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Comparative Evaluation of Demineralized and Mineralized Xenogeneic Bovine Bone Powder and Chips on the Healing of Circumscribed Radial Bone Defects in the Dog

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Healing of circumscribed defects made in the radius of dogs were evaluated using demineralized and mineralized xenogeneic bovine bone powder and chips at 2, 4, 6, 8, 12 weeks. No foreign body reactions and postoperative complications were encountered.

Presence of more bone formation in the defects filled with demineralized xenogeneic bovine bone powder compared to other graft-filled defects explains the importance of demineralization process and smallness of the graft size.

Comparison of the appearance of bone healing and formation pattern in demineralized and mineralized xenogeneic bovine bone powder and chips shows the remarkable histologic difference of field phenomenon bone formation in favour of demineralized bone powder. Based on these findings, demineralized xenogeneic bovine bone powder being not species specific may be a good implant with respect to availability of wide variety of animal resources as animal donor, easy preparation and storage in advance.

Key Words: *Circumscribed defect, Xenogeneic implant, Demineralized powder and chip, Mineralized powder and chip, Dog.*

Köpeklerde Xenogenik Demineralize ve Mineralize Kemik Tozu ve Partiküllerinin Dairesel Kemik Defeklerinin İyileşmesi Üzerindeki Etkilerinin Karşılaştırmalı Olarak Değerlendirilmesi

Köpeklerin radiusunda yapılan dairesel defeklerde demineralize ve mineralize xenogenik sığır tozu ve partiküllerinin 2, 4, 6, 8 ve 12. haftalardaki iyileşme seyirleri karşılaştırmalı olarak değerlendirildi. Yabancı cisim reaksiyonu ve herhangi bir postoperatif yan etkiye rastlanmadı.

Demineralize sığır xenogenik kemik tozu ile doldurulan defeklerin diğer formlardaki graf ile doldurulan defek iyileşmelerine göre daha fazla kemik dokusu oluşturması demineralizasyon ile birlikte graf ölçü küçüklüğünün önemini ortaya koymaktadır.

Demineralize ve mineralize xenogenik kemik tozu ile partiküllerinin kemik iyileşmesi ve oluşum özelliklerinin karşılaştırılması demineralize kemik tozu lehine açık bir şekilde alan fenomeni farklılığını göstermektedir.

Bu bulgulara dayanarak, türe özgü olmayan demineralize xenogenik sığır kemik tozunun verici olarak çok geniş hayvan kaynaklarının bulunması, kolay uygulanması, hazırlanması ve depolanabilme özelliği iyi bir graf materyali olduğunu ifade etmektedir.

Anahtar Kelimeler: *Dairesel defek, Xenogenik graf, Demineralize toz ve partikül, Mineralize toz ve partikül, Köpek.*

Introduction

Autogeneneic and allogeneic bone grafts have been widely used for stimulation of fracture healing (1), delayed and nonunion of fractures, infected and contaminated fractures, filling of defects formed after osteomyelitis, following removal of bone neoplasms, arthrodesis, and large segmental defects (1, 2), and prevention of refracture of long bones after plate removal as a result of stress shielding effect of a plate (3).

Prolongation of anesthesia and surgery, risk of infection, limited quantities for harvest, structural defects at the donor site along with severe postoperative pain have been encountered with autogeneic bone grafting (4, 5). It is generally in agreement that fresh autogeneic grafts are superior to fresh or stored allogeneic and xenogeneic grafts (6-9) but, the delay in the incorporation of allogeneic bone graft as compared to that of auogeneic bone graft has not caused to hamper its clinical use.

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Although there is tremendous amount of efforts to find the best potential bone graft substitutes including, demineralized bone matrix or bone derivatives as bone morphogeneic protein (BMP), osteogenin (10) to be used, synthetic graft materials lack osteoinductive or osteogenic elemental properties to the host (11, 12).

It is the fact that the use of xenogeneic bone (13) is increasing as being advocated to be a suitable solution to tissue shortage in near future (14).Though tremendous amount of research have been carried out related to allograft without or with regard to demineralization process, search of the literature revealed no information on the effectiveness of xenogeneic bovine bone with demineralization process in different forms.

