always uncovered. Comparisons were then made to determine if bone contact was maintained or lost during the next 4 weeks.
Histologic Preparation

Paraffin embedding

Under deep anesthesia, rats were perfused transcardially with 60 mL of 10% formalin solution until death. The maxilla was dissected by using a low-speed saw (Isomet Buehler Ltd, Lake Bluff, Ill) under constant water irrigation. The soft tissue was removed from the bone and each sample resulted in a hemi-maxillae. The samples were decalcified for 6 weeks in 0.1 M ethylenediamine tetraacetic acid and 0.1 M sodium hydroxide. The sections were processed through series of alcohol and xylene before paraffin embedding.

Serial sections of 5 μm thick were cut sagitally with a rotary microtome (Microm, Walldorf, Germany). Sections were placed on slides and oven-heated overnight at 50°C. Tissue sections were stained by routine hematoxylin-eosin staining and coverslipped with Permount (Biomeda Corp, Foster City, Calif).

Plastic embedding

Under deep anesthesia, rats were killed, soft tissue was removed, and the hemi-maxillae samples containing the implant were dissected. Samples were immersed in a series of solutions consisting of ethanol, xylene, and methyl methacrylate. After the samples were polymerized, they were cut using a Well 4240 Diamond Wire Saw (Norcross, Ga) in a mesio-distal direction. The sample sections were 300 μm thick, and constant water irrigation was used to prevent overheating.

Radiographic bone density

Samples were selected for each rat by choosing the methacrylate samples that represented the most complete implant sections. Samples were randomized to blind the investigator as to the experimental group. All samples were placed on a single cephalometric film and X-rayed to ensure that the exposure and scanning conditions were identical. The X ray was scanned and radiographic bone density was measured using UTHSCA Image Tool software (San Antonio, Tex). Radiographic bone density was determined by quantifying pixels at 3 points: mesial, apical, and distal to each implant. The 3 regions of interest were selected by using a standardized box (1 mm by 1 mm) placed adjacent to but not in contact with the implant (Figure 3). Average radiographic bone densities of the 3 regions were considered together to characterize osseointegration. These values were compared for the 4 groups.

Statistical analyses

A repeated measures 1-factor analysis of variance (ANOVA) model was used for both bone contact ratio and bone density. For bone contact ratio, pairwise comparisons with 2-sample t tests were made for each timepoint-treatment group combination. For bone density, pairwise comparisons with 2-sample t tests were made for each treatment group. The Bonferroni adjustment was made for each set of pairwise comparisons to preserve the 5% family-wise error rate. All statistical analyses were performed using Statistical Analysis System (SAS) 9.1.3 software (SAS Institute, Cary, NC), and statistical significance was assessed using an alpha level of .05.
RESULTS

Histology

Paraffin samples with hematoxylin-eosin staining were observed by light microscopy (Figure 4). More bone was observed around ALD-OVX and ALD samples than in control and OVX samples. The OVX samples presented almost no bone in contact with the implants and no osseointegration. Inflammatory infiltrate was observed at the implant apex in some samples from all 4 groups. Osteoclasts were observed associated with bone in the control and OVX samples only (data not shown).

Observations

In the OVX group, 3 rats lost the implant at 2 weeks; 1 OVX rat did not lose the implant, but it was submerged into the floor of the nose. It was evident that there was no osseointegration in these rats. One rat died in the ALD-OVX group and 1 in the ALD during the experiment, but implants were maintained. In the control group none of the implants were lost during the 4 weeks. When the data were collected, 7 rats were analyzed for the ALD-OVX group, 8 rats for the OVX group (lost implants equal zero contact), 7 rats for the ALD group, and 8 rats for the control group.

Estimated bone contact ratio

The least square means of the contact ratio values were measured at 0, 7, 14, and 28 days. Mean values plus standard error are shown for the 4 groups in Figure 5 and P values are recorded in the Figure 5 legend. The ALD-OVX group presented the highest bone contact ratio at 4 weeks with 85% of initial contact (day 0/day 28 = 0.50/0.59), followed by ALD with 63% (0.41/0.65), control with 57% (0.34/0.60), and OVX with 13% (0.07/0.56). In Figure 5, it is shown that after 1 week there was more contact in the ALD-OVX group than in the OVX group. At week 4, all 4 groups had diminished contact ratios. However, the OVX group at 2 weeks showed a significant and dramatic reduction in the bone contact ratio.

From the repeated measures 1-factor ANOVA model, a borderline (at the 10% level) significant difference was found between the 4 treatment groups. There was, however, a significant difference between the 4 times measurements were made, which indicates that the contact ratio decreased over time for all groups. From the pairwise comparisons with 2-sample t tests, the OVX group at 4 weeks had a significantly lower contact ratio than the other groups (OVX vs ALD-OVX, P = .002; OVX vs control, P = .04; OVX vs ALD, P = .15). Furthermore, within the OVX group, there was a significant difference between the implantation date and the measurement taken at 4 weeks (P = .0006), and between 1 week and 4 weeks (P = .0009). Thus, in the OVX group, there is a large decrease in the contact ratio after the second week.

