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Bioglass as a carrier for reindeer bone protein extract in the healing of rat femur defect

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Abstract Bioactive glasses have been developed as scaffolds for bone tissue engineering but combination with reindeer bone protein extract has not been evaluated. We investigated the effects of bone protein extract implants (5–40 mg dosages) with bioglass (BG) carrier on the healing of rat femur defects. Bioglass implants and untreated defects served as controls. All doses of extract increased bone formation compared with the control groups, and bone union was enhanced with doses of 10 mg or more. In comparison with untreated defect, mean cross-sectional bone area at the defect site was greater when implants with BG + 15 mg of extract or bioglass alone were used, bone density at the defect site was higher in all bioglass groups with and without bone extract, and the BG + 15 mg extract dosage marginally increased bone torsional stiffness in mechanical testing. Bioglass performed well as a carrier candidate for reindeer bone protein extract.

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1 Introduction

Bone morphogenetic proteins (BMPs) are important growth factors in bone and cartilage regeneration [1, 2]. Despite modern surgical techniques, segmental long bone defects are often difficult to manage. Thus, the efficacy of bone protein extracts (containing bone morphogenetic proteins [BMPs]) with carrier materials in healing of long bone defects have been studied experimentally using segmental long bone defects in animal models with close similarity to clinical situations.

An optimal carrier matrix should fulfil several criteria. First, the matrix should be biocompatible and protect bone proteins from non-specific lysis. The ideal matrix should also be bioabsorbant, malleable, and withstand sterilization [3–6]. The carrier matrix should bond to the host bone without the formation of scar tissue, and resorb at the same rate as the bone is regenerated [7, 8]. Inorganic matrices have the advantage of being structurally strong, immunologically inert, osteoconductive, and biodegradable to various degrees [3, 4]. Bioactive glasses are inorganic materials that have been used in granular form to fill bone defects. They meet many criteria of optimal carrier matrices including biocompatibility and osteoconductivity, and are biodegradable. Thus, they are called surface-bioactive ceramics [9–13]. Bioactive glasses have been used as carriers of BMP, especially recombinant human BMP (rhBMP) [14–17]. In some studies, they have also been used as a carrier of native bone matrix [18, 19].

In the present study, we investigated the effect of reindeer bone protein extract combined with bioglass carrier on the healing of critical-sized bone defects in rat femurs. Our hypothesis was that bioglass could act as a carrier for reindeer bone protein extract and that this combination would heal the bone trauma in 8 weeks. The aim was to

evaluate the healing effects of different doses of reindeer bone protein extract combined with bioglass carrier evaluated using native radiology, quantitative peripheral computer tomography (pQCT), and mechanical torsion tests.

2 Materials and methods

2.1 Bone protein extract

Native reindeer (Rangifer tarandus) protein extract was prepared from diaphyseal bone of the reindeer. Cortical bones from each animal were chilled immediately after death. The epiphyseal ends, bone marrow, and periosteum were mechanically removed and, after freezing in liquid nitrogen, the cleaned cortical bones were ground to a particle size of 1.0 mm^3. The pulverized bone was demineralized in 0.6 M HCl and extracted in 4 M guanidine hydrochloride (GuHCl) at <9°C. The GuHCl-extracted solution was filtered through a tangential flow system and concentrated. The concentrated solution was dialyzed against deionised water and the water-insoluble material was collected. After re-dissolving in 4 M GuHCl solution, the water-insoluble material was dialyzed against 0.25 M citrate buffer, pH 3.1. The citrate-buffer-insoluble material was washed with deionised water and deep-frozen [20].

2.2 Reconstitution of implants

A dose of 5, 10, 15, or 40 mg of reindeer bone extract (BBS Ltd, Finland) was added to carboxymethyl cellulose (CMC, Sigma-Aldrich) to obtain a 3.2% (water/weight, w/w) gel. Bioglass granules (S53P4, size 500–800 μm, Vivoxid, Turku, Finland) were combined with extract-CMC-gel, and the mixture was shaped into a rod (diameter 5 mm) and lyophilized. Before drying, one 8 mm implant included 40% w/w of CMC-gel with a propriety amount of bone extract, and 60% (w/w) bioglass granules. The amount of CMC in the dried product was 2% (w/w). The doses of bone extract were chosen according to our previous studies [21–26]. Control implants were constructed in an identical fashion, but contained only bioglass carrier.