The purpose of this study was to find out and compare the histologic character of bone formed using demineralized and mineralized xenogeneic bovine bone powder and chips in circumscribed defects of radial bone in dogs.

Materials and Methods

All animals used in this study, were treated in accordance with national or local animal welfare legislation based on European Council Directive.

Xenogeneic bovine bone powder and chips were prepared from freshly obtained diaphysis of tibia of approximately a 2-year-old-bovine. All soft tissue, including periosteum, bone marrow and blood was removed mechanically and the bone was washed in sterile deionized water. The cortical bone was fragmented with a hammer and then freed from fat by extraction in a 1:1 mixture of chloroform and methanol (30 mL of per g of bone) for 1.5 h and dried overnight (15).

The fragmented bone was pulverized in a mill and sieved to a size of 75 to 450 µm of powder. The powdered implants were demineralized in 0.6 N hydrocloric acid per g of bone for 3 h at 4 ºC, dehydrated in 70% alcohol (7) for 15 min with magnetic stirring and centrifuged at 5000 rpm for 15 min (16). The chips (3 x 6 mm) were decalcified for 4 days (17). Finally the demineralized powder and chips were stored in 95% alcohol in vials at room temperature until used. Before use, they were rinsed in sterile saline for 1 h. Mineralized powder and chips, stored in vials, were sterilized at 120°C for 20 min in auotoclave.

A total of 20 adult dogs in different sex were used and allowed two weeks of acclimation period prior to surgery. The dogs as 2, 4, 6, 8 and 12 weeks were divided into 5 experimental groups of 4 animals each. Because betadine inactivates the biopeptides in demineralized bone (7, 18), isopropyl alcohol was used for sterilization of surgical field (7).

Five circumscribed bone defects only over the near cortex, 5 mm in diameter and 5 mm apart were made. Starting proximally, the 1st and 2nd defects were filled with mineralized xenogeneic bovine bone chip and minereralized xenogeneic bovine bone powder, respectively. The 3rd defect unfilled, kept as a contol. The 4th and 5th defects were filled with demineralized xenogeneic bovine bone chip and demineralized xenogeneic bovine bone powder, respectively. Anteroposterior and lateral radiographs were made of all animals postoperatively and at the time of euthanasia. After decalcification of the specimens, using standard histopathological techniques, the tissues were stained with hematoxylin and eosin (H and E), Masson's trichrome and van Gieson stains.

Results

Histopathological Findings

Healing of Unfilled Defect: At 2 weeks, trabecular bone formation through periosteal and endosteal reactions with fibrovascular tissue over the defect (Figure 1A) were present which continued with new bone formation at 4, 6 and 8 weeks. Remodeling with resorption of trabecular bone and formation of bone marrow were evident beneath the vascularized defect area at 12 weeks (Figure 1B).

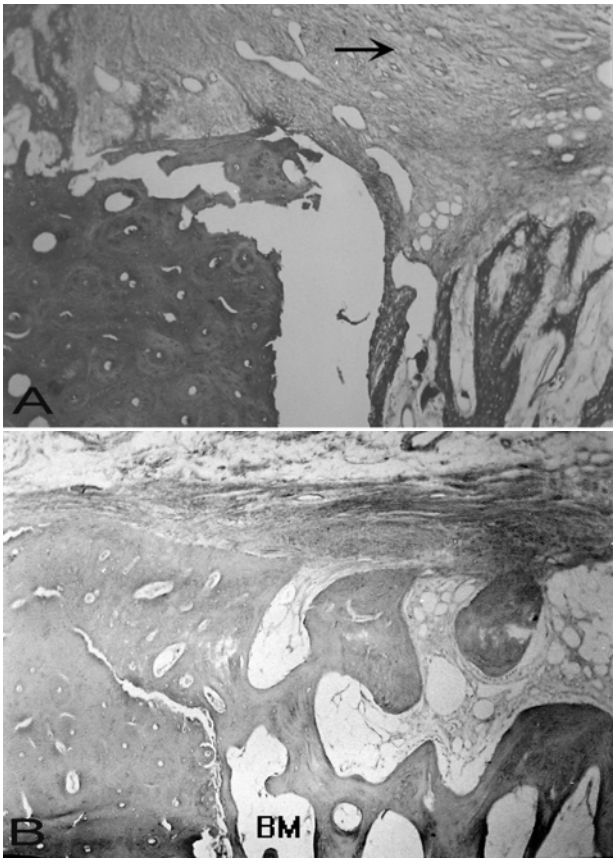

Figure 1. (A) Unfilled defect at 2 weeks showing trabecular bone formation induced by periost and endost under fibrovascular tissue (arrow), Masson's trichrome X50. (B) Remodeling and bone marrow formation (BM) at 12 weeks, van Gieson X50.

