



The effect of equine-derived bone protein extract (Colloss-E) in the treatment of cavitory bone defects: an experimental study

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Objective: Bone protein extract (BPE) usually requires a carrier or a scaffold for implantation. We aimed to compare the effect of equine-derived BPE, an osteoinductive agent composed of a high amount of type-I collagen and other bone proteins (Colloss-E), with that of human demineralized bone matrix (DBM) for treating cavitory bone defects not requiring scaffold use.

Methods: Rabbit distal femoral condyle was used as a stable cavitory bone defect model. Bone defects of 6-mm diameter and 10–12-mm depth were created in the femoral condyles. Rabbits were assigned into the equine-derived BPE (BPE), human-derived DBM (DBM), and control (C) groups. Approximately 20 mg of BPE was implanted into the defect in the equine-derived BPE group (n=6), whereas 0.3 cc of DBM was implanted in the DBM group (n=6). Defects were left empty in the C group (n=6). The defect area was histologically examined after 6 weeks.

Results: There were no instances of macroscopic defect collapse or failure. Histopathological examination revealed that the BPE group had better scores (statistically significant) than both the other groups in terms of quality of union. The BPE group also had higher scores than the DBM group in terms of graft incorporation and new-bone formation.

Conclusion: The current study revealed results consistent with those of the previous studies concerning BPEs. Equine-derived BPE was found to be successful for treating cavitory bone defects not requiring scaffold use.

Keywords: Animal study; bone defect; bone protein extract; Colloss-E; distal femur.

Bone grafts and substitutes are frequently used for treating bone loss and conditions that require new-bone formation. The major problems encountered with allografts

are limited donor sources and high cost. Therefore, the search for alternative bone sources of different species (xenografts) became popular.

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Bone protein extract (BPE) derived from bovine bone is a biomaterial that consists of purified triple helix type-I collagen and other noncollagenous bone proteins. It was demonstrated that collagen content stimulates chondrocyte differentiation and mineral deposition throughout collagen fibers via the action of growth factors (transforming growth factor [TGF]- β , insulin-like growth factor [IGF]) and cytokines (fibronectin, osteoclastin). BPE is different from autologous bone graft, and it has osteoinductive characteristics via the stimulation stimulating osteogenesis without the osteoclastic process.^[1-3] Moreover, it was reported that collagen shows chemotactic activity on mesenchymal cells and enhances cell adhesion by forming complexes with growth factors.^[4,5] Because of its osteoinductive and osteoconductive characteristics, successful outcomes have been reported with bovine-derived BPE because of its low immunogenicity and minimal disease transmission risk.^[6-8] Although bovine bone is readily available, spongiform encephalopathy, a transmissible disease, is a possible complication. To date, there is no case of spongiform encephalopathy reported that was shown to be transmitted by prions from equine species. Therefore, equine bone can be considered a safe alternative to bovine bone with regard to disease transmission risk.^[9,10]

Equine-derived BPE (E-BPE), commercially available as Colloss-E (OSSACUR AG, Oberstenfeld, Germany), is a cotton wool-like biomaterial manufactured from horse bone. E-BPE was shown by the enzyme-linked immunosorbent assay method that E-BPE contains 55 ± 11 mg/g of TGF- β 1, 2.6 ± 0.2 mg/g of bone morphogenetic protein (BMP)-2, 3.8 ± 2.7 mg/g of BMP-7, and 2.9 ± 0.8 mg/g of IGF-1.^[11] E-BPE has been reported to show osteoinductive characteristics by stimulating bone formation in the ectopic tissue, similar to bovine-derived BPE.^[12,13] However, it has unfavorable aspects, including the lack of structural support and precipitation on exposure to body fluids. Therefore, a carrier or scaffold is usually required during utilization. In a rat model of ectopic bone formation, in which a mineralized collagen scaffold was used, it was reported that a massive inflammatory process was observed with E-BPE compared with the results obtained by titanium cages. In addition, no new-bone formation was reported.^[12,14] Therefore, carrier substances may alter the biological environment and the biological behavior of the bone graft substitute.

We aimed to investigate efficacy of E-BPE (Colloss-E) in a stable cavitory bone defect model. The effect of E-BPE was compared with demineralized bone matrix (DBM), which is also an osteoinductive substance. The

goal of using a cavitory bone model is to demonstrate the biological behavior of E-BPE without the possible favorable or unfavorable impacts of mediator or scaffolds on the osteogenic process.

