Research Article

Ilkka Saarenpää, Patricia Stoor, and Janek Frantzén*

BAG S53P4 putty as bone graft substitute – a rabbit model

https://doi.org/10.1515/bglass-2017-0003
Received Mar 20, 2017; revised Jun 30, 2017; accepted Jul 23, 2017

Abstract: Bioactive glass (BAG) S53P4 granules represent a bone augmentation biomaterial for the surgical treatment of bony defects, even in challenging conditions such as osteomyelitis. The aim of this eight-week rabbit implantation study was to evaluate the biocompatibility and bone regeneration performance of a BAG S53P4 putty formulation following its implantation into the proximal tibia bone of twenty-eight New Zealand white rabbits.

BAG S53P4 putty was compared to BAG S53P4 granules (0.5–0.8 mm) to evaluate whether the synthetic putty binder influences the bone regeneration of the osteostimulative granules. The putty formulation facilitates clinical use because of its mouldability, injectability and ease of mixing with autograft.

Implantation of putty and granules into proximal tibia defects resulted in good osseointegration of the two groups. Both biomaterials were biocompatible, showed high new bone formation, high vascularization and periosteal growth. No signs of disturbed bone formation were observed due to the PEG-glycerol binder in the BAG S53P4 putty. Instead, intramedullary ossification and stromal cell reaction were more advanced in the putty group compared to the control group ($p = 0.001$ and $p < 0.001$).

In conclusion, the novel mouldable BAG S53P4 putty showed reliable bone regeneration in bony defects without adverse tissue or cell reactions.

Keywords: Bioactive glass, putty, bone formation, vascularization

***Corresponding Author: Janek Frantzén:** Division of Clinical Neurosciences, Department of Neurosurgery, Turku University Hospital, Turku, Finland, 20521 Turku, Finland; Department of Clinical Medicine, University of Turku, 20014 Turku, Finland; Email: janek.frantzen@tyks.fi; Tel.: +35823130617; Fax: +35823131709
Ilkka Saarenpää: Division of Clinical Neurosciences, Department of Neurosurgery, Turku University Hospital, Turku, Finland, 20521 Turku, Finland; Department of Clinical Medicine, University of Turku, 20014 Turku, Finland
Patricia Stoor: Department of Oral and Maxillofacial Surgery, Helsinki University Hospital, 00029 Helsinki, Finland; University of Helsinki, 00014 Helsinki, Finland

1 Introduction

Development of synthetic bone graft substitutes is necessary due to the drawbacks associated with autogenous and allograft bone materials [1]. Although autografting is generally considered as the graft of choice, its use is complicated by the limited supply and donor site morbidity [2–8]. In allografting, the disadvantages include possibility for disease transfer, supply limitations and loss of osteoinductivity in harvesting and treatment [9]. As bone graft substitute materials, bioactive glasses (BAGs) are biocompatible and osteoconductive, and thus have a significant potential for being used as implants in bone grafting [10–13]. Also, bioactive glasses are able to bond chemically with the surrounding bone [11].

Bioactive glass works by leaching out ions that react with the body fluids, transforming the glass surface chemically into one that by its chemical composition and structure resembles the mineral phase found in natural bone. In contact with an aqueous solution, bioactive glass develops a silica-gel layer which acts as a template for calcium phosphate (CaP) precipitation. The CaP crystallizes *in vivo* into natural hydroxyapatite, and this layer provides a suitable reaction surface for the following processes to occur: (a) adhesion of osteoblasts to the surface layer, (b) chemical bonding between the surface layer and the surrounding bone, and (c) promotion of new bone formation in the implanted area [10, 13, 14]. In addition, the leached ions affect the bone cells in their vicinity by activating osteoblast proliferation and differentiation [11]. However, it has been demonstrated in *in vivo* preclinical studies that BAG particles do not stimulate bone formation if migrated into ectopic areas, such as soft tissues. This might lead to alterations in the internal structure of the BAG particles resulting in increase of the reaction surface that can be associated with a certain degree of inflammation in the surrounding soft tissue [15–17].

Consisting of a total of 60% by weight bioactive glass, BAG S53P4 putty is a synthetic, bioactive, osteoconductive and osteostimulative paste-like bone void filler that is easy to handle and implant. In the chemical composition, the putty-form of BAG S53P4 differs from the granule-

form by its synthetic binder. Both the binder compounds of BAG S53P4 putty, glycerol and PEGs, are well known and widely used *e.g.* in pharmaceutical and food industry. *In vivo*, glycerol is rapidly absorbed in the intestine and the stomach, and thus distributed over the extracellular space and excreted [18]. Prior to excreting, glycerol is phosphorylated to alpha-glycerophosphate by glycerol kinase predominantly in the liver (80–90%) and kidneys (10–20%), and incorporated in the standard metabolic pathways to form glucose and glycogen [18]. After *in vivo* exposure, PEGs are excreted mainly unchanged in the urine and feces through liver [19]. A minor part of PEGs of > 400 g/mol is absorbed [19]. Part of the absorbed PEGs is metabolized to lower oligomers, glycolic acid, hydroxyl glycolic acid and diglycolic acids homologs, carbon dioxide and, in very minor quantities, harmful oxalic acid [19]. Glycolic acid further decomposes to glyoxylic acid and oxalic acid [19].

Preclinical studies with an antibacterial formulation of BAG S53P4 putty and clinical studies with BAG S53P4 granules have demonstrated its significant potential as bone substitute for the treatment of infections caused by multiresistant microorganisms and excellence as an adjuvant in the treatment of prosthetic infections related to biofilm [20–23]. On the other hand, the BAG S53P4 putty formulation that has been investigated in this study and studies on a comparable device (NovaBone Putty®) did not show any inhibitory effect in any of the bacteria tested [24].

An eight-week rabbit proximal tibia bone implantation study was carried out to confirm that BAG S53P4 putty has no harmful effects or toxicity, neither locally nor systemically, and also that it forms new bone. The primary aim of the study was to evaluate if the synthetic binder has an impact on the safety and efficacy of using BAG S53P4 putty as a bone graft substitute. In addition, the level of vascularization, growth of periosteum and occurrence of connective tissue were studied.