Healing of Mineralized Bovine Bone Chip Filled Defect: At 2 weeks, periosteal over the defect and endosteal reactions were evident (Figure 2A). While envelopment of bone chips within fibrous tissue and newly forming bone were observed from the edges at 4 weeks, decreased periosteal and marked endosteal reactions took place at 6 weeks. The most prominent observation was the finding of many unresorbed bone chips within the newly formed bone at 8 weeks (Figure 2B), resorbtion of bone chips along with absence of periosteal and endosteal reactions and remodeling of trabecular bone at 12 weeks.

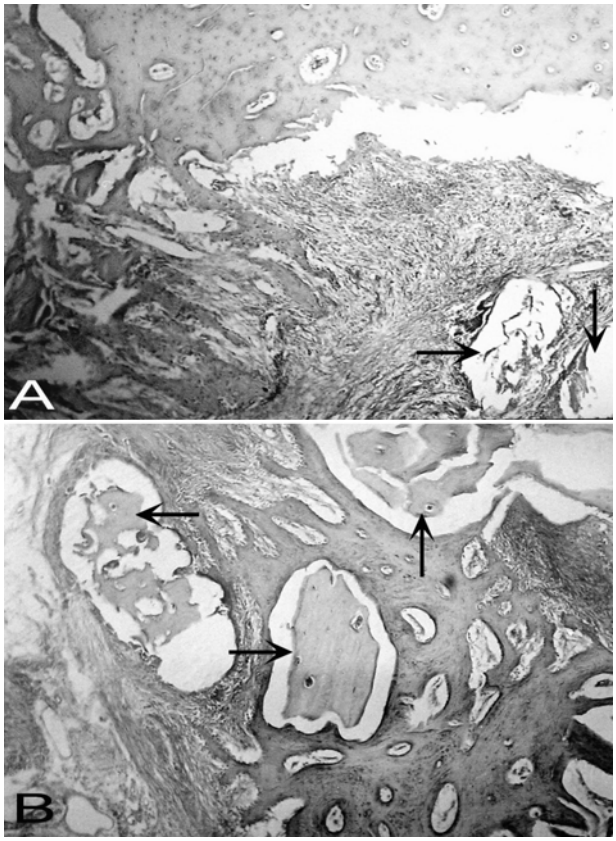

Figure 2. (A) Mineralized chip filled defect at 2 weeks with periosteal and endosteal reactions and empty spots (arrows) due to missing bone chips surrounded by fibrous tissue, van GiesonX50. (B) Presence of unresorbed bone chips (arrows) within the fibrous tissue and newly formed bone at 8 weeks, van Gieson X50.

Healing of Demineralized Bovine Bone Chip Filled Defect: At 2 weeks, periosteal reaction was similar to those of the mineralized chips. Embedment of demineralized chips within the fibrovascular tissue was observed along with trabecular bone formation at 4 weeks (Figure 3A). Cartilage formation was also present in some of the dogs at different times. Trabecular bone and fibrous tissue formation with increased activitiy at 6 (Figure 3B) and 8 weeks and residual demineralized chips at 8 and 12 (Figure 3C) weeks with bone marrow formation were observed.

