Histomorphometric Study of New Bone Formation Comparing Defect Healing with Three Bone Grafting Materials: The Effect of Osteoporosis on Graft Consolidation

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Purpose: *Bone grafting materials are frequently utilized in oral surgery and periodontology to fill bone defects and augment lost or missing bone. The purpose of this study was to compare new bone formation in bone defects created in both normal and osteoporotic animals loaded with three types of bone grafts from different origins.* ***Materials and Methods:*** *Forty-eight female Wistar rats were equally divided into control normal and ovariectomized animals. Bilateral 2.5-mm femur defects were created and filled with an equal weight of (1) natural bone mineral (NBM, BioOss) of bovine origin, (2) demineralized freeze-dried bone allograft (DFDBA, LifeNet), or (3) biphasic calcium phosphate (BCP, Vivoss). Following 3 and 6 weeks of healing, hematoxylin and eosin and TRAP staining was performed to determine new bone formation, material degradation, and osteoclast activity.* ***Results:*** *All bone substitutes demonstrated osteoconductive potential at 3 and 6 weeks with higher osteoclast numbers observed in all ovariectomized animals. NBM displayed continual new bone formation with little to no sign of particle degradation, even in osteoporotic animals. DFDBA particles showed similar levels of new bone formation but rapid particle degradation rates with lower levels of mineralized tissue. BCP bone grafts demonstrated significantly higher new bone formation when compared with both NBM and DFDBA particles; however, the material was associated with higher osteoclast activity and particle degradation. Interestingly, in osteoporotic animals, BCP displayed synergistically and markedly more rapid rates of particle degradation.* ***Conclusion:*** *Recent modifications to synthetically fabricated materials were shown to be equally or more osteopromotive than NBM and DFDBA. However, the current BCP utilized demonstrated much faster resorption properties in osteoporotic animals associated with a decrease in total bone volume when compared with the slowly/nonresorbing NBM. The results from this study point to the clinical relevance of minimizing fast-resorbing bone grafting materials in osteoporotic phenotypes due to the higher osteoclastic activity and greater material resorption.* Int J Oral Maxillofac Implants 2018;33:645–652. doi: 10.11607/jomi.5879

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There is an increasing demand for bone substitute materials in oral therapies to fill bone voids resulting from tooth loss, trauma, biochemical disorders, infections, and abnormal skeletal development.[1–4] Ideally, bone substitute material should provide an osteoconductive surface that allows three-dimensional tissue ingrowth as well as intrinsic osteoinductive properties further facilitating future new bone formation.[5] Other requirements of ideal bone grafts are their biocompatibility, mechanical strength, lack of potential disease transmission, and ability to be degraded over time and replaced by host bone.[6–8] While autogenous bone harvested from the same person is considered the gold standard due to its incorporation of host living cells,[9–11] drawbacks including lack of availability, longer surgical times, and donor site morbidity have necessitated alternative strategies.[12,13]

Natural bone mineral (NBM; Bio-Oss, Geistlich) has been utilized in the fields of oral surgery and periodontology for more than two decades. NBM is a highly purified anorganic bone matrix mineral derived from bovine origin and is one of the most widely utilized and well-documented grafting materials.[10,14–16] It has excellent biocompatibility and osteoconductivity with the added advantage of being nonresorbable, and is favored during certain indications in guided bone regenerative procedures in implant dentistry.[10,14–16] A second grafting material with osteoinductive potential and approved by the U.S. Food and Drug Administration (FDA) is demineralized freeze-dried bone allograft (DFDBA; LifeNet).[5] This material contains osteoinductive growth factors (bone morphogenetic protein 2) that promotes mesenchymal progenitor cell recruitment and their differentiation toward bone-forming osteoblasts.[17–19] In some aspects, both of these materials have limitations. NBM is not osteoinductive and is unable to further speed up the rate of new bone formation, whereas osteoinductive DFDBA lacks ideal mechanical properties due to its processing technique, which requires demineralization and is therefore resorbed rapidly. Thus, it becomes relevant to develop new bone graft substitutes in light of technologic advancements to further enhance graft consolidation.[5,20,21]

Recently, synthetically fabricated biphasic calcium phosphates (BCP; Vivoss, Straumann) bone grafts sintered at low temperatures have been shown to have the potential to form ectopic bone in muscle.[22,23] The material possesses better mechanical properties when compared with DFDBA, yet maintains osteoinductive potential (whereas NBM does not).[24] Despite these findings, research from another group has reported the fast resorption rates of BCP particles.[25] While much research has been performed in recent years on graft consolidation using a variety of bone biomaterials,[25–28] much less attention has been placed on graft consolidation in systemically compromised patients.