2.3 Animals and study groups

Fifty 2.5-month-old male Sprague–Dawley rats were used. Each animal was randomised to one of four groups for unilateral treatment with an implant: (1) bioglass (BG) + 5 mg bone protein extract (BG + 5 mg extract group); (2) bioglass + 10 mg bone protein extract (BG + 10 mg extract group); (3) bioglass + 15 mg bone protein extract (BG + 15 mg extract group); (4) bioglass + 40 mg bone protein extract (BG + 40 mg extract group); (5) bioglass alone (bioglass (BG) group); and (6) no implant (untreated group). Ten rats died within 24 h of the operation of no obvious cause. Thus, 40 of 50 rats survived to the end of the study (9 rats in the 40 mg extract group, and 6–7 rats in each of the other groups).

The study protocol was approved by the institutional animal experiment and ethical committee.

2.4 Surgical procedure

Surgery was performed under general anaesthesia with a blend of fentanylcitrate 80 μg/kg–fluanisone 2.5 mg/kg (Hypnorm®, Janssen Pharmaceutica, Inc., Beerse, Belgium) and midazolam 1.25 mg/kg (Dormicum®, Roche, Basel, Switzerland). Preoperatively, the animals were administered cefuroksim 20 mg/kg (Zinacef®, GlaxoSmithKline Manufacturing S.p.A., Verona, Italy) subcutaneously. A transverse skin incision was made over the posterior aspect of the thigh after shaving the hair around the left hind limb. The muscles were elevated circumferentially from the femoral diaphysis. A 25 mm × 4 mm × 2.5 mm polyacetyl plaster plate (POM, Vink Finland Ltd) was placed on the surface of the femur. The plate was temporarily secured in place with a stainless steel holder, which was exclusively designed and manufactured for this operation (Technical Services Unit, University of Oulu). The plate was fixed using 0.8-mm threaded Kirschner-wires, which have a 5-mm long threaded distal end (Synthes Oy, Helsinki, Finland). The canal for the threaded K-wire was predrilled through the plate and through both cortices of the femur using a 0.7 mm drill (Dremel 400, The Netherlands). The threaded K-wire was bent so as to easily screw the threaded end of the wire through the plate and into the bone. The correct depth for inserting the wire was determined by carefully dissecting the other side of the bone with a probe to confirm that the wire was penetrating the distal cortex of the bone. A total of 6 threaded K-wires were used for each plate, three on each sides of the bone defect, each screwed through two cortices. The excess wire was removed to the level of the plate using side-cutting pliers.

An 8-mm diaphyseal critical-sized defect was created using a stainless steel mini bur. After a thorough wash with saline, the implant was applied to the defect, or the defect was left empty for controls. The muscles and skin were closed in two layers using absorbable 4.0 sutures (Dexon®, Covidien, Mansfield, MA, USA). The pain medication after the operation consisted of buprenorfin (Temgesic®, Reckitt & Colman Pharmaceuticals, Inc, Richmond, England) at 0.01–0.05 mg/kg administered subcutaneously. In case of respiratory problems during anaesthesia, animals were treated with 1 mg of furosemid (Furesis® Orion, Espoo, Finland) subcutaneously. Eye gel (Viscotears®, Novartis Healthcare, Kobenhavn, Denmark) was applied to avoid

eye dehydration during anaesthesia. The animals were allowed full activity in their cages postoperatively.

The rats were killed in a carbon dioxide (CO_2) chamber after 8 weeks (untreated, bioglass alone, or 5, 10, and 15-mg extract groups), or after 10 weeks (40 mg extract group). The left leg of each rat was dissected from the body, wrapped in a saline wetted serviette, and frozen at −20°C until biomechanical analysis.