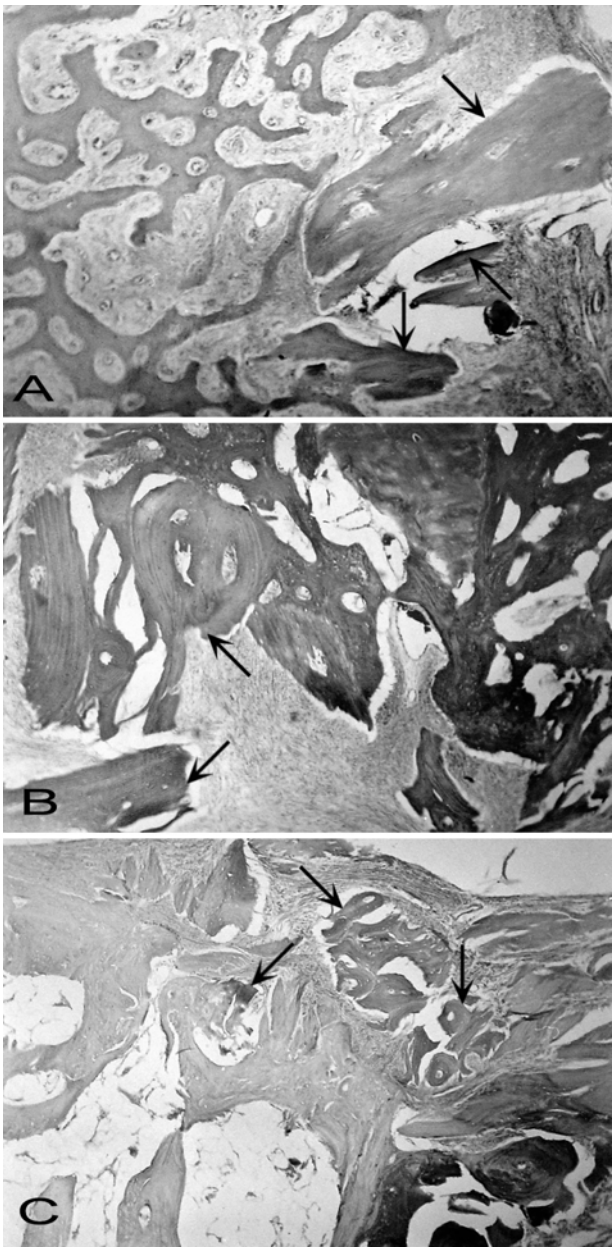

Figure 3. Appearance of trabecular bone and fibrous tissue formation around decalcified chips (arrows) in demineralized chip filled defect at 4 (A) van GiesonX50, and 6 weeks (B), Masson's trichrome. (C) Unresorbed demineralized chips (arrows) at 12 weeks, van Gieson X50.

Healing of Mineralized Bovine Bone Powder Filled Defect: Increased fibrous tissue surrounding the mineralized powder with periosteal and endosteal bone formation and high vascularization at 2 weeks (Figure 4A) and trabecular bone formation with persistence of mineralized powder at 4, 6 and 8 weeks (Figure 4B) within the fibrous tissue and remodeling along with formation of bone marrow at 12 weeks (Figure 4C) were the characteristic pictures.