Worldwide, osteoporosis now affects more than 200 million people.[29,30] It has been shown that the disease is caused by an imbalance between bone-resorbing osteoclasts and bone-forming osteoclasts commonly associated with estrogen deficiencies in postmenopausal women, leading to lower bone mineral density and higher fracture rates.[31,32] Despite the great deal of research that has been performed to diagnose and utilize preventative medication in osteoporotic women (such as bisphosphonates and receptor activator of nuclear factor kappa-B ligand [RANKL] inhibitors), little research has been performed investigating the effect of osteoporosis on graft consolidation of various biomaterials. Therefore, the purpose of this study was to compare new bone formation, material resorption, and osteoclastic activity in bone defects filled with either *(1)* NBM, *(2)* DFDBA, or *(3)* BCP in both normal and osteoporotic animals.

MATERIALS AND METHODS

Bone Graft Selection

Xenograft Bone. A bovine-derived grafting material commonly used in dentistry (NBM, Geistlich) was used in this study due to its widespread use and the present group's numerous publications using NBM.[15]

Allograft Bone. DFDBA (LifeNet) was utilized as the allograft due to the present group's previous characterization of this specific DFDBA that has osteoinductive potential as confirmed by its ability to form ectopic bone in vivo.[17]

Alloplast Bone. BCP grafts were fabricated from a 10:90 ratio of hydroxyapatite to beta-tricalcium phosphate sintered at approximately 1,000°C (Vivoss, Straumann).

Animals and Surgical Protocols

Forty-eight 8-week-old Wistar rats were used in this study. Animal handling and animal surgical procedures were conducted in accordance with the guidelines for animal care and use committee of Wuhan University, People's Republic of China, and approved by the Ethics Committee at the School of Dentistry, before the start of the experiment. The animals were kept under a 12-hour light/dark cycle at 20°C to 25°C and with food and water ad libitum. All the operations were carried out under sterile conditions. The surgeon (Y.Z.) was blinded to the treatment. Postoperatively, 40,000 IU/mL of penicillin was injected intramuscularly. No preoperative or postoperative infections or fractures were observed.

Osteoporotic Animal Model

After 1 week to acclimatize to the laboratory environment, the osteoporotic model was established with bilateral ovariectomy (OVX).[33] Briefly, when general anesthesia was achieved, a 10-mm linear incision was made in the lumbar lateral skin bilaterally. Thereafter, the bilateral ovaries were gently removed. The tissues were repositioned by layers and sutured. Following surgery, buprenorphine (0.05 mg/kg) was administered by subcutaneous injections for pain management.

Femur Defect Model

After injection of sodium pentobarbital (40 mg/kg body weight) intraperitoneally, the defect model was performed under general anesthesia as previously described.[16] A 10-mm linear incision was made in the distal femoral epiphysis bilaterally, the muscle was