2.5 Radiographic evaluation of bone formation

Radiographs of the left femur (26 kV, 9.00 mAs, 0.09 s/exp, Mamex dc® ami, Orion Ltd., Soredex) were taken 3, 6, and 8 weeks postoperatively, and also 10 weeks postoperatively in the BG + 40 mg extract group. The radiographs were taken under neuroleptic analgesia (Hypnorm-Dormicum, 0.15–0.3 ml/100 g). Osiris 4.19 (Digital Imaging Unit, Geneva) software was used in the analysis of the radiographs. The percentage of orthopic new bone formation (BF) and the development of a bone union (BU) or non-union was estimated as described previously [26] according to the scoring system developed by Sciadini et al. [27].

2.6 Computed tomography

After the soft tissue on the frozen legs thawed it was removed from the bone and all left femurs were scanned using a pQCT device (Stratec XCT 960A, 5.21 software version, Norland Stratec Medizintechnik GmbH, Birkenfeld, Germany). A voxel size of 0.148 mm × 0.148 mm × 1 mm was used. One cross-sectional slice from the middle of the defect in each sample was scanned in two directions: first with a fixation system above the bone, then turned 90° axially. Average values of the two scans were used in the statistical analysis. Cross-sectional bone areas (mm^2) and bone densities (mg/cm^3) from the scanned slices of the samples were recorded using the pQCT software with a threshold of 169 mg/cm^3 to distinguish the bone from the surrounding soft tissue, and a threshold of 464 mg/cm^3 for the inner surface of the bone.

2.7 Mechanical tests

After pQCT imaging, the fixation system was removed from the samples. The bone ends were embedded into the moulds of the sleeves with dental stone (GC Fujirock, Improved Dental Stone, G-C Dental Industrial Corp., Tokyo, Japan). The torsional shaft was adjusted to 2 cm. After the cast hardened, the samples were placed in the torque machine [28] and torsionally loaded at a constant angular speed of 6°/s until failure. Maximum breaking load (Nm) and torsional stiffness (Nm/°) were recorded [29]. Mechanically unstable bones were not tested, and their values were considered to be zero in the statistical analysis.

2.8 Statistics

Statistical analysis was performed using SPSS for Windows ver. 15.0 statistical package (SPSS Inc., Chicago, IL, USA). The non-parametric Kruskall–Wallis test was used to evaluate the statistical differences between the groups. The Mann–Whitney test was used for pairwise comparisons between the treatment groups and the control groups (BG and untreated). The Benjamini–Hochberg procedure was used to correct P values for multiple comparisons [30]. Values of $P < 0.05$ were considered statistically significant. Results of pQCT are reported as the mean ± standard deviation, and results of mechanical tests are given as medians and quartiles. Results of the radiographic assessment are given as cross tabulations.

3 Results

3.1 Radiographs

All doses of extracts with bioglass increased bone formation (BF) observed on radiographs compared with the bioglass alone and untreated groups ($P < 0.002$ to $P < 0.03$). With extract doses of 10–40 mg + bioglass, bone formation was superior as soon as 3 weeks postoperatively when compared with the plain bioglass and untreated groups ($P < 0.03$). Details of the BF data evaluated by radiographical analysis 3–8 weeks after the surgery are presented in Table 1. Typical radiographs showing new bone formation in different study groups after 8 weeks are shown in Fig. 1.

Bone union (BU) at 6 weeks was better in the groups receiving bioglass + 10–40 mg of the extract compared with the plain bioglass and untreated groups ($P < 0.009$ to $P < 0.03$, respectively). Detailed BU data of are presented in Table 2.

3.2 pQCT

pQCT showed cross-sectional bone areas to be greater in the BG + 15 mg of the extract group and in the bioglass alone group than in the untreated group ($P < 0.03$) (Table 3). Bone density at the defect site was greater in all groups that received bone protein extract with bioglass and the bioglass alone group than in the untreated group ($P < 0.004$). There were no significant difference in bone density between the plain bioglass group and any of the extract groups (Table 3).