Materials and methods

The study was carried out with the approval and supervision of Gazi University, Animal Experiments Local Ethics Committee, Ankara, Turkey, numbered B.30.2nd DAY.0.EU.00.00/51-8294.

In the present study, we used a distal femur cavitory defect model in rabbits. Rabbit bone offers adequate bone size and a haversian system similar to human bone, precluding the need for fixation and decreasing the likelihood of spontaneous union. E-BPE was compared with human-derived DBM, a biomaterial being used mainly for its osteoinductive properties, to investigate their impact on the healing of cavitory bone defects. Although autogenous cancellous bone grafting is the gold standard, osteoconductive behavior and cellular content of autografts may complicate the interpretation of the results. The study included 3 groups; i.e., E-BPE, DBM, and control (C) groups. A total of 18 male, mature, New Zealand rabbits aged 10–12 months and weighing 800–1000 g were used. Six rabbits were included in each group.

Surgical procedures were performed and follow-up was conducted at Gazi University, Laboratory for Experimental Animals. General anesthesia was provided with intramuscular ketamine and xylazine. After providing adequate anesthesia and analgesia, followed by appropriate surgical preparation and draping, a 1.5-cm incision was made on the lateral aspect of the left lateral femoral condyles. After incising the skin, subdermal tissue, and fascia, the lateral femoral condyle was exposed through the lateral aspect of the quadriceps muscle. A defect of 6-mm diameter and 10–12-mm depth was created (volume: mean 0.3 cc) in a lateral to medial direction using a cannulated drill with stopper. The drill center was located 6 mm from the anterior joint surface and 8 mm proximal to the distal joint surface, leaving an intact bone of 3 mm anteriorly and 5 mm distally (Figures 1 and 2a). Integrity of the cavity surface and medial wall was checked by palpating with a 4-mm arthroscopic probe. Thereafter, 20 mg of Colloss-E, which is the maximum amount to be impacted, was implanted into the defect in the E-BPE group. On the other hand, 0.3 cc of gel DBM (MTF, Edison, NJ) was injected into the defect in the DBM group (Figures 2b and 2c). The defect was left empty in the control group. The periosteum and fascia over the defect were sutured. None of the rabbits died or developed complications either during or after the

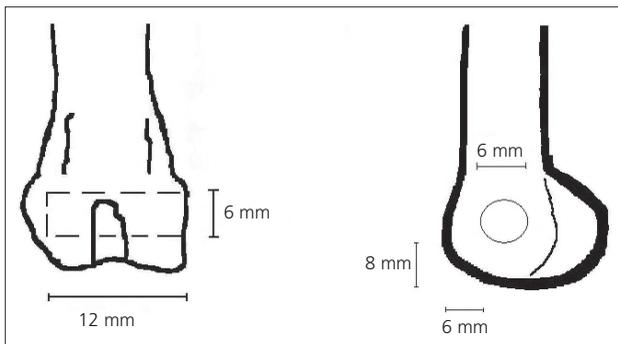


Fig. 1. Anteroposterior and lateral images of the created defect.

surgery. It was observed that the rabbits continued their routine feeding the same day and were freely mobilized after the first day. To avoid spontaneous ossification, the rabbits were sacrificed at the end of the 6-week observation period and femora were removed by hip and knee disarticulation. Macroscopic and histological examination was performed after the removal of soft tissues.

Macroscopically, drill-hole surface was covered by fibrous tissue in all specimens. Fracture or collapse was not seen in any specimen. Histological examination included subjective assessment by means of routine microscopy with the use of a histomorphometric scale^[15] (Table 1). Because the sections for histological examination were obtained from the femoral condyle, in the sagittal plane and only from the middle of the cylindrical cavity (5–6 mm deep from the surface), cortex development and cortical remodeling were not taken into consideration in scoring. Data on the quality of the union, graft incorporation, and new-bone formation were evaluated. The data obtained were statistically analyzed by Kruskal–Wallis and Mann–Whitney U tests, using the software SPSS 12.0 for Windows.

Results

Histologically, the defect area was completely differentiated into bone tissue in the E-BPE group. Additionally, complete cellular maturity with mature osteocytes were noted with mature collagen fiber arrangement and mineralization-associated staining characteristics (Figure 3a). High magnification revealed bone lamellae with circular arrangement in patches, but it was not organized yet and osteocytes were relatively bulgy and elliptical compared with those in the mature bony tissue. This finding was interpreted as newly bone tissue formed by primary bone healing.