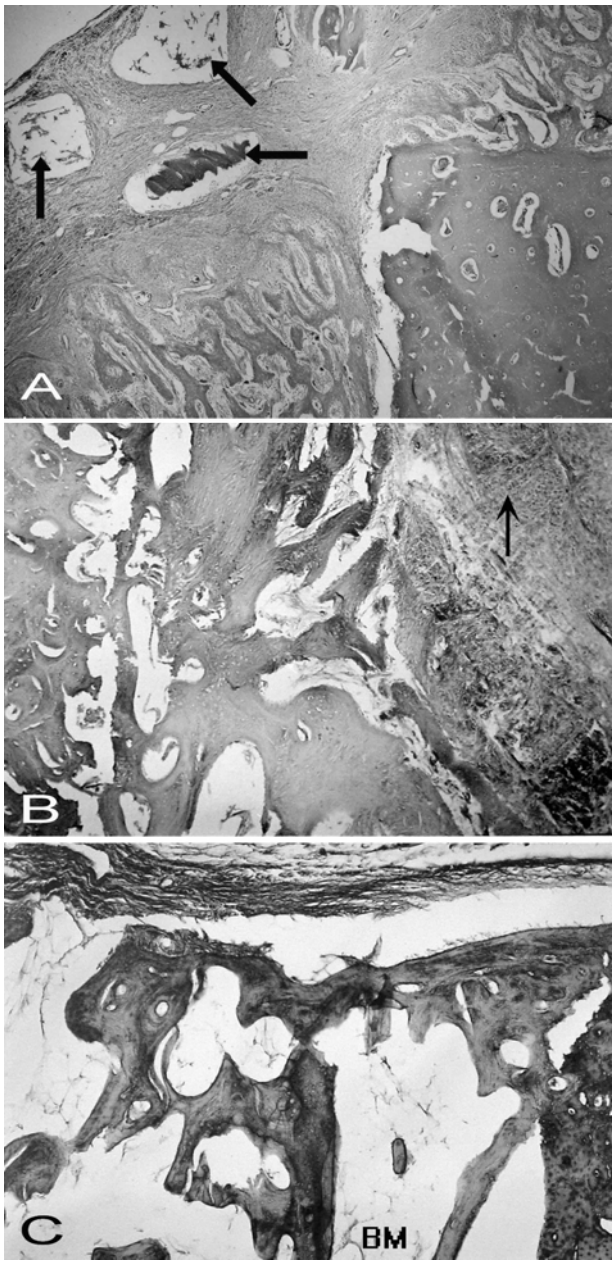

Figure 4. (A) Mineralized powder (arrows) in the fibrous tissue at 2 weeks showing the trabecular bone within the highly vascularized area, H.E X50. (B) Mineralized powder (arrow) is present within the fibrous tissue at 8 weeks, van Gieson X50. (C) Remodeling activity with bone marrow (BM) at 12 weeks, van Gieson X50.

Healing of Demineralized Bovine Bone Powder Filled Defect: Demineralized bone powder induced newly formed extraordinary amount of new trabecular bone within the defect which was covered with highly fibrovascular tissue with presence of demineralized powder at 2 (Figure 5A) and 4 weeks (Figure 5B).

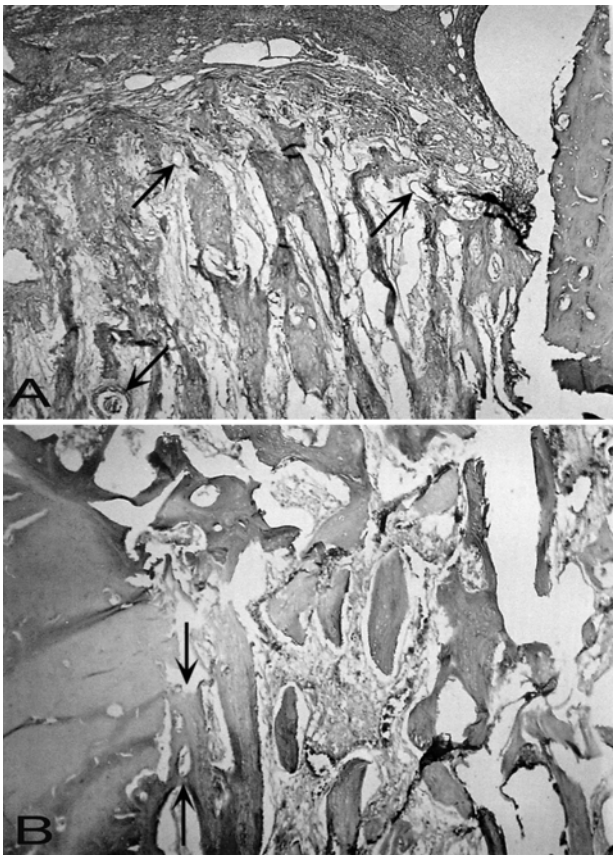

Figure 5. (A) Very nice appearance of field phenomenon in demineralized bone powder. Extensive bone formation in the highly vascularized (arrows) area at 2 weeks, van Gieson X50. (B) Extensive amount of new bone amalgamated (arrows) into the host cortex at 4 weeks, van Gieson X50.

The union of new trabecular bone to the host cortex started at 4 weeks (Figure 5B) and increased at 6 weeks (Figure 6A). Complete filling of the defect with numerous trabecular bone formation and union to the host cortex were remarkable without observation of bone powder at 6, 8 and 12 weeks. There was evidence of remodeling, formation of bone marrow with collagen formation over the defect at 12 weeks (Figure 6B). Cartilage formation was observed in some of the defects at different time intervals.