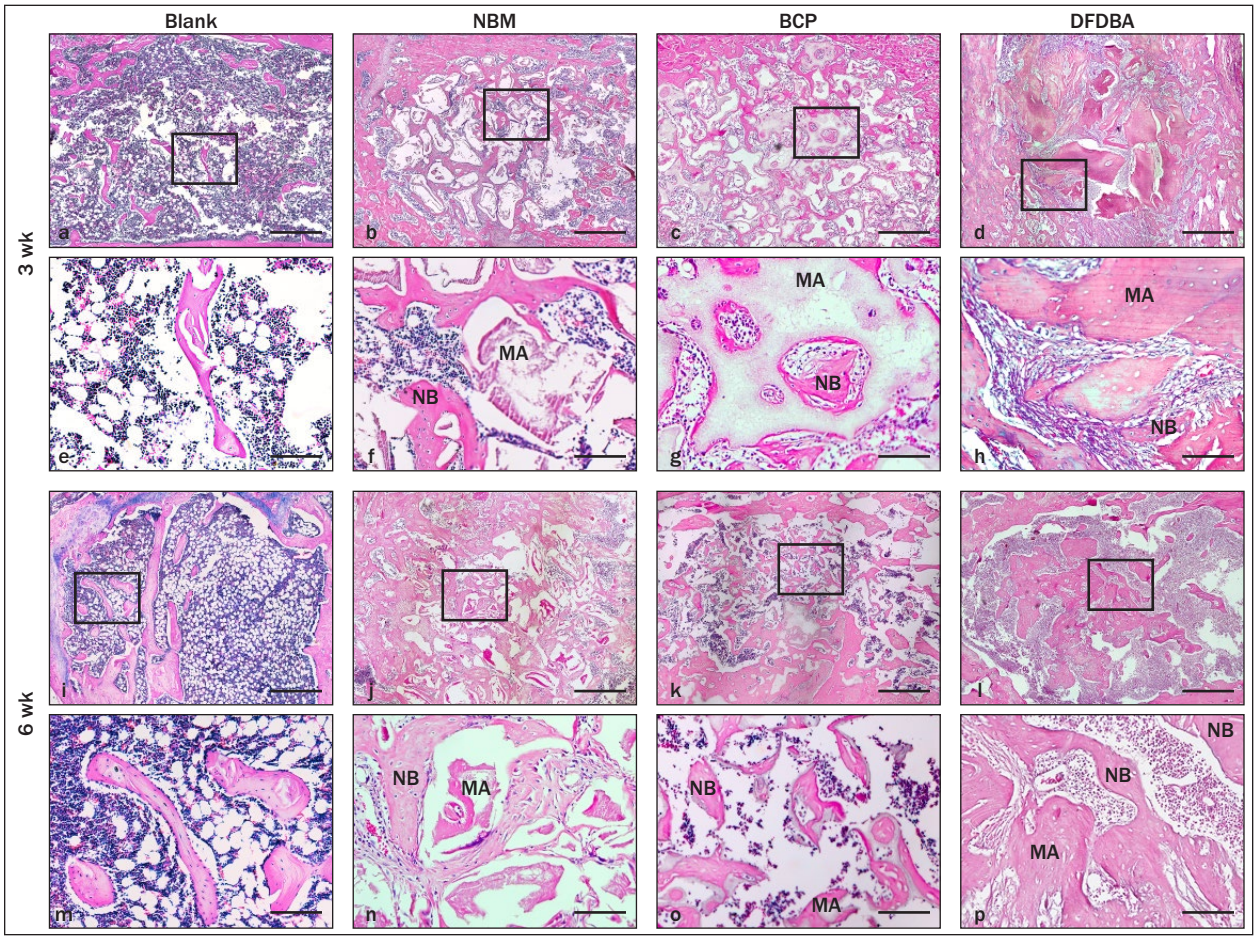

Fig 1 Representative sections of hematoxylin and eosin (H&E) staining demonstrating the healing femoral defects at 3 and 6 weeks postoperation at low (×10; bar = 500 μm) and high (×20; bar = 100 μm) magnification in control nonosteoporotic animals. New bone formation was continuously formed in all groups receiving a bone graft. NB = new bone; MA = material.

blunt dissected, and the femoral condyle was exposed. Then, an anteroposterior bicortical channel of 2.5-mm diameter perpendicular to the shaft axis was created, and cancellous trabecular bone was removed. To avoid thermal necrosis, a trephine bur was utilized at a slow speed and irrigated with saline solution. The saline solution was then injected into the drilled holes to remove bone fragments and rinse the defects. Bone substitute materials were then gently placed according to the group allocation to fill the defects as previously described.[34–36] The groups included: empty controls (n = 12), drilled defect + NBM (n = 12), drilled defect + DFDBA (n = 12), and drilled defect + BCP (n = 12) (total of 48 defects in 24 rats). All bone grafting materials ranged in size between 600 and 1,000 μm and could gently be easily placed into the 2.5-mm defects. Furthermore, an equal number of surgeries (total of 48 defects in 24 osteoporotic animals) were performed in ovariectomized rats. At 3 weeks and 6 weeks after femur surgery, six rats in each group were sacrificed, accordingly. All femurs were separated and assigned to histologic studies.