2 Materials and Methods

This study was a biocompatibility and performance test of BAG S53P4 putty following its implantation into the defect of the proximal tibia bone of the New Zealand white rabbits. The study was performed in a laboratory accredited by The Association for Assessment and Accreditation of Laboratory Animal Care International in accordance with the Swiss Federal Act on Animal Protection under the license number of 409/2010. BAG S53P4 putty (BonAlive® putty; BonAlive Biomaterials Ltd., Turku, Finland) was compared to BAG S53P4 granules (BonAlive® granules; 0.5–0.8 mm) to evaluate whether the synthetic binder in putty has potential effects on the performance of the granules and formation of the new bone.

BAG S53P4 putty is composed of osteostimulative calcium-phosphorous-sodium-silicate BAG S53P4 granules (size 0.5–0.8 mm, 48 weight-%) mixed with spherical (BAG S53P4) granules (size 0.09–0.425 mm, 12 weight-%) and a synthetic binder (mix of glycerol and three chain lengths of PEG). The composition of the bioactive glass granules of BAG S53P4 putty is (by weight-%): SiO_2 53%, Na_2O 23%, CaO 20% and P_2O_5 4%. The binder acts as a temporary binding agent for the granules. The granules and the binder are provided as a premixed extrudable, pliable cohesive material, packed in a syringe-like applicator and sterilized by gamma irradiation. The closest comparable device of BAG S53P4 putty on the market is BAG 45S5 putty (NovaBone Putty®). The composition of BAG 45S5 putty is (by weight-%): SiO_2 45%, N_2O 24.5%, CaO 24.5% and P_2O_5 6% that slightly differs from BAG S53P. In addition, the size range of the bioactive glass particulates in NovaBone putty (0.03–0.71 mm) is slightly different from BonAlive putty.

Twenty-eight male and female New Zealand white rabbits were anaesthetized, the back legs of the animals were shaven, and the area for the incision was disinfected with a standard solution of Betadine. An incision was made in the skin directly above the tibia of each leg and the proximal tibial area was isolated. Using a water-cooled drill, two holes (diameter 2 mm and depth 6 mm) were drilled per tibia (penetrating through the cortex into the cancellous bone area), 8–10 mm apart from each other. The periosteum was destroyed during the drilling. The holes were filled with either the BAG S53P4 putty or with the control BAG S53P4 granules (size 0.5–0.8 mm) using a sterile instrument (*e.g.* a thin spatula). All the four defects of the same animal were filled with the same material. Special attention was paid to filling the holes completely with the test or the control material. The wound was finally closed with sutures, which were removed approximately 10 days later. The implantation was performed by the same experienced surgeon. As BAG S53P4 granules resorb slowly, complete new bone formation was not expected during the eight-week study.

The animals were observed for clinical signs throughout the pre- and post-operative period. Food consumption and body weight were determined, and at either 4 or 8 weeks post-implantation blood and urine were sampled for extensive clinical laboratory investigations. Animals were euthanized and necropsied at 4 or 8 weeks post-

implantation, and selected specimens were weighed, histologically processed and read.

From each tibia, the tissue for histopathology was collected so that both implanted holes were included as a whole, and forwarded for histological analyses. Each tibial sample was fixed in 10% buffer formalin solution and reduced in size so that each block contained one implanted hole. These small samples were dehydrated in growing alcohol series and embedded in methyl methacrylate (MMA). After polymerization, thin sections were produced by the cutting-grinding technique. The plane of sectioning was along the long axis of the implanted hole. The histological sections were then stained with Paragon stain and evaluated in a light microscope. Representative photographs were taken with both low magnification (to show the whole implanted holes) and high magnification (to show details; individual granules of the implanted area). Structural changes in bioactive glass material, tissue integration, bone formation, level of vascularization, growth of the periosteum and biocompatibility of the BAG S53P4 putty and granules were assessed from each tibial bone tissue samples. The test items were compared to the controls to find out whether the synthetic binder (PEG-glycerol) had any effects on the performance of the granules and the formation of new bone. In addition, the filling volume of the granules in the defect was assessed after the binder was diluted away, during the specimen preparation. Special attention was paid to osteogenesis, occurrence of connective tissue, the amount of new bone and structural changes in the remaining test or control materials.

The data gained were described using absolute and relative values. In the statistical part, a normal distribution of the body weight, the clinical laboratory data and the grading of microscopic findings was firstly tested visually from histograms and secondly by the Shapiro-Wilk test [25]. The normally distributed test and control values were analyzed by the parametric two-sample Student's t-test. If the values could not be assumed to follow a normal distribution, the analysis was performed by the non-parametric Mann-Whitney U-test [26]. P-values less than 0.05 were considered statistically significant. Statistical analyses were performed using SPSS system for Mac, version 24 (IBM Corp., Armonk, NY, USA).

3 Results

3.1 Clinical and macroscopic findings

Three female animals died prematurely before the scheduled necropsy. One died 13 days after the implantation of the test material, the other 29 days after the implantation of the control implant, and the third 34 days after the implantation of the control implant. Histopathological results for the animals living 29 and 34 days were reported as at 4 weeks. Overall, there were no clinical signs associated with complications, such as cervical scabs, sores, hair loss, nodules or wounds.

No effects on food consumption or body weight were considered to be related to the treatment with the test or control sample. Furthermore, no differences in haematology, clinical biochemistry or urinalysis parameters considered to be related to the treatment with the test or control sample were recorded.

Mean values and standard deviations of the measured body weight, haematology and clinical biochemistry parameters are presented in Tables 1, 2 and 3 (periodically in the case of body weight, and otherwise at the moment of necropsy). Some intergroup variations occasionally achieved statistical significance, but these were considered to reflect the normal biological variation. All clinical and macroscopic findings were considered to be incidental and commonly occur in rabbits of this strain and age under the experimental conditions used in this study.

3.2 Microscopic findings

Microscopic findings of the samples from the tibial bone implantation site were recorded during the histopathologic examination by the study pathologist. The histological gradings were defined to current situation in every case of histological changes [27, 28]. The descriptions of the distinguishing features of each severity grade are presented in the following.