Table 1 Bone formation (BF) in the defect area of rat femur 3–8 weeks after the operation evaluated by radiographical analysis using the scoring system presented in the study of Sciadini et al. [27]

Group	BF score																	
	N	3 weeks					P	6 weeks					P	8 weeks					P
		0	1	2	3	4		0	1	2	3	4		0	1	2	3	4	
Untreated	6	6	0	0	0	0		6	0	0	0	0		3	2	1	0	0	
Bioglass (BG)	6	6	0	0	0	0		6	0	0	0	0		5	1	0	0	0	
BG + 5 mg extract	6	6	0	0	0	0		1	4	1	0	0	[b,e]	0	5	1	0	0	
BG + 10 mg extract	6	2	4	0	0	0	[a,d]	0	5	1	0	0	[c,f]	0	0	5	1	0	[b,f]
BG + 15 mg extract	7	0	7	0	0	0	[c,f]	0	1	5	1	0	[c,f]	0	0	2	5	1	[b,f]
BG + 40 mg extract	9	3	3	3	0	0	[a,d]	0	3	4	2	0	[c,f]	0	0	4	3	2	[c,f]

[a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.005$ versus untreated after Benjamini–Hochberg corrections

[d] $P < 0.05$, [e] $P < 0.01$, [f] $P < 0.005$ versus bioglass after Benjamini–Hochberg corrections