In the DBM group, it was observed that DBM implanted into the wound site differentiated into cartilage tissue, which is a bone precursor, in many areas and

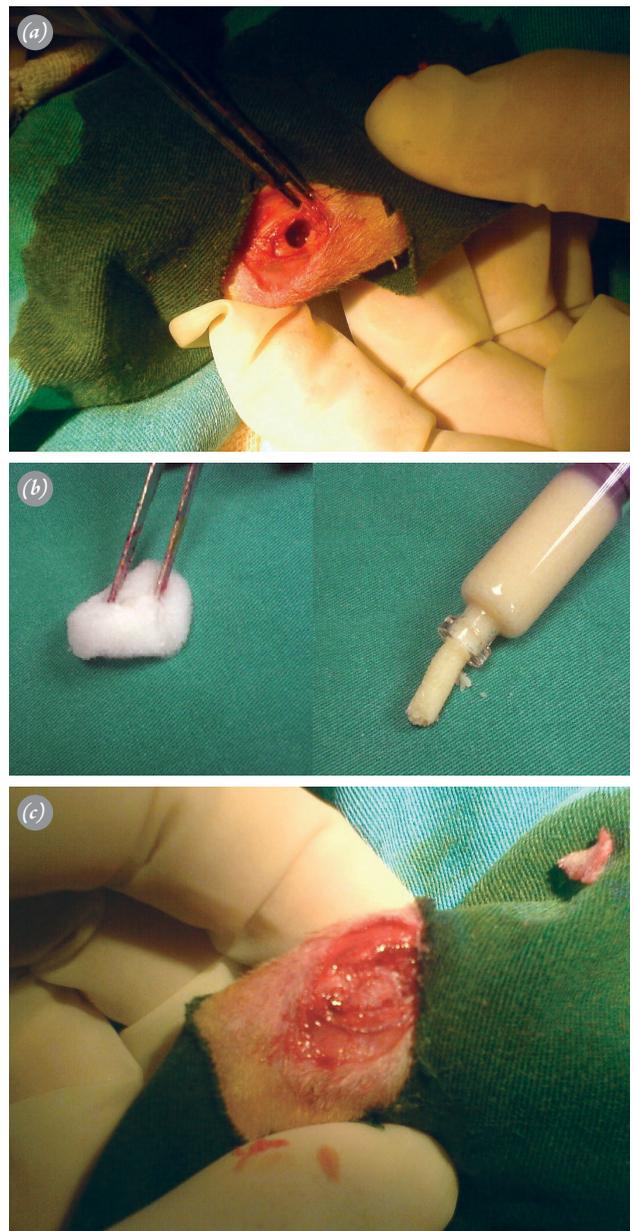


Fig. 2. (a) Defect created by approaching the distal femur through the lateral aspect of the knee. (b) Graft materials: Colloss-E (left) and human demineralized bone matrix (right). (c) Filled defect. [Color figures can be viewed in the online issue, which is available at www.aott.org.tr]

mimicked the epiphyseal tissue. It was observed that cartilage tissue differentiated into bone tissue in the majority of the defect, and DBM continued to differentiate into bone tissue in some areas, and mature bone tissue was not formed yet in these areas (Figure 3b).

In the control group, it was observed that the wound site was completely filled with fibrous tissue (fibrous connective tissue rich in collagen fibers) (Figure 3c). High-magnification examinations demonstrated fibroblasts

Table 1. Histological Assessment Scale.^[15]

Union quality	
No sign of union	0
Fibrous union	1
Fibrocartilage or cartilage union	2
Mineralized cartilage and bone union	3
Complete union	4
Cortex development and remodeling	
No cortex formation	0
Cortical density at the outer margins	1
Remarkable outer cortex and medulla formation	2
Cortex has formed but there is no bridging	3
Complete bridging of defect and cortex formation	4
Graft persists, no incorporation, no new bone	0
Bone graft incorporation and new-bone formation	
Graft persists, no incorporation, no new bone	0
Graft persists, incorporation with a small amount of new bone	1
Graft persists, moderate amount of new bone and incorporation	2
Graft persists, there is new bone and early remodeling	3
Graft is decreased, graft incorporation is good, remarkable new bone	4
Amount of graft is quite decreased, bone incorporation is good, new bone	5
Complete bone incorporation, advanced remodeling	6

separated from the bone tissue, characterized by collagen fibers in all directions and in fusiform shape, and clusters were not formed yet. Notably, neighboring bone tissue appeared as regular bone lamellae and osteocytes in the lacunae that had been aligned among the lamellae. Quality of union scores are summarized in Table 2.