Radiographic bone density

All 3 measurement sites (mesial, distal, and apical) were considered together as indicative of osseointegration. For the 3 measured sites together, the average least square means with standard error for the 4 groups is shown in Figure 6. The ALD group had
the highest bone density, followed by ALD-OVX, control, and OVX.

The average radiographic bone density was significantly higher for the ALD-OVX group than the OVX group \((P = .0003)\). The radiographic bone density was 50% more in the ALD-OVX group at all 3 sites. Significant differences were found between the 4 treatment groups. From the pairwise comparisons with 2-sample \(t\) tests, there was no significant difference between the control group and the OVX group \((P = .2676)\). However, all the other pairs displayed significant differences (Table).

**DISCUSSION**

The ovariectomized rat model is widely known to develop osteopenia after ovariectomy. The OVX rats is, therefore, widely used as a model for postmenopausal osteoporosis. Osseointegration of implants was impaired in the estrogen-deficient OVX rats compared with the ALD-OVX group. After 1 week, ALD-OVX displayed an increase in bone-implant contact compared with the OVX rats. Fifty percent of the implants (4 of 8) were lost from the OVX group at the second week. In the fourth week, the ALD-OVX rats still had 85% of bone in contact with the implants \((P = .0009)\). Data are presented as mean ± SE.

**FIGURE 6.** Radiographic bone density was measured by placing all samples on a single cephalometric X-ray film and determined by quantifying pixels at 3 points: mesial, apical, and distal. Significant difference from control. For other significant differences found between the 4 groups, see the Table. Data are presented as mean values ± SE.

<table>
<thead>
<tr>
<th>Comparison</th>
<th>(P) value</th>
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<tbody>
<tr>
<td>ALD-OVX vs OVX</td>
<td>.0003*</td>
</tr>
<tr>
<td>ALD-OVX vs control</td>
<td>.0072*</td>
</tr>
<tr>
<td>ALD-OVX vs ALD</td>
<td>.0008*</td>
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<tr>
<td>OVX vs control</td>
<td>.2676</td>
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<tr>
<td>ALD vs OVX</td>
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<td>ALD vs control</td>
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\(\text{ALD}\) indicates aldesterone; \(\text{OVX}\), ovariectomized.
Radiographic evidence suggests that none of the implants in the O VX group osseointegrated. The bisphosphonates, including ALD, are known to increase bone mineral density, reducing bone resorption by inhibiting osteoclastic activity. In our study, the ALD-O VX group presented 50% more radiographic bone density in all 3 sites compared with the O VX group. Both the ALD-O VX and ALD groups had significantly higher radiographic bone density than the control and O VX groups. This confirms that ALD is increasing the mineral density in the bone. Also, there were no significant differences between the O VX and CTRL groups. Radiographic bone density was higher in the ALD group followed by ALD-O VX, control, and O VX groups. These results were expected and confirmed by the histology, where more bone was observed around the implant sites of the ALD and ALD-O VX groups.

Furthermore, these results support our hypotheses that estrogen deficiency affects implant osseointegration. Implants placed in rats with estrogen deficiency did not osseointegrate. The probability of losing the implants at 2 weeks is 50%, and at 4 weeks just 13% of the implant surface remained in contact with bone. Administration of ALD helped to improve the bone-implant contact to 85% of the initial value. None of the implants were lost when ALD was administered, and the quality of the bone density improved to 50%.

The placement of implants in patients taking bisphosphonates is now controversial because of the recent reports of osteonecrosis of the jaw in these patients. These reports are mainly associated with cancer patients under treatment with the intravenous forms of bisphosphonates (pamidronate and zolendronic acid). Some cases of osteonecrosis of the jaw have been reported with oral bisphosphonates for the treatment of osteoporosis, but these are very few. Jeffcoat reported 100% implant success and 0 cases of osteonecrosis of the jaw in 335 patients taking ALD for a 2-year period.

We evaluated implant osseointegration on the maxilla in an estrogen-deficient model with the administration of ALD on an acute environment. A future study should focus on chronic treatment of ALD, as is more common to treat patients who have been taking the medication for long periods. The rat model might be a poor model for osteonecrosis of the jaw, because rats only live for 2 years. We concluded that implant osseointegration on maxilla was improved with ALD. Results of previous studies in humans find that implant placement on osteoporotic women without treatment is not recommended. The osseointegration is slower and compromised, and the rate of implant failure is high. Our studies suggest that ALD may improve the quality and quantity of bone available for a successful implant.

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