Incidence of cortical, intramedullary and periosteal granules, and spheres in the BAG S53P4 putty group, were estimated as present, not present and not assessable. Structural changes in the granules and in the spheres (in the BAG S53P4 putty group) were graded as no structural change (grade 0), < 40% of granules/spheres shrunken, fractured/slight opaque and/or slight dark blue to deep violet in staining (grade 1), 40–70% of granules/spheres shrunken, fractured/slight opaque and/or moderate dark blue to deep violet in staining (grade 2) and > 70% of gran-

Table 1: Summary of body weights and standard deviations (SD) of the test animals in the test and control groups as a function of time.

Time point	Test group						Control group					
	Male			Female			Male			Female		
	N	Mean (kg)	SD (kg)	N	Mean (kg)	SD (kg)	N	Mean (kg)	SD (kg)	N	Mean (kg)	SD (kg)
Acclimatization												
Day 1	7	3.6–	0.4	7	4.4–	0.5	7	4.0	0.5	7	4.6	0.4
Day 8	7	3.5*	0.3	7	4.3–	0.4	3	4.3	0.4	3	4.7	0.4
Treatment	7	3.5–	0.2	7	4.2–	0.3	7	3.8	0.5	7	4.4	0.3
Recovery												
Day 1	7	3.4–	0.3	7	4.1–	0.3	7	3.8	0.5	7	4.3	0.4
Day 8	7	3.4–	0.3	7	3.9–	0.3	7	3.6	0.5	6	4.2	0.5
Day 14	2	3.4–	0.4	4	3.9–	0.2	3	4.0	0.3	3	4.3	0.7
Day 15	4	3.3–	0.2	5	3.8–	0.2	6	3.4	0.5	5	4.0	0.2
Day 16	7	3.5–	0.4	6	3.9–	0.2	5	3.4	0.7	5	4.1	0.4
Day 17	4	3.3–	0.2	5	3.9–	0.2	6	3.5	0.6	5	4.1	0.5
Day 18	7	3.6–	0.4	6	3.9–	0.2	6	3.5	0.6	4	3.8	0.2
Day 19	7	3.6–	0.4	6	3.9–	0.2	7	3.6	0.6	5	4.1	0.5
Day 22	7	3.6–	0.5	6	4.0–	0.3	7	3.7	0.7	6	4.0	0.5
Day 28	7	3.6–	0.5	6	4.0–	0.4	7	3.7	0.7	6	3.9	0.6
Day 29	4	3.7–	0.5	4	4.2–	0.3	4	3.6	0.5	4	4.2	0.7
Day 30	4	3.7–	0.5	4	4.2–	0.3	4	3.6	0.5	4	4.2	0.7
Day 33	4	3.7–	0.5	4	4.2–	0.3	4	3.7	0.5	4	4.2	0.8
Day 36	4	3.8–	0.5	4	4.3–	0.3	4	3.8	0.5	3	4.6	0.3
Day 43	4	3.7–	0.6	4	4.4–	0.4	4	3.8	0.5	3	4.7	0.4
Day 50	4	3.7–	0.6	4	4.4–	0.3	4	3.9	0.5	3	4.7	0.4
Day 56	4	3.7–	0.6	4	4.3–	0.3	4	3.8	0.4	3	4.6	0.4

N: number of animals, SD: standard deviation, *: p-value < 0.05, -: p-value ≥ 0.05

ules/spheres shrunken, fractured/slight opaque and/or marked dark blue to deep violet in staining (grade 3). Integration of granules/spheres in the new bone tissue was scored as no integration (*i.e.* no granule/sphere in cortical defect surrounded by new bone) (grade 0), < 40% (grade 1), 40–70% (grade 2) and > 70% (grade 3) of the surface of granules/spheres in the cortical defect surrounded by the new bone.

In addition, the filling volume, *i.e.* the percentage of the volume of the cortical defect filled by the implants and new bone, was graded as none (grade 0), < 40% (grade 1), 40–70% (grade 2) and > 70% (grade 3). Cortical ossification was graded as no new bone formation (grade 0), new bone formation around the implants (grade 1), around the implants and at margins of the drilled hole (grade 2), around the implants and at the margins of drilled hole including proximal inner and outer tibial bone borders with presence of thin bridging bone trabeculae (grade 3), medium–large bone trabeculae bridging the whole defect and the surrounding implants, slight new bone formation extending along inner and outer tibial bone borders (grade 4) and large bone trabeculae bridging the whole defect and the surrounding implants, prominent new bone formation extending along inner and outer tibial bone borders (grade 5).

Intramedullary ossification was graded 0 with no new bone formation, 1 with thin new bone formation around the implants covering less than 50% of the granules surface, 2 when covering more than 50% of the granules surface, 3 with new bone formation around the implants along with bone trabeculae and 4 when bridging the bone trabeculae. Stromal cell reaction was scored as not existent (grade 0), and slight (grade 1), moderate (grade 2) or marked (grade 3) sheet of fibres and cells around the implants. Periosteal growth over the defect graded as not existent (grade 0), slight fibrosis (grade 1), moderate fibrosis (grade 2) and marked fibrosis (grade 3). Moreover, vascularization in the defect was scored as not existent (grade 0), few blood vessels (grade 1), moderate blood vessels (grade 2) and many blood vessels (grade 3). The compila-

Table 2: Summary of hematological parameters of the tested animals both 4 and 8 weeks after the implantation. P indicates that parametric tests and N that non-parametric tests were used in calculating the possibility of intergroup variations between the groups. A diagonal mark separates the test used between the test and the control group.