Radiographic findings provided no discernible differences regarding the graft healing. Therefore they were not included in the study.

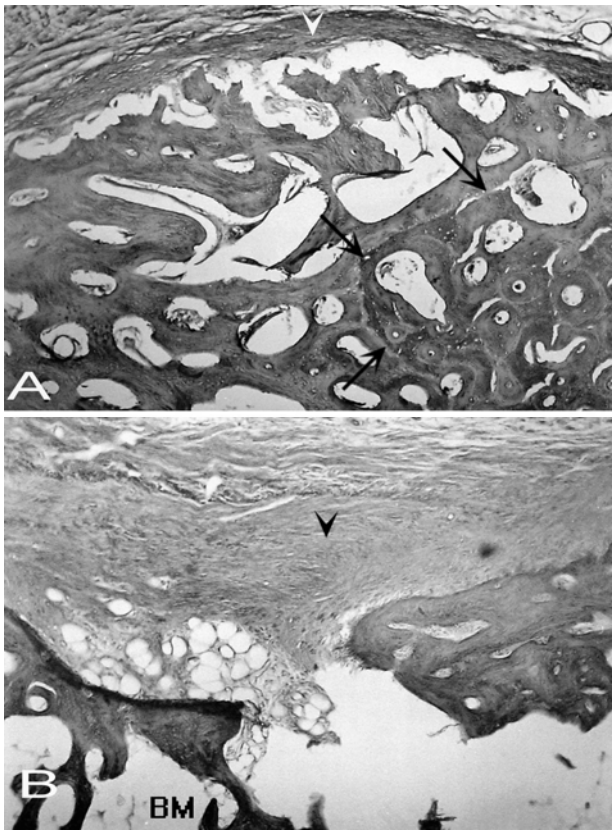

Figure 6. (A) New bone amalgamation into the host cortex increased (arrows) with collagen formation (arrow head) at 6 weeks, van Gieson X50. (B) Remodeling with collagen (arrow head) and bone marrow (BM) formation at 12 weeks, van Gieson X50 in demineralized bone powder.

Discussion

No foreign body reaction and postoperative complications were encountered in this study. Radiographic evaluation did not provide any means of useful information in the current study.

The healing of the unfilled defects as controls mainly through endosteal trabecular and less with periosteal bone formations because of superior immobilization of a circumscribed defect in the current study is in agreement with the reports of others (9, 19). While depression which is the weakened area predisposing to cyst formation in the centre of the unfilled defects has been reported (9), there was no depression in our study at the end of 12 week period as reported by others (19).

The remarkable histological difference of wide spread appearance of bone formation in demineralized xenogeneic bovine bone powder (Figure 5) compared with mineralized xenogeneic bovine bone powder, other forms and controls in our study, is compatible with the healing throughout the defect as a field phenomenon-induced bone formation in an orderly sequence of endochondral bone formation (16, 17, 20) utilizing demineralized allograft (18, 20, 21).

One must be cautious when talking about BMP because the results obtained through BMP may not be comparable to those of demineralized bone powder. The use of BMP extracted from demineralized bone and residuals itself loose and hampers the capability of induced bone formation (22, 23). It is our opinion that, this is because demineralized bone powder has naturally everything in it and that BMP except one, lost every factor/factors that would be responsible to play a part to induce bone in a very complex environment. The problem of using BMP is the lack of an ideal delivery system (24). Taking into account these in demineralized graft, it also shows the importance of a suitable delivery system as the whole bone itself other than other delivery systems for BMP. At the present time, there is no ideal carrier to substitute bone powder and therefore, the demineralized bone powder is superior to deliver BMP with other preserved factor/factors.