Histologic Analysis

The separated femurs were decalcified in 10% ethylene diaminetetraacetic acid (EDTA) for 3 weeks and changed every 3 days, and then dehydrated in a series of graded concentration of ethanol from 70% to 100%. The femurs were then embedded in paraffin, and the alignment and orientation was adjusted to get a distinct view as previously described.[16,37] To analyze the bone regeneration process within the defect, the central region of the defect was defined by analyzing a circular contour as the area of measurement per slice to obtain a consistent area of interest (AOI) and to avoid including the native bone margins. Five-micrometer serial sections were cut and then mounted on polylysine-coated microscope slides. Hematoxylin and eosin (H&E) staining and tartrate-resistant acid phosphatase (TRAP) staining (Sigma #387A; Sigma-Aldrich) were performed with the specimens according to the manufacturer's protocol and then examined under light microscopy (Olympus DP71 microscope, Olympus). The analysis was repeated at least three times to confirm the results. H&E staining of the sections was used to perform

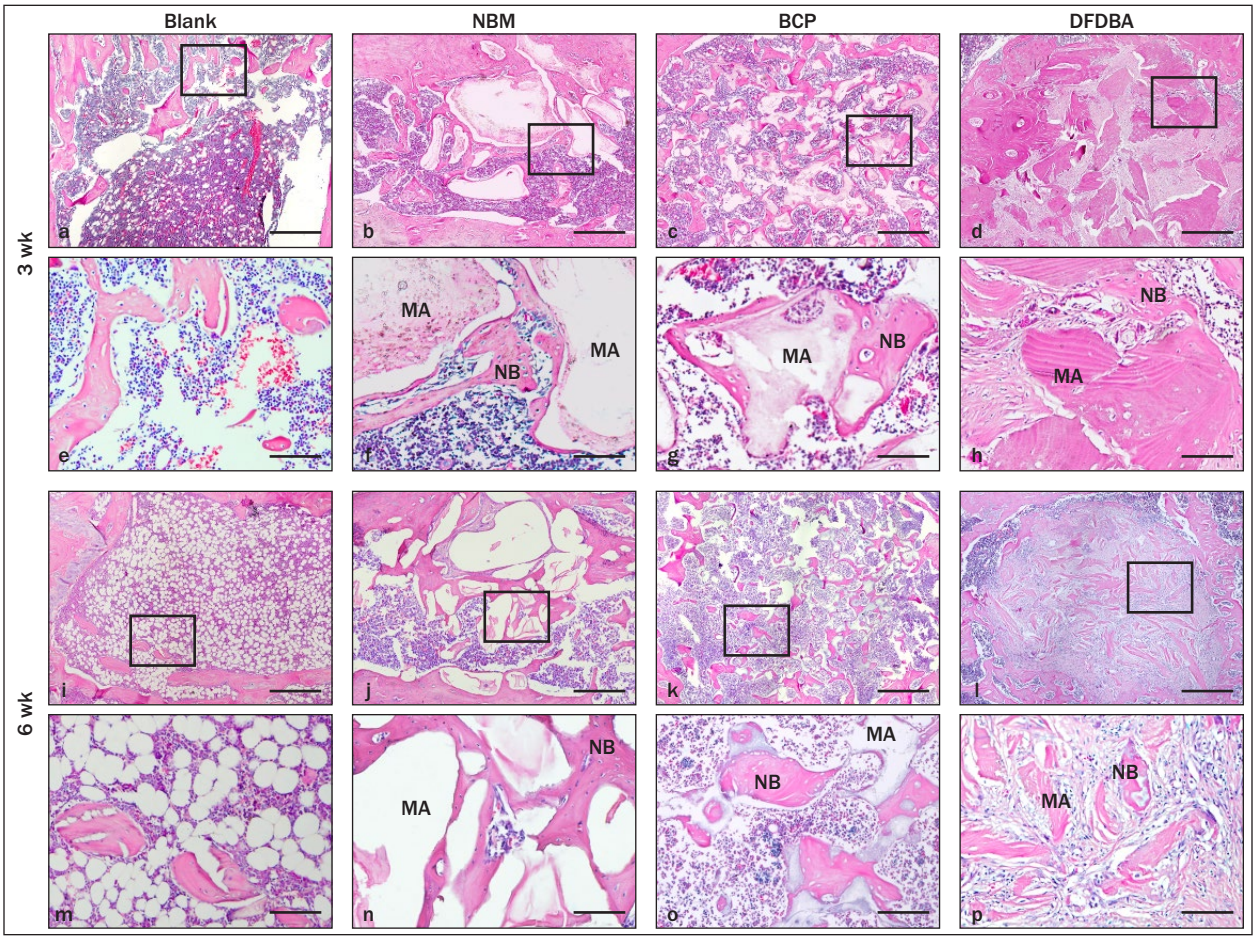

Fig 2 Representative sections of H&E staining demonstrating the healing femoral defects at 3 and 6 weeks postoperation at low (×10; bar = 500 μm) and high (×20; bar = 100 μm) magnifications in osteoporotic animals. New bone formation was continuously formed in all groups receiving a bone graft (NB = new bone; MA = material).