Parameter	Unit	4 weeks after surgery					8 weeks after surgery				
		Test	Test group		Control group		Test	Test group		Control group	
			Male	Female	Male	Female		Male	Female	Male	Female
Erythrocytes	cells/µl	P/N	6.37–	5.61–	5.33	6.59	P/P	7.11–	6.59–	6.71	6.67
Hemoglobin	mmol/l	P/N	8.70–	7.30–	7.23	8.30	P/P	9.57*	8.75–	8.85	8.70
Hematocrit	-	P/N	0.40–	0.33–	0.34	0.40	P/N	0.45*	0.41–	0.41	0.41
MCV	fl	P/N	61.7–	57.9–	63.9	60.8	P/P	63.2–	61.5–	60.5	62.3
RDW	-	P/N	0.125–	0.133–	0.147	0.135	N/P	0.127–	0.124–	0.118	0.127
MCH	fmol	P/N	1.36–	1.30–	1.35	1.26	P/P	1.35–	1.33–	1.33	1.31
Reticulocyte relative count	-	P/N	0.014–	0.016–	0.022	0.015	P/P	0.018–	0.014–	0.015	0.027
Reticulocyte absolute count	g/l	P/N	88–	90–	122	93	P/P	130–	91–	95	179
Maturity index (L-reti)	-	P/N	0.982–	0.981–	0.980	0.983	P/N	0.975–	0.986–	0.848	0.888
Maturity index (M-reti)	-	P/N	0.016–	0.018–	0.015	0.017	P/N	0.023–	0.013–	0.134	0.102
Maturity index (H-reti)	-	N/N	0.003–	0.002–	0.005	0.001	P/N	0.001–	0.001–	0.019	0.010
Leukocytes	g/l	N/N	7.15–	5.62–	6.87	6.73	P/P	7.16–	7.20–	8.77	6.13
Neutrophils	-	P/N	0.166–	0.404–	0.175	0.282	P/P	0.141–	0.155–	0.138	0.193
Eosinophils	-	P/N	0.011–	0.013–	0.031	0.018	P/P	0.025–	0.019–	0.019	0.022
Basophils	-	P/N	0.026–	0.041–	0.047	0.033	P/P	0.046–	0.051–	0.050	0.060
Lymphocytes	-	P/N	0.784–	0.507–	0.728	0.647	P/P	0.777–	0.749–	0.770	0.679
Monocytes	-	P/N	0.012–	0.035–	0.018	0.019	N/N	0.010–	0.021–	0.021	0.040
Neutrophils	g/l	P/N	1.20–	2.23–	1.10	1.94	P/P	0.89–	1.12–	1.18	1.21
Eosinophils	g/l	P/N	0.08*	0.07–	0.17	0.11	P/P	0.16–	0.13–	0.16	0.14
Basophils	g/l	P/N	0.18–	0.23–	0.28	0.21	P/P	0.32–	0.38–	0.41	0.37
Lymphocytes	g/l	P/N	5.61–	2.88–	5.22	4.32	P/P	5.70–	5.37**	6.80	4.11
Monocytes	g/l	P/N	0.01*	0.20–	0.09	0.14	P/N	0.07–	0.16–	0.20	0.27
Thrombocytes	g/l	P/N	430–	491–	420	375	P/P	428–	388–	373	533
PT	s	P/N	6.37–	5.61–	5.33	6.59	P/P	7.11–	6.59–	6.71	6.67
PTT	s	P/N	8.70–	7.30–	7.23	8.30	P/P	9.57*	8.75–	8.85	8.70

MCV: mean corpuscular volume, RDW: red cell volume distribution width, MCH: mean corpuscular haemoglobin, PT: prothrombin time, PTT: partial thromboplastin time, P: parametric test, N: non–parametric test, **: p–value < 0.01, *: p–value ≥ 0.01 and < 0.05, –: p–value ≥ 0.05

tion of the findings in the implantation site is shown in Table 4.

Moreover, in the soft tissues outside the implantation site, the microscopic examination revealed slight to moderate lymphoid atrophy of the spleen, higher degrees of lymphoid atrophy in the thymus and one case of slight acute tubular necrosis in the kidneys. All of these disorders were considered to be secondary and related to stress in the laboratory animals.

4 and 8 weeks after implantation, in both the BAG S53P4 putty group and in the control group with BAG S53P4 granules, there was generally a high cortical ossification along with a high integration of granules in the new bone, the volume in the cortical defect was abundantly filled by the implants and the new bone, and the granules showed high progressed structural changes (Table 4). All these findings were slightly more prominent 8 weeks after implantation when compared to 4 weeks after implantation (Figures 1 and 2, Table 5). The remaining volume consisted of stromal cell reaction (mainly around implants) or connective tissue containing blood vessels and occasionally bone marrow cells. Intramedullary ossification and

Table 3: Summary of biochemical parameters of the tested animals both 4 and 8 weeks after the implantation. P indicates that parametric tests and N that non-parametric tests were used in calculating the possibility of intergroup variations between the groups. A diagonal mark separates the test used between the test and the control group.