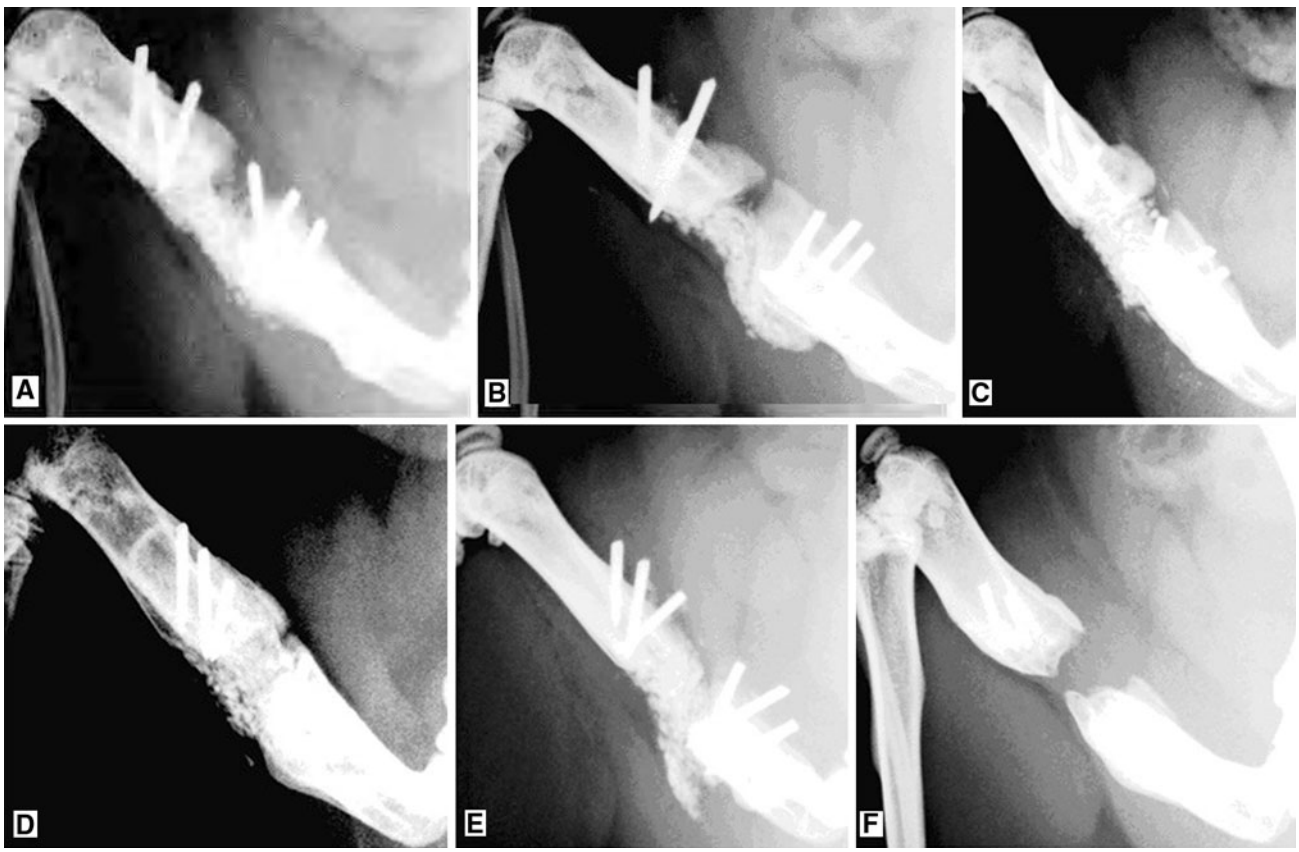

Fig. 1 Radiographs showing new bone formation induced by different amounts of reindeer bone protein extract in the rat femur after 8 weeks follow-up. **a**: 5 mg of bone protein extract, **b**: 10 mg of bone protein extract, **c**: 15 mg of bone protein extract, **d**: 40 mg of bone protein extract, **e**: bioglass, **f**: untreated defect

3.3 Torsion testing

Torsional stiffness of the bone was marginally higher in the group receiving BG + 15 mg of extract compared with the untreated group ($P = 0.066$) (Table 3; Figs. 2, 3).

4 Discussion

This study shows for the first time that bioglass is a candidate carrier for reindeer bone protein extract when used in a rat femur defect model. The results suggest that bone

Table 2 Bone union (BU) in the defect area of rat femur 3–8 weeks after the operation evaluated by radiographical analysis using the scoring system presented in the study of Sciadini et al. [27]

Group	BU score												
	N	3 weeks				P	6 weeks				P	8 weeks				P
		0	1	2	3		0	1	2	3		0	1	2	3	
Untreated	6	6	0	0	0		6	0	0	0		6	0	0	0	
Bioglass (BG)	6	6	0	0	0		6	0	0	0		6	0	0	0	
BG + 5 mg extract	6	6	0	0	0		5	1	0	0		5	1	0	0	
BG + 10 mg extract	6	6	0	0	0		2	4	0	0	[a]	1	5	0	0	[b]
BG + 15 mg extract	7	6	1	0	0		2	5	0	0	[a]	1	2	4	0	[b]
BG + 40 mg extract	9	6	3	0	0		1	4	4	0	[b]	0	3	3	3	[c]

[a] $P < 0.05$, [b] $P < 0.01$, [c] $P < 0.005$ versus untreated and bioglass after Benjamini–Hochberg corrections

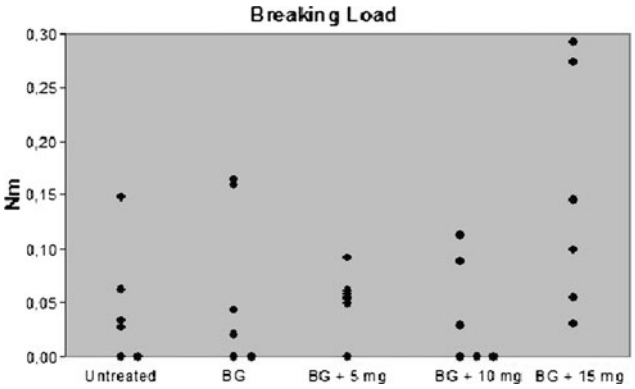

Fig. 2 Maximum breaking load (Nm) of defect site after 8 weeks follow-up. The *dots* indicate individual samples

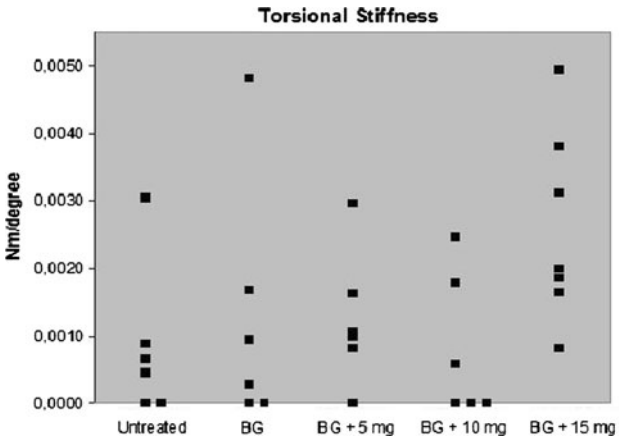

Fig. 3 Torsional stiffness (Nm/°) of defect site after 8 weeks follow-up. The *squares* indicate individual samples