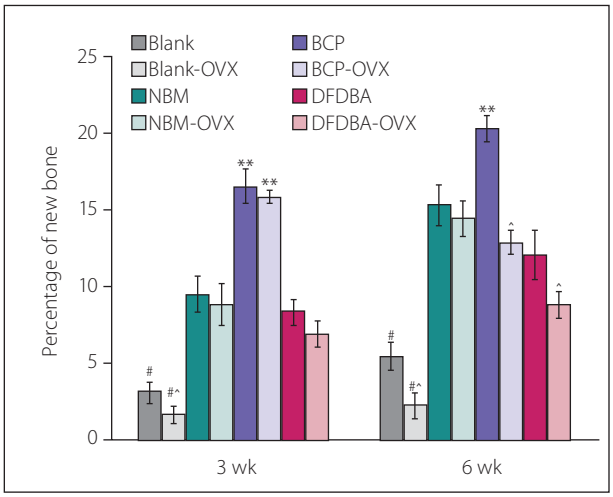

Fig 3 Analysis of the H&E staining images reveals that significantly higher new bone formation was observed in drilled defects filled with BCP when compared with blank, NBM, and DFDBA groups at 3 and 6 weeks postimplantation in both control and osteoporotic animals (**P < .01). ^ = significant difference between the control and the OVX group; # = significantly lower than all other groups.

the bone regeneration by an individual blinded to the group allocation as previously described.[16,34,35] The areas of new bone formation and residual bone grafting material were delineated manually and then calculated as the percentage of new bone area and residual content of material in total cross-sectional area [(bone area/total area) × 100%] and [(material area/total area) × 100%] as previously described.[34] The number of osteoclasts (cells positively stained for TRAP containing 3+ nuclei) was then calculated under light microscopy (Olympus DP71, Olympus). The bone histomorphometry and osteoclast measurements were performed on three consecutive sections of each specimen. From each section, three representative fields (1,024 × 1,536 pixels) were identified (original magnification ×10) and averaged as previously described.[38]

Statistical Analysis

SPSS 17.0 software (SPSS) was used for the statistical analysis. The mean and standard deviation were analyzed using one-way analysis of variance with post hoc