Parameter	Unit	4 weeks after surgery					8 weeks after surgery				
		Test	Test group		Control group		Test	Test group		Control group	
			Male	Female	Male	Female		Male	Female	Male	Female
Glucose	mmol/l	P/N	6.00–	6.76–	5.45	5.77	P/P	7.88–	7.00–	6.60	6.59
Urea	mmol/l	P/N	6.87–	8.13–	6.54	8.84	P/P	6.22–	9.61–	7.93	8.49
Creatinine	µmol/l	P/N	92.2–	93.3–	76.2	98.0	P/P	97.9–	122.9–	94.8	134.2
Bilirubin	µmol/l	N/N	1.56–	1.24–	1.05	1.79	P/N	0.74*	0.88–	2.08	2.04
Cholesterol	mmol/l	N/N	0.84–	1.26–	1.12	1.07	P/N	0.42*	1.52–	0.72	1.21
Triglycerides	mmol/l	P/N	0.56–	0.88–	0.39	1.11	P/N	0.54–	0.56–	0.50	0.42
Phospholipids	mmol/l	N/N	0.63–	1.08–	0.69	1.05	P/P	0.58–	1.30–	0.71	1.02
ASAT	IU/l	N/N	60.7–	40.1–	24.3	172.3	P/P	16.0–	22.1–	32.5	12.3
ALAT	IU/l	N/N	35.6–	42.7–	46.4	80.5	P/P	30.6–	61.9–	37.0	38.1
LDH	IU/l	P/N	136.9–	102.1–	173.3	245.6	N/P	105.3–	103.4–	815.2	84.6
ALP	IU/l	P/N	97.2–	116.9–	83.8	110.9	N/P	60.7–	67.2–	58.1	70.3
GGT	IU/l	P/N	15.0–	12.0–	4.3	9.5	P/P	9.1–	11.7*	10.2	6.5
CK	IU/l	P/N	698.4–	504.8–	1110.1	902.5	P/P	380.2–	282.3–	997.8	361.5
Sodium	mmol/l	N/N	136.4–	134.1–	139.0	142.8	P/P	143.6–	141.6–	142.3	144.6
Potassium	mmol/l	P/N	3.51–	2.63–	4.32	3.17	P/P	4.01–	3.92*	5.22	5.19
Chloride	mmol/l	P/N	96.6–	94.9–	102.2	99.9	P/P	103.0–	101.3–	100.9	103.3
Calcium	mmol/l	P/N	3.30–	3.23–	3.23	3.25	P/N	3.34–	3.27–	3.34	3.41
Phosphorous	mmol/l	P/N	1.43–	1.17–	1.46	1.24	P/P	1.20–	1.48–	1.78	1.50
Protein	g/l	P/N	59.39–	62.93–	58.69	61.33	P/P	59.41–	59.12–	60.53	61.37
Albumin	–	P/N	0.740–	0.724–	0.699	0.690	P/P	0.760–	0.746–	0.745	0.704
Alpha-1-globulin	–	P/N	0.059–	0.069–	0.078	0.070	P/P	0.038–	0.041–	0.046	0.049
Alpha-2-globulin	–	P/N	0.041–	0.047–	0.050	0.055	P/P	0.046–	0.051–	0.044	0.057
Beta-1-globulin	–	P/N	0.057*	0.052–	0.078	0.074	N/P	0.059–	0.046–	0.049	0.053
Beta-2-globulin	–	N/N	0.059–	0.070–	0.048	0.061	P/P	0.056–	0.066–	0.062	0.078
Gamma-globulin	–	P/N	0.044–	0.040–	0.047	0.052	P/P	0.031–	0.050–	0.052	0.059

ASAT: aspartate aminotransferase, ALAT: alanine aminotransferase, LDH: lactate dehydrogenase, ALP: alkaline phosphatase, GGT: gamma–glutamyltransferase, CK: creatine kinase, P: parametric test, N: non–parametric test, *: p–value < 0.05, –: p–value ≥ 0.05

stromal cell reaction were present at high incidence in both groups (Table 4). In the BAG S53P4 putty group, both findings were slightly more advanced when compared to the control group ($p = 0.001$ for intramedullary ossification and $p < 0.001$ for stromal cell reaction at 8 weeks, respectively) indicating a slightly better formation of new bone in the medullary cavity (Figures 1 and 2, Table 5). In addition, the spheres in BAG S53P4 putty composition showed less structural changes and integration in the new bone when compared to granules in BAG S53P4 putty at 4 and 8 weeks after implantation (Table 4). The spheres were mainly surrounded by fibrosis along with inflammatory cell infiltrates, mainly round cells, as well as fibroblasts and fibrocytes (*i.e.* stromal cell reaction; a normal and wanted process in formation of new bone).

In both groups, the periosteum grew back over the defect showing variable degrees of fibrosis depending on the presence of the periosteal implants (the more implants, the more fibrosis). The degree of periosteal growth and fibrosis was slightly higher at 4 weeks after implantation, when compared to 8 weeks after implantation (Figures 1 and 2, Tables 4 and 5). High vascularization was observed

Table 4: Incidence (%) and mean grade of the findings in the implantation site in proximal tibia. Mean grade is expressed depending on the parameter.

	Test group				Control group			
	4 weeks		8 weeks		4 weeks		8 weeks	
Number of animals	3 M	2 F	4 M	4 F	3 M	3 F	4 M	3 F
Number of implants	12	8	16	16	12	12	16	12
Cortical granules (incidence, %)	100	100	100	94	100	100	100	100
Cortical spheres (incidence, %)	100	100	100	94	-	-	-	-
Intramedullary granules (incidence, %)	100	100	100	94	100	92	69	92
Intramedullary spheres (incidence, %)	100	100	100	94	-	-	-	-
Periosteal granules (incidence, %)	58	88	25	56	42	83	25	67
Periosteal spheres (incidence, %)	83	100	13	50	-	-	-	-
Structural changes in granules	100	100	100	100	100	100	100	100
(incidence, %/mean grade)	/2.0	/2.0	/3.0	/2.9	/2.1	/2.3	/2.9	/3.0
Structural changes in spheres	100	100	100	100	-	-	-	-
(incidence, %/mean grade)	/1.2	/1.5	/1.5	/1.2				
Integration of granules in new bone	100	100	100	94	100	100	100	100
tissue (incidence, %/mean grade)	/2.8	/2.6	/3.0	/3.0	/2.4	/2.4	/2.9	/2.8
Integration of spheres in new bone	100	100	100	94	-	-	-	-
tissue (incidence, %/mean grade)	/0.9	/1.1	/0.8	/1.3				
Filling volume	100	100	100	94	100	100	100	100
(incidence, %/mean grade)	/2.7	/2.2	/2.7	/2.9	/2.7	/2.1	/2.7	/2.5
Cortical ossification	100	100	100	94	100	100	100	100
(incidence, %/mean grade)	/4.2	/3.5	/4.4	/4.7	/3.6	/3.6	/4.4	/4.2
Intramedullary ossification	100	100	100	100	100	100	100	100
(incidence, %/mean grade)	/1.2	/1.1	/1.6	/1.2	/1.0	/0.9	/0.6	/1.0
Stromal cell reaction	100	100	100	100	100	100	100	100
(incidence, %/mean grade)	/1.8	/1.9	/2.0	/1.9	/1.5	/1.7	/0.9	/1.2
Periosteal growth	100	100	75	94	67	75	44	75
(incidence, %/mean grade)	/1.7	/2.5	/1.2	/1.5	/2.0	/2.2	/1.3	/1.4
Vascularization	100	100	100	94	100	100	100	100
(incidence, %/mean grade)	/3.0	/3.0	/3.0	/3.0	/3.0	/3.0	/3.0	/3.0

F: female, M: male

in all sites of implantation (Table 4). There were no cytotoxic cells or signs in either group, hence, both the test and control items were considered to be biocompatible. No cartilage tissue was seen in any of the tibial bone samples examined.