It was reported that unless supplemented with demineralized allogeneic bone matrix, autoclaved autoclaved autogeneic grafts were inferior (25, 26). If this is the case as seen clearly in our study, that autoclaving per se show the potential of demineralized xenogeneic bovine bone powder for osteoinductive properties because in the autoclaved group, bony formation was suppressed as reported due to the inactivation of BMP (27). In our study, mineral containing implants were sterilized in autoclave which removed the any capacity of osteoinduction serving only as a scaffold and bone formation took place as early as 2 weeks like controls with almost no beneficial difference as to use of mineralized implant which is in agreement with observation of no new bone formation with the use of mineralized allogeneic powder and cortical chips for the augmentation of rat mandibular ridge even at 24 weeks (20). These results show the effects of active periosteal and endosteal bone formation in radial defects in our study. Bony formation was more suppressed in chip compared to powder form in the autoclaved grafts. At 2 weeks, the appearance of mineralized powder filled defects (Figure 4A) resembled of demineralized powder filled defects (Figure 5A) like field phenomenon most likely due to the smallness of the powder other than demineralization. This resemblance disappeared at 4 weeks and thereafter. This explains the importance of size in addition to demineralization in the early phase as early as 2 weeks. In the following weeks, the advantage of demineralization process in the powder form has become remarkable over mineralized powder. No resemblance was observed in either type of filled defects compared to the controls. This shows the importance of necessity of filling of the defects at least for the scaffolding effect.

Because in mineralized implants bone occurs through creeping substitution, it needs clarification that is there something to prevent the same type of bone formation in demineralized implants apart from the field phenomenon? In our study, excluding the healing of controls, occurrence of bone formation even at 2 weeks through creeping substitution with periosteal and endosteal bone formation in mineralized powder (Figure

4A) just like field phenomenon, may be considered as an obvious indication of additive healing that also occurred with demineralized bone powder (Figure 5A). Therefore, considering above and our results, it would be reasonable and reliable to say that bone formation occurs through mainly field phenomenon, secondarily to a lesser extent with suppressed periosteal and endosteal bone formation in demineralized xenogeneic bovine bone powder as in our study.

Longer exposure time to the acidic bath for the reduction of larger chips destroys the bioactive peptides decreasing the effectiveness of the chips to induce bone formation (16, 21). Therefore it would be interesting to find out the critical size of the chips to obtain better results comparable to powder form in the future for demineralized implants. In addition to this, the formation of neovascularization is difficult with the use of large chips (16) which is in accordance with the findings in our study due to a barrier to vascularization (20).

Long-term resorption of demineralized allogeneic bone was reported (18). While mineralized xenogeneic bovine bone chips were resorbed by 8 weeks, demineralized bovine bone chips could still be identified at 12 weeks with no osteoclast observation around the chips in the current study. It was reported that induced osteogenesis had slow progression in demineralized allogeneic bone blocks as compared with allogeneic bone powder (16) which is in agreement with our results of demineralized bone chips undergoing less resorption with slowly proceeding osteogenesis in circumscribed radial defects. Presence of mineralized powder at the end of 12 weeks and absence of demineralized bone powder at the end of 4 week period along with formation of new bone much more in demineralized bone powder than mineralized bone powder at all time intervals may explain that demineralization promote new bone formation in powder form. In our study, resorption of demineralized bone powder at 4 weeks, in contrast to persistence of demineralized bone powder at 12 weeks as an onlay graft reported in a study (20) may reflect the effect of stress and compressional factors acting in the radius as being the long bone to decrease the resorption time in our study.

In our study, remodeling took place at 12 weeks in unfilled defect as well as mineralized chip and powder filled defects and demineralized powder filled defect. The advantage of demineralization process for the healing occurs as early as 2 weeks and it is lost or become equal to each other for those remodeled at 12 weeks. Therefore, regarding these and our results, if one prefers early bone formation in a particular defect should decide to use demineralized bovine bone powder due to superior rapid bony union compared to other forms.

Because induced bone is not species specific as was seen in this study and reported by others (17, 28), wide variety of xenogeneic bone implant as far as animal resources are concerned makes the donor availability more versatile in contrast to allogeneic implants. It was concluded that demineralized xenogeneic bovine bone powder was superior to demineralized and mineralized bovine bone chips and mineralized bovine bone powder. It is obvious that demineralization overcome the disadvantages of the xenogeneic mineralized bovine bone powder and chips. Taking into account the ethical problems and difficulties as far as owner consent is concerned for the use of allogeneic bone for example (dog vs dog and cat vs cat), demineralized xenogeneic bovine bone powder may be a good choice to be used as an alternative to other types of grafts as being more versatile as animal resources are concerned.

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