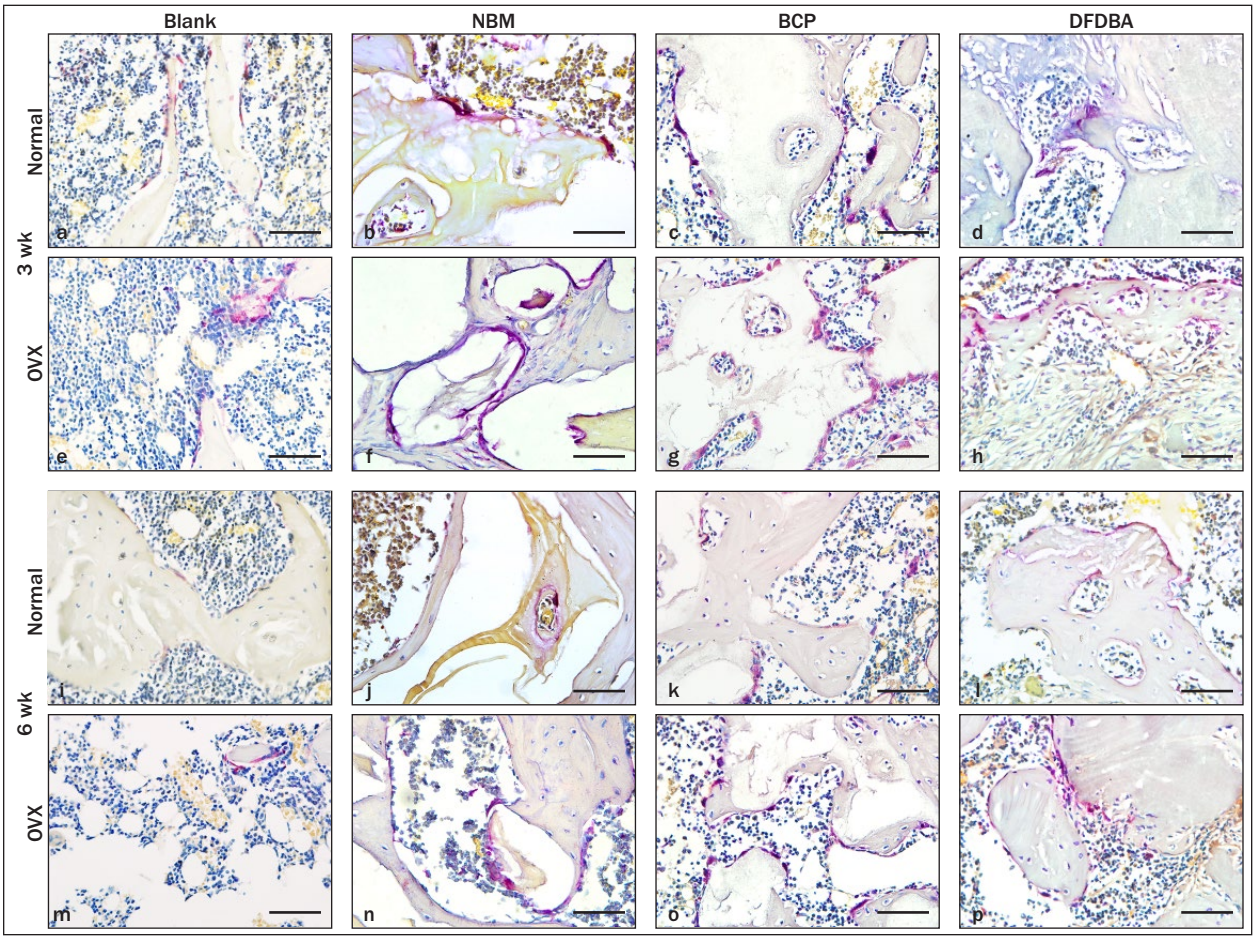

Fig 4 Representative TRAP staining of osteoclasts around the bone grafting particles at *(a, b, c, d)* 3 and *(e, f, g, h)* 6 weeks post-implantation in both control and osteoporotic animals. Bar = 50 mm.

test; $P < .05$ was considered as the statistically significant difference.

RESULTS

Histologic and Immunohistochemical Observation of the Femoral Defect

All animals healed normally without any complications or infections after the surgical techniques. While H&E staining of sections from the three groups in control nonosteoporotic and osteoporotic animals revealed that all the bone grafts were osteoconductive by demonstrating new bone growth, differences in either new bone formation or material degradation were observed (Figs 1 and 2). For the NBM group, a small increase in bone formation was observed between 3 and 6 weeks with little to no sign of particle degradation over a 6-week period (Figs 1b, 1f, 1j, and 1n). Contrarily, a similar amount of new bone formation was observed in the DFDBA group; however, much faster particle degradation was observed (Figs 1 and 2). Of all the groups tested, BCP bone grafts had the highest level of new bone formation as depicted by the bone area/tissue area (BA/TA) in Fig 3. At 3 weeks, there were similar levels of new bone formation between control and osteoporotic animals; however, at 6 weeks, it was observed that the osteoporotic phenotype tended to be associated with less new bone formation, surprisingly mostly observed in the BCP group (Fig 3). TRAP staining was then utilized to detect the presence of multinucleated giant cells and osteoclastic activity (Fig 4). It was found that the BCP group showed higher levels of TRAP staining as well as a significantly higher number of multinucleated cells (osteoclasts), and this was significantly higher in the osteoporotic animals (Figs 4 and 5). In general, all osteoporotic groups showed elevated levels of osteoclasts when compared with their group's respective controls (Fig 5). Interestingly, no signs of particle degradation were apparent after a 3- and 6-week healing period for groups treated with NBM in both control and osteoporotic animals