4 Discussion

In this study, we compared the use of the novel BAG S53P4 putty to BAG S53P4 granules for filling bony defects in the tibias of twenty-eight rabbits to find out whether the synthetic binder has any effects on the performance of the granules and formation of new bone. The granules in the putty are embedded in a matrix, which is a water-soluble synthetic binder made of a blend of polyethylene glycols (PEGs) and glycerol. For this reason, the granules in the putty will not immediately be exposed to the aqueous environment, and there will thus be a delay in the surface reactions. Another detail is that there will be a sequential reaction cascade with granules reacting firstly on the surface of the putty mass in the defect. The granules in the middle of the putty mass will only react after a certain delay, when the body fluids have reached the middle.

An earlier study using a sheep vertebral defect model to compare NovaBone Putty® with or without autograft and NovaBone BAG particulates did not show any significant difference in new bone formation at 6 or 12 weeks after implantation [19]. However, a sheep vertebral body defect model used to compare the local response of Nova-

Table 5: Comparison of grades of the microscopic parameters in the test and in the control groups at 4 and 8 weeks after the implantation. P-value indicates the significance level of the difference between the test and control groups in each case.

	Time point	Test group			Control group			N	p-value
		Median	Lower quartile	Upper quartile	Median	Lower quartile	Upper quartile		
Structural changes	4 w	2.0	2.0	2.0	2.0	2.0	2.0	44	0.053
in granules	8 w	3.0	3.0	3.0	3.0	3.0	3.0	60	0.594
Integration of granules	4 w	3.0	2.3	3.0	2.0	2.0	3.0	44	0.058
in new bone tissue	8 w	3.0	3.0	3.0	3.0	3.0	3.0	59	0.101
Filling volume	4 w	3.0	2.0	3.0	2.0	2.0	2.0	44	0.009*
	8 w	3.0	3.0	3.0	2.0	3.0	3.0	59	0.056
Cortical ossification	4 w	4.0	4.0	4.0	4.0	3.0	4.0	44	0.070
	8 w	4.0	4.0	5.0	4.0	4.0	5.0	59	0.380
Intramedullary	4 w	1.0	1.0	1.8	1.0	1.0	1.0	44	0.131
ossification	8 w	1.0	1.0	2.0	1.0	0	1.0	60	0.001*
Stromal cell reaction	4 w	2.0	2.0	2.0	2.0	1.0	2.0	44	0.097
	8 w	2.0	2.0	2.0	1.0	1.0	1.0	60	0.001*
Periosteal growth	4 w	2.0	1.0	3.0	2.0	2.0	3.0	37	0.097
	8 w	1.0	1.0	2.0	1.0	1.0	2.0	43	0.935

w: weeks, N: number of animals, *: p-value < 0.05

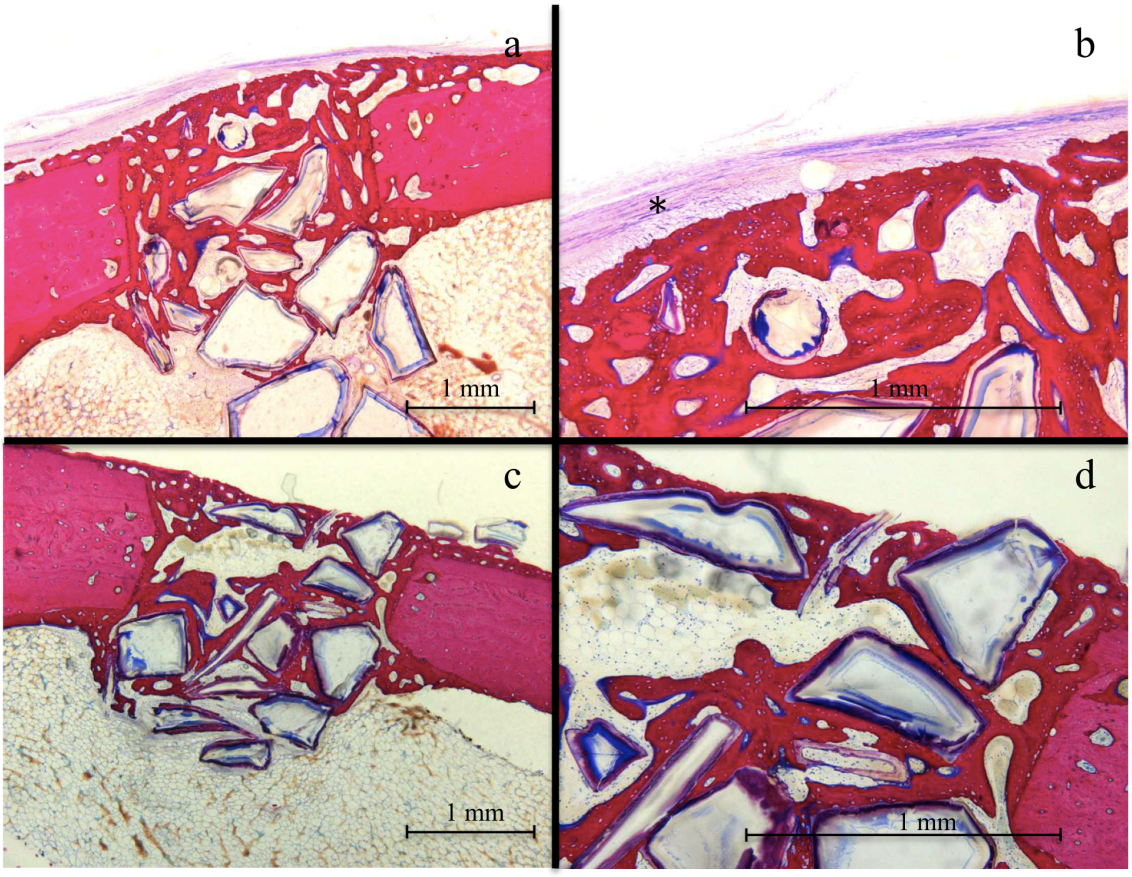

Figure 1: Microphotographs 4 weeks postoperative. Histological examples of bone defects filled with the test BAG S53P4 putty (a, b) or with the control BAG S53P4 granules (c, d) (paragon stain, rabbit tibia) 4 weeks after the implantation. Four times magnification in a and c. Ten times magnification in b and d. Asterisk indicates periosteal growth and fibrosis. Scaled bars indicate the size of 1 mm.