extract enhances the healing of the bone, compared with bioglass alone, 8 weeks after lesion formation, which is in agreement with previously published studies. Although bioglass is used alone in bone surgery, its healing effect is increased when it is combined with BMP products [15, 16, 18, 19, 31, 32] or with other inorganic materials like tricalcium phosphate (TCP) or a calcium sulphate barrier [13, 33], or with various organic materials [32, 34–37].

This was the first test of bioglass granules combined with reindeer bone protein extract. Previously, bioglass has been used as a carrier for bovine bone extract, demineralised bone matrix, and allografts [18, 19, 31]. Many other inorganic carrier materials have been used with native reindeer bone extracts. Pekkarinen et al. [24] used TCP, hydroxyapatite (HA), and coral as scaffolds for native reindeer bone extract in a mouse model of ectopic bone formation. TCP cylinders and coral composite implants with moose bone protein extract and type IV collagen have been used in the sheep segmental defect model [38, 39]. Canine segmental defects have been treated with bovine bone extract and HA or coral [40, 41]. Bovine bone extract and Plaster of Paris have been used clinically to treat femoral shaft non-unions in patients in a study by Bai et al. [42], and in scaffold and ulnar non-union with coral implants in a study by Kujala et al. [43, 44]. Potential advantages of bioglass over HA, TCP, or polymers include its superior biomechanical strength, higher osteoconduction, biocompatibility, and faster degradation [9, 45–47].

Table 3 Bone healing in the defect area of rat femur as medians or means, evaluated by pQCT densitometry and mechanical testing

Group	N	pQCT analysis (mean value)		Mechanical tests (median value)	
		Cross-sectional area	Density	Breaking load	Stiffness
		(mm^2 (std dev))	(mg/cm^3 (std dev))	(Nm (1 and 3 quartiles))	(Nm/° (1 and 3 quartiles))
Untreated	6	28.72 (13.47)	166.45 (37.37)	0.0311 (0.0000–0.0835)	0.0005 (0.0000–0.0014)
Bioglass (BG)	6	51.75 (15.94)[a]	445.77 (81.51)[b]	0.0317 (0.0000–0.1606)	0.0006 (0.0000–0.0025)
BG + 5 mg extract	6	43.83 (6.07)	432.29 (36.66)[b]	0.0563 (0.0373–0.0691)	0.0010 (0.0006–0.0020)
BG + 10 mg extract	6	47.29 (15.81)	374.18 (27.60)[b]	0.0145 (0.0000–0.0949)	0.0003 (0.0000–0.0020)
BG + 15 mg extract	7	59.19 (20.29)[a]	348.35 (120.81)[b]	0.0995 (0.0551–0.2732)	0.0020 (0.0016–0.0038)[c]

[a] $P < 0.05$, [b] $P < 0.005$, [c] $P = 0.066$ versus untreated after Benjamini–Hochberg corrections

For about 20 years, our research group has studied the osteoinductivity of extracted reindeer bone protein (containing BMPs). We have shown that reindeer bone protein extract is an effective inducer of new bone formation in a muscle pouch model in mice [21, 22, 24, 25]. Furthermore, the good healing capacity of the reindeer bone extract with a collagen carrier in a segmental bone defect was previously demonstrated in the rabbit [26]. BMPs have been extracted from the bone materials of a variety of animal species, humans, and from bone tumours. The ability of reindeer bone protein extract to heal various bone traumas has been noted as being better than bovine or ostrich BMP extract, for example, which is attributed to reindeers' ability to renew their antlers annually [20, 48]. Furthermore, it was suggested that more of the protein material extracted from the reindeer bone is in monocomponent form compared with that of bovine, ovine, or porcine protein material [20]. Surprisingly, the effect of native reindeer bone extract in the rat femur defect model was not as good as in the rabbit ulnar defect model owing to differences in animal species and experimental model [26]. It could also imply that native reindeer bone extract is immunogenic for the rat model [49, 50]. The used doses of native bone extracts are milligrams compared to recombinant products that are in micrograms. This is because the bone extracts and demineralised bone matrix products involve a wide spectrum of all bone proteins and collagens while recombinants only one specific protein with specific task in bone regeneration [51, 52]. The difference in the response of recombinant and native reindeer bone extract might partly be explained by the different effective doses, and more studies on the optimal dosing are needed.