There was significant difference between the E-BPE and DBM groups in terms of quality of union ($p=0.002$), and the difference between the E-BPE group control groups was also significant ($p=0.002$). In addition, a significant difference was observed between the DBM and control groups ($p=0.007$) (Figure 4a).

Bone-graft incorporation scores were compared be-

tween the E-BPE and DBM groups. The average was 5.33 (standard deviation [SD]=0.516) in the E-BPE group and 4.0 (SD=0.632) in the DBM group. Accordingly, both groups obtained high values, especially the E-BPE group, in terms of incorporation ($p=0.002$) (Figure 4b).

Discussion

The difficulty encountered in obtaining raw materials is the major problems in manufacturing grafts and substitutes. Another problem is the failure to decrease the risk of contamination to zero, despite the advanced technologies implemented during the preparation period. The aim of development of E-BPE is to obtain a cheap and rela-

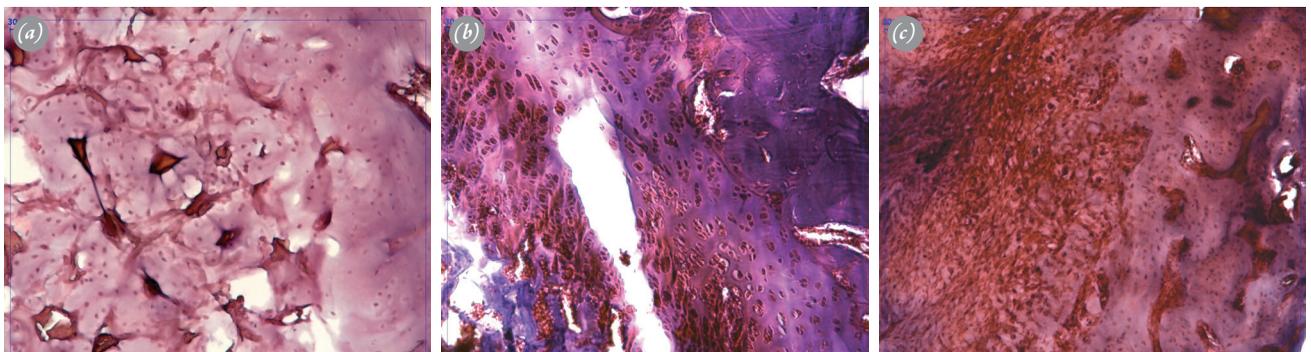


Fig. 3. (a) Bone protein extract. Lamellar formation that shows patchy circular array in the implantation area. (b) Demineralized bone matrix. Bone precursor cartilage and the area that cartilage differentiates into bone tissue. (c) Control group. Irregular collagen fibers, fibroblasts, regular bone lamellae, and osteocytes among lamellae. [Color figures can be viewed in the online issue, which is available at www.aott.org.tr]

Table 2. Scores of quality of union.

Group	n	Score (SD)
E-BPE	6	4.0 (± 0)
DBM	6	2.66 (± 0.516)
Control	6	1.33 (± 0.516)

N: Number; SD: Standard deviation; E-BPE: Equine-derived bone protein extract; DBM: Demineralized bone matrix.

tively readily available biomaterial that shows good osteoinductive behavior without the need for human bone and minimize the risk of transmission of infectious disease.

Overall, EBP-E shows an osteoinductive effect because of the growth factors it contains. It was demonstrated that E-BPE leads to osteoblastic differentiation in a rat bone-marrow-cell culture early as well as increased activity of alkaline phosphatase. It also induces proliferation and matrix calcification by means of TGF- β 1, BMP-7, BMP-2, and IGF-1 and possibly vascular endothelial growth factor that it contains.^[11,16] Another study investigated the effects of E-BPE, BMP-2, and TGF- β on differentiation and proliferation in cell cultures and found that BMP-2 more effectively enhanced the proliferation, whereas, E-BPE and TGF- β (which is the main growth factor of E-BPE) showed similar efficacy and enhanced the differentiation in a subcutaneous ectopic bone formation model in rats.^[17]