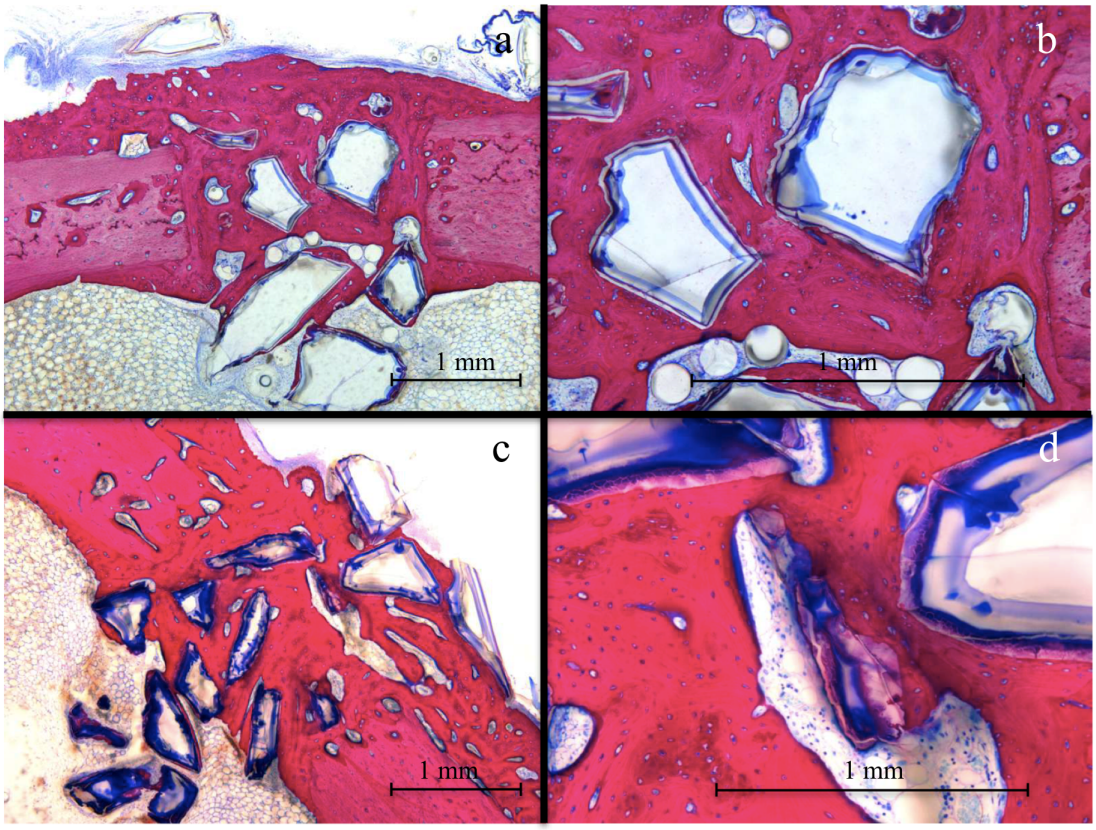

Figure 2: Microphotographs 8 weeks postoperative. Histological examples of bone defects filled with the test BAG S53P4 putty (a, b) or with the control BAG S53P4 granules (c, d) (paragon stain, rabbit tibia) 8 weeks after the implantation. Four times magnification in a and c. Ten times magnification in b and d. Scaled bars indicate the size of 1 mm.

Bone Putty® and NovaBone BAG particulates indicated that the putty group had a greater bone content than the particulate group at 6 and 12 weeks post-implantation [29]. On the other hand, in comparison, when utilizing a rabbit cranial defect model, the OsteoSelect demineralized bone matrix (DBM) putty was associated with significantly more bone formation than the synthetic NovaBone Putty® [30]. In micro-computed tomography and histomorphometric comparison of two synthetic bone graft products using a rabbit posterolateral fusion model, Signafuse® Bioactive Bone Graft Putty showed greater new bone formation than the Actifuse® ABX sculptable synthetic bone graft substitute at 6 weeks post-surgery, but no further differences were detected at 12 weeks [31].

Despite the observation that the bioactive glass particles pack into a defect easily and stay in place, even when the site is bleeding in the periodontal treatment model, several studies report difficulties in keeping the bioactive glass particles within experimental defects [15, 16, 32]. Once the particles migrate, they start to degrade in the soft tissue and are associated with an intense inflammatory reaction [15]. Further, varied degrees (from acute to chronic) of inflammation are associated with collection of either lymphocytes and plasma cells, or macrophages and giant cells [15, 17]. In this study, neither signs of inflammation nor migration of the particulates were observed. Due to its physical properties, unlike the granules, the putty can be shaped so that the bone defect can be filled easily with little residual implant migrating into the undesirable areas [29]. Besides, the increase in early bone deposition rate might also allow subjects to start functional recovery training as early as possible [29].

Our results revealed ossification and integration of both the BAG S53P4 putty and the granules within the new bone in the cortex and medullar. Both the test and the control samples were biocompatible and showed high new bone formation along with high vascularization and periosteal growth. With the BAG S53P4 putty, no indication of possible disturbed bone formation by the PEG-glycerol binder was seen, which is in line with earlier studies [17, 29]. We also found that the bioactive glass granules in the BAG S53P4 putty showed a slightly higher in-

tramedullary ossification than the controls, but no difference in the cortical ossification was seen. Similar findings at 6 and 12 weeks after surgery with NovaBone putty® have been shown revealing a greater bone content in the putty group than in the particulate group [29]. As stated by Wang *et al.*, a possible explanation for the increased bone formation with the putty material could be that addition of the carrier maintains a better spatial distribution of the particles [29]. Altogether, the novel BAG S53P4 putty can be considered to be biocompatible and non-toxic as earlier demonstrated for BAG in general [33].