An ideal bone implant would be osteoconductive, osteoinductive, radiolucent, and resorbable to allow easy radiographic evaluation of bone formation and healing [3, 5, 7, 53]. The bone graft substitute should also avoid being rejected or inducing a foreign body response [27] and should retain BMPs at the implant site for a period of time sufficient to induce bone formation [15]. Some properties of bioglass granules can affect the bone-healing capacity of BMP extracts. In this study, we used traditional bioglass granules. SiO_2 (53% in bioglass granules used in the present study) worked as a network former, and had a slow and incomplete resorption property. Na_2O (23%), CaO (20%), and P_2O_5 (4%) are compounds found in bioglass granules, and the variation in their ratios can be changed to adjust the dissolution rate [30, 31, 54, 55]. Variations in local pH levels also strongly influence the osteoclastic activity and reactions of bioglass in bone trauma [56].

Granulometry is influenced by the biological properties of bioactive glass [9, 13–15, 57, 58]. The size of the bioglass granules used in this study was 500–800 μm, which appeared to be a good size because the granules could be impacted firmly and stably in the bone defect and because granules of this size are not resorbed too quickly. In our preliminary study (results not shown) we used smaller granule size of bioglass (<300 μm), but after 3 weeks the granules had resorbed, and no bone formation could be seen even after 8 weeks. Different ratios of the compounds of bioglass granules could have been used to adjust the degradation process, allowing more time for osteoconduction and a larger pore size would also have yielded slower resorption of the bioglass; thus pore sizes > 300 μm are recommended [30, 58–60].

We used an 8-week follow-up time in this study. However, because bioglass resorbs slowly, we decided to extend the follow-up time of the bioglass + 40 mg extract group from 8 to 10 weeks to assess longer-term healing. The results of bone formation and bone union in this group were significantly better after 10 weeks compared with all the other groups after 8 weeks follow-up. However, there was plenty of bioglass material remaining even after 10 weeks follow-up when granule sizes of 500–800 μm were used. It is known that bioglass is radiopaque and can have influence on the pQCT results, the density of bioglass being slightly higher than that of bone [31]. This may explain why there were no statistically significant differences in bone density between any implant groups, although the bone extract groups displayed more bone formation than bioglass group (which displayed the highest density value). Thus, bioglass may overcome the density of new bone. On the other hand, plain radiographs showed signs of increased resorption of bioglass in the bone extract groups which could decrease the density values.

Furthermore, the lack of histological quantification could have limited the estimation of the results and comparing the bone extract groups to control groups. A longer follow-up time should be used when bioglass is used as the carrier compared with collagen, for example [15]. We also found that in the group 40 mg + BG biomechanical results were better after 10 weeks follow-up than in each other groups (these results not shown).

In this study, 10 rats died soon after surgery of no obvious cause. No relationship with bone extract or bioglass could be inferred, because deaths also occurred in the control groups. The deaths may have been reactions to anaesthesia, although the anaesthesia and methods used are well accepted in animal testing. Upon necropsy, some rats that died showed signs of pulmonary oedema that is a typical phenomenon after great bone surgery.

Native bone protein extract proved to be functional in the bioglass carrier. Theoretically, particles containing bone proteins could adhere to the surface of the bioglass granules and be released when the bioglass granules dissolve around the defect. The bone-healing effect of native bone extract combined with bioglass granules was encouraging, but the

optimization of the carrier and formulated bone protein extract is required before native reindeer bone protein extract implants are ready for clinical use.

5 Conclusions

Bioglass granules seem to perform well as a carrier of reindeer bone protein extract at doses of bioglass + 10–40 mg, and a dose-dependent effect of the extract on the healing of the bone was observed.

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