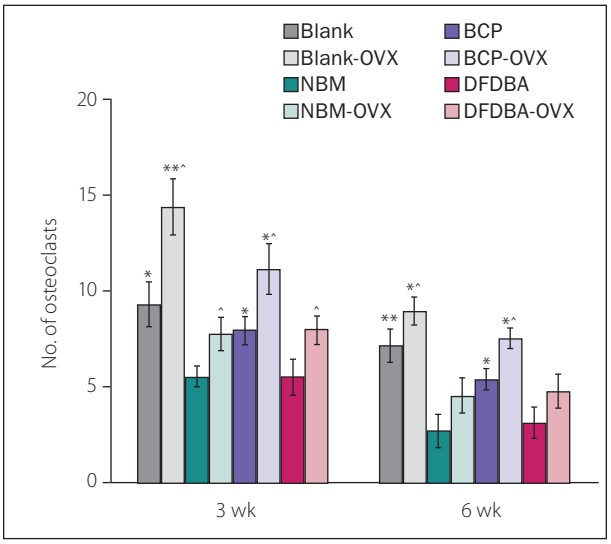

Fig 5 The number of osteoclasts in the blank, NBM, BCP, and DFDBA group at 3 and 6 weeks in both control and osteoporotic animals (*$P < .05$, **$P < .01$).

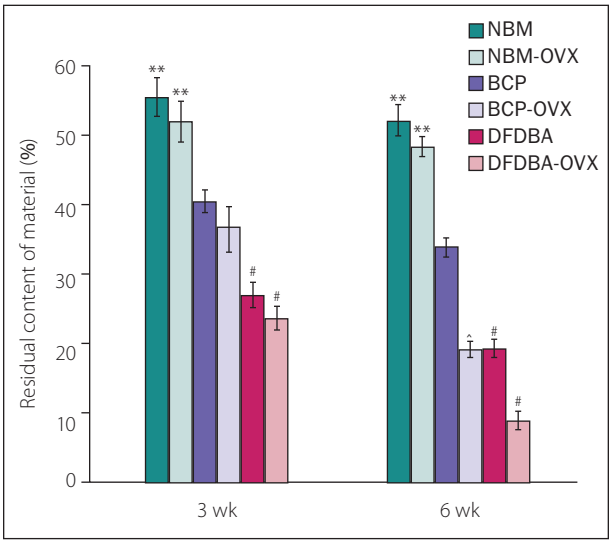

Fig 6 Material degradation of NBM, BCP, and DFDBA particles at 3 and 6 weeks in both control and osteoporotic animals.

(Fig 6). The most surprising finding was the synergistically negative effect of osteoporosis on graft consolidation of BCP and DFDBA bone grafting materials (Fig 6). At 6 weeks, a much-higher-than-anticipated particle loss was observed in both BCP and DFDBA, whereas the osteoporotic phenotype had virtually no effect on NBM material degradation/resorption (Fig 6).

DISCUSSION

The cellular basis for osteoporosis is associated with a marked increase in osteoclast activity leading to disruption in the bone remodeling cycle. Upregulated formation and activation of osteoclasts and a decreased lifespan and activation of osteoblasts induces an imbalance of bone remodeling. Estrogen deficiencies has been suggested as one of the main reasons for such phenotypes by decreasing the expression of insulin-like growth factor 1 (IGF-1) and transforming growth factor beta (TGF-β) in osteoblasts, leading to their decreased proliferative and differentiation potential.[39] Estrogen is also related to type I collagen expression, and its deficiency has been shown to decrease the production of a functional extracellular matrix.[39] Estrogen increases the lifespan of osteoblasts through suppressing osteoblast apoptosis, which is how osteoporosis with estrogen deficiency affects bone formation.[39]

The aim of this study was therefore to compare the effects of three different bone grafting materials on new bone formation in a 2.5-mm femur defect in both control and osteoporotic animals. Many studies to date have previously investigated the osteogenic capability of various bone grafts with little effect, comparing how systemically compromised diseases might affect regenerative outcomes.[17–20,38,40–44] Therefore, the purpose of this study was to investigate graft consolidation in an osteoporotic phenotype to further characterize the regenerative potential of three bone grafting materials commonly utilized in implant dentistry.