In the current study the effect of E-BPE for treating a stable cavitory bone defect model of the distal femur of rabbit was evaluated without the need for a specific carrier to retain the biomaterial in the defect. Previous experimental studies investigating new-bone formation

with the use of E-BPE revealed similar radiological and histological results both with the use of autografts and allografts; however, they were used in different amounts and doses and with a different scaffold (Table 3). In a swine model of interbody fusion, at the end of 3 months after implantation, similar radiological and histological results have been obtained with E-BPE, implanted at a dose of approximately 100 mg/cc using a titanium cage, to those obtained with an autograft.^[18] In another study, the use of E-BPE in combination with ceramic filler provided new-bone formation a rate of 20 mg/cc by preventing fibrous tissue formation.^[19] In a swine model of interbody fusion using a polyetheretherketone (PEEK) cage, E-BPE with a dose of approximately 35 mg/cc was compared with BMP-2 and an autograft and similar metabolic activity was observed at the end of 8 weeks. The study also reported significant endochondral ossification with E-BPE.^[20] However, in another study, different trabecular orientations were demonstrated in a finite-element model comparing BMP, E-BPE, and an autograft. In the study, the maturity of bone tissue with BMP was more than that with an autograft and E-BPE; treatment with both autograft and E-BPE showed similar levels of mature bone tissue.^[21] On the other hand, another study demonstrated central bone formation with E-BPE used at a dose of 35 mg/cc in a mandibular defect using a goat model, but it was not considered more effective than the control group. This result was interpreted as excessive edema caused by growth factors released by E-BPE and a biomembrane sutured over the defect preventing cellular migration.^[22] In a study conducted using a sheep proximal humerus model, carboxymethyl cellu-

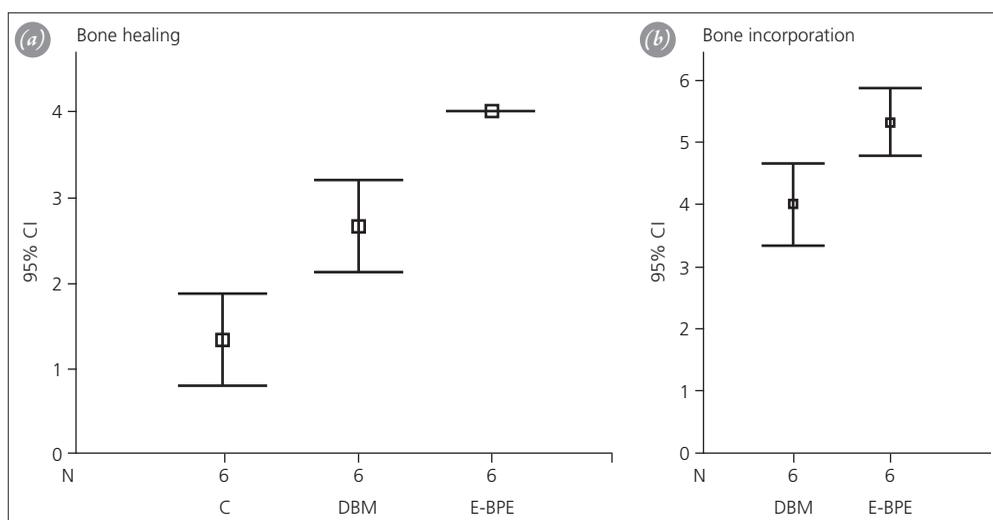


Fig. 4. (a) Significantly high scores of bone healing with equine-derived bone protein extract (confidence interval = 95%). (b) Significantly high graft incorporation scores with equine-derived bone protein extract (confidence interval = 95%).

Table 3. Previous reports comparing the effect of equine-derived bone protein extract (E-BPE) and other bone grafts or substitutes.

Author (year)	Model (bone)- scaffold	Concentration	Result
Li et al. (2007) ^[18]	Swine (interbody fusion) titanium cage	100 mg/cc	Equivalent to autograft
Baas et al. (2008) ^[19]	Dog (bone defect) titanium implant + β TCP granule	20 mg/cc	Better than allograft
Foldager et al. (2008) ^[20]	Swine (interbody fusion) PEEK cage	35 mg/cc (approx.)	Equivalent to autograft
Foldager et al. (2009) ^[21]	Swine (interbody fusion) PEEK cage	35 mg/cc (approx.)	Equivalent to autograft (inferior to BMP)
Nienhuijs et al. (2010) ^[22]	Goat (mandibular defect) plain BPE with no scaffold and BPE + β TCP granule	35 mg/cc	E-BPE attenuated effect of β TCP granule. Control defect healed with more bone.
Jensen et al. (2010) ^[23]	Sheep (proximal humerus defect) BPE + carboxymethyl cellulose collagen + autologous blood with no scaffold	20 mg/cc	A viable osteogenic bone filler.
Ding et al. (2012) ^[24]	Sheep (distal femur defect) Hydroxyapatite-b-tricalciumphosphate (HA/b-TCP) was reinforced with poly(D,L)-lactic acid (PDLLA) + BPE with a titanium implant	100 mg/cc	Equivalent to sheep allograft.
Baas et al. (2012) ^[25]	Dog (proximal tibia defect) Titanium implant fixation with and without BPE	20 mg/cc	Low fibrous encapsulation and better pull out resistance with BPE treated implant.