In conclusion, the novel BAG S53P4 putty showed reliable bone regeneration in the bony defects without adverse cell or tissue reactions.

Funding: No funding was received for this research.

Conflict of Interests: Dr Frantzén is a Member of Board at Bonalive Ltd. He has no shares or options in the company. Other authors certify that they have no affiliations with or involvement in any organization or entity with any financial interest (such as honoraria; educational grants; participation in speakers' bureaus; membership, employment, consultancies, stock ownership, or other equity interest; and expert testimony or patent-licensing arrangements), or non-financial interest (such as personal or professional relationships, affiliations, knowledge or beliefs) in the subject matter or materials discussed in this manuscript.

Ethical approval: All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

Abbreviations

BAG	bioactive glass
DBM	demineralized bone matrix
MMA	methyl methacrylate
PEG	polyethylene glycol

References

[1] El-Ghannam A., Bone reconstruction: from bioceramics to tissue engineering, Expert. Rev. Med. Devices, 2005, 2, 87-101
[2] Coventry M.B., Tapper E.M., Pelvic instability: a consequence of removing iliac bone for grafting, J. Bone Joint Surg. Am., 1972, 54, 83-101
[3] Younger E.M., Chapman M.W., Morbidity at bone graft donor sites, J. Orthop. Trauma, 1989, 3, 192-195
[4] Goulet J.A., Senunas L.E., DeSilva G.L., Greenfield M.L., Autogenous iliac crest bone graft, complications and functional assessment, Clin. Orthop. Relat. Res., 1997, 339, 76-81
[5] Ubhi C.S., Morris D.L., Fracture and herniation of bowel at bone graft donor site in the iliac crest, Injury, 1984, 16, 202-203
[6] Heary R.F., Schlenk R.P., Sacchieri T.A., Barone D., Brotea C., Persistent iliac crest donor site pain: independent outcome assessment, Neurosurgery, 2002, 50, 510-516
[7] Sawin P.D., Traynelis V.C., Menezes A.H., A comparative analysis of fusion rates and donor-site morbidity for autogenic rib and iliac crest bone grafts in posterior cervical fusions, J. Neurosurg., 1998, 88, 255-265
[8] Kurz L.T., Garfin S.R., Booth Jr. R.E., Harvesting autogenous iliac bone grafts - a review of complications and techniques, Spine, 1989, 14, 1324-1331
[9] Giannoudis P.V., Dinopoulos H., Tsiridis E., Bone substitutes: an update, Injury 2005, 36, S20-S27
[10] Hench L.L., Xynos I.D., Polak J.M., Bioactive glasses for *in situ* tissue regeneration, J. Biomater. Sci. Polymer Edn., 2004, 15, 543-562
[11] Hench L.L., The story of Bioglass®, J. Mater. Sci.: Mater. Med., 2006, 17, 967-978
[12] Hench L.L., Genetic design of bioactive glass, J . Eur. Ceram. Soc., 2009, 29, 1257-1265
[13] Jones J.R., Review of bioactive glass: from Hench to hybrids, Acta Biomater., 2013, 4457-4486
[14] Fagerlund S., Hupa L., Hupa M., Dissolution patterns of biocompatible glasses in 2-amino-2-hydroxymethyl-propane-1,3-diol (Tris) buffer, Acta Biomater., 2013, 9, 5400-5410
[15] Moreira-Gonzalez A., Lobocki C., Barakat K., Andrus L., Bradford M., Gilsdorf M., et al., Evaluation of 45S5 bioactive glass combined as a bone substitute in the reconstruction of critical size calvarial defects in rabbits, J. Craniofac. Surg., 2005, 16, 63-70
[16] Amato M.M,, Blaydon S.M., Scribbick F.W. Jr., Belden C.J., Shore J.W., Neuhaus R.W., et al., Use of bioglass for orbital volume augmentation in enophthalmos: a rabbit model (oryctolagus cuniculus), Ophthal. Plast. Reconstr. Surg., 2003, 19, 455-465
[17] Kobayashi H., Turner A.S., Seim H.B., Kawamoto T., Bauer T.W., Evaluation of a silica-containing bone graft substitute in a vertebral defect model, J. Biomed. Mater. Res. Part A, 2010, 92A, 596-603
[18] Lin E.C.C., Glycerol utilization and its regulation in mammals, Ann. Rev. Biochem., 1977, 46, 765-795
[19] Webster R., Didier E., Harris, P., Siegel N., Stadler J., Tilbury L., et al., PEGylated proteins: evaluation of their safety in the absence of definitive metabolism studies, Drug Metab. Dispos., 2007, 35, 9-16
[20] Drago L., Vassena C., Fenu S., De Vecchi E., Signori V., De Francesco R., et al., In vitro antibiofilm activity of bioactive glass S53P4, Future Microbiol., 2014, 9, 593-601
[21] Drago L., De Vecchi E., Bortolin M., Toscano M., Mattina R., Romano C.L., Antimicrobial activity and resistance selection of different bioglass S53P4 formulations against multidrug resistant strains, Future Microbiol., 2015, 10, 1293-1299
[22] Kankare J., Lindfors N. C., Reconstruction of vertebral bone defects using an expandable replacement device and bioactive glass S53P4 in the treatment of vertebral osteomyelitis: three patients and three pathogens, Scand. J. Surg., 2016, 105, 248-253

[23] Lindfors N., Romano C., Scarponi S., Lorenzo D., Monica B., Frantzén J., et al., Chapter 14: Bioactive glasses in infection treatment, In: Boccaccini A.R., Brauer D.S., Hupa L. (Eds.), Bioactive glasses: fundamentals, technology and applications, The Royal Society of Chemistry, Croydon, 2017

[24] Wren A.W., Cummins N.M., Towler M.R., Comparison of antibacterial properties of commercial bone cements