Significant improvements have been made over the years with respect to developing novel bone substitute materials fabricated from synthetic sources. However, to date, autogenous bone has remained the gold standard for bone grafting procedures.[10] Despite this, the utilization of synthetic materials as bone substitutes has been a desired end goal for many clinicians to prevent the necessity of harvesting autogenous bone and facilitating the ease of use of bone grafts for local transplantation into bone defects. Therefore, the present study investigated a newly developed synthetic bone graft fabricated from a biphasic calcium phosphate in a 10:90 ratio of hydroxyapatite and beta-tricalcium phosphate.[23,42]

Previously, Yip et al compared BCP with NBM and also found that BCP promoted significantly more new bone formation at 3 months in a rabbit calvarial defect model.[7] At 6 months, however, no differences were found between the two groups, and both were recommended for clinical use.[7] The results from this study further confirm the satisfactory results for BCP in normal graft consolidation; however; in an osteoporotic phenotype, the rapid material degradation rates combined with the higher average number of osteoclasts found in an osteoporotic phenotype led to a significant reduction in total BA/TA and far too much material loss after only a 6-week healing period. Therefore, it may be clinically recommended that such fast-resorbing bone

grafting materials be avoided specifically in osteoporotic animals.

In terms of their bone-forming capacity, the present study confirms the previous reports indicating that BCP grafts do possess excellent bone-inducing properties by displaying higher levels of new bone formation when compared with NBM and DFDBA.[7,24,45] Interestingly, the present results further found that at 3 weeks, both the control and osteoporotic animals showed high levels of new bone formation, whereas at 6 weeks, the results were statistically higher in the normal non-osteoporotic animals. Therefore, it was confirmed in the present study that even in an osteoporotic phenotype, changes in new bone formation were reported, and this was most likely caused by a decrease in the bone remodeling cycle seen in osteoporotic phenotypes. It was also interesting to report that in control animals, significantly higher new bone formation was reported in the BCP group when compared with NBM, but at 6 weeks, the nonresorbing NBM showed similar levels of new bone formation to BCP in osteoporotic animals, yet was not resorbed over time.

One of the main advantages of NBM particles is their low substitution rate, which has allowed its widespread use in dentistry for a variety of treatments such as sinus elevation procedures, alveolar bone reconstructions, and guided bone regeneration.[46–48] The material degradation rate of NBM has also generated controversial results, as some clinical results from Tadjoedin et al indicated that osteoclasts are very active on NBM particles and result in a degradation rate of 10% per year.[49] However, in other studies, the material has shown no evidence of substantial resorption after a healing period of 4.5 years.[50] The results of this present study demonstrated a presence of multinucleated cells around NBM particles. Despite demonstrating a high number of multinucleated giant cells resembling osteoclasts, little to no evidence of material resorption was observed. Recent evidence suggests that multi-nucleated giant cells (MNGCs) may contribute to graft stability by acting as poly-nuclear macrophages capable of polarizing toward M1 tissue inflammatory or M2 tissue repairing macrophages.[51] There remains great interest in better understanding their role in biomaterial graft consolidation, especially given they remain on the surface of bone biomaterials years after their implantation.[50] Although the search for the ideal graft material is still ongoing, these newly developed BCP bone grafting particles do show some early promise for the repair of femur defects; however, their faster-than-ideal resorption, especially in osteoporotic animals, greatly limits their potential for clinical use. Future research should focus primarily on the material stability of BCP over a longer healing period while maintaining their osteoinductive properties.

CONCLUSIONS

The results from the present study demonstrate that all bone grafting particles were osteoconductive by improving new bone formation in 2.5-mm cylindrical defects in rat femurs. The ability to form new bone in defects treated with NBM, DFDBA, and BCP demonstrated signs of new bone formation over 3- and 6-week healing periods with BCP grafts supporting higher levels of newly formed bone when compared with NBM and DFDBA. However, BCP grafts were also associated with much faster particle degradation when compared with NBM, and this finding was significantly more severe in osteoporotic animals. Interestingly, the effects of an osteoporotic phenotype had little effect on graft consolidation of NBM particles, as the increase in osteoclastic activity had no effect on particle degradation when defects were treated with the nonresorbing NBM. Therefore, it may be clinically recommended that patients with osteoporosis avoid treatment with fast-resorbing bone grafting materials, as the increase in osteoclastic activity associated with osteoporosis leads to material degradation that is too high at early time periods.

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