lose was used as the scaffold and a successful outcome was obtained with a dose of 20 mg/cc.^[23] In a previous study in which critical size cortical defect repair was performed in a sheep femur, similar mechanical robustness and new-bone formation was obtained with 100 mg/cc E-BPE implanted with a hydroxyapatite- β tricalcium phosphate-poly-D-lactic acid implant in comparison with allogenic sheep bone.^[24] Another study investigated bone osteointegration with a hydroxyapatite-coated implant, and high osteointegration and low fibrous encapsulation was observed with the use of 20 mg/cc E-BPE.^[25] In the present study, a concentration of approximately 67 mg/cc E-BPE was administered into a stable cavitory bone defect without using any scaffold. The periosteum and fascia over the defect area were sutured to prevent dissolution and expansion after contact with body fluids. Histological evaluation was performed at approximately 6–16 weeks after implantation in previous studies. Therefore, the time of sacrifice of rabbits and histological data in the current study are comparable with those of previous reports. In the present study, higher bone formation scores were obtained in the DBE group than in the DBM (which has similar osteoinductive activity) group and the control group. Although previous reports have shown the effectiveness of DBE within a wide range of doses and concentrations (20–100 mg/cc), a titration study to assess the optimal concentration in a clinical setting is needed. In the current study a marked difference among the study groups was found. The difference can be explained by the higher growth factor content of E-BPE than that of DBM, the possible inhibition of osteogenesis by other matrix proteins in DBM, and a possible foreign-body reaction to DBM.

In previous studies, many carrier implants (titanium meshes, cages, and poly-L-lactide [PLLA] implants), scaffolds (β TCP, carboxymethyl cellulose-collagen), synthetic patches, and marking devices (K-Wires) were utilized. These devices may inhibit or delay osteogenesis because of foreign-body and inflammatory reactions, which were previously reported as inhibitors of osteogenesis induced by E-BPE when used with mineralized collagen.^[14] In the present study the defect was closed directly by using the fascia and periosteum, and anatomical landmarks were used to assign the defect area for histological evaluation; therefore, possible confounding effects of the devices were avoided.

Another potent osteoinductive biomaterial is recombinant human BMP-2 (rhBMP-2). However, routine use of rhBMP-2 is debatable because of reported potential complications.^[26] In previous studies, rhBMP-2 resulted in higher osteogenic function radiologically and histologically.^[17,20,21] Because of its high cost and complications, rhBMP-2 was not within the scope of the current study.

The most striking finding of the present study was primary bone healing observed with the use of E-BPE, new-bone formation in the form of healing with endochondral ossification in the DBM group, and findings that could be considered fibrous union in the control group. These findings are different from those reported by other previous studies;^[17,18] however, the different results may be because of the studies being performed at different doses and in different types of subjects using different models. Mature central bone formation unrelated from edges was reported by Nienhuijs et al., which

supports the current findings^[22] The other significant finding in the current study was more mature bone tissue obtained with the use of E-BPE than with DBM. This finding is consistent with previous studies that reported increased cell differentiation and increased bone turnover with the use of E-BPE resulting in a shorter time required for union.^[19]

A limitation of the present study is that the histological data was not verified by microtomography and biomechanical studies. Previous studies evaluating new bone produced radiologically mainly focused on trabeculation and the percentage of new-bone formation. Because most of the histological results of the studies were concordant with the radiological results, we believe the current results still have a value in terms of quality of new bone produced. Further clinical studies are warranted for wide range use regarding osteoinduction in humans.

Better bone formation was observed with the use of E-BPE (Colloss-E) than in human-derived DBM in a cavitory bone defect model in rabbits. Therefore, E-BPE can be used as an alternative bone graft for treating cavitory defects not requiring scaffold use. Although available data and literature are promising, further descriptive studies regarding dosage and concentration and safety studies, as well as *in vitro* (human-cell culture) and *in vivo* studies on humans are necessary before routine use in a clinical setting.

Conflicts of Interest: No conflicts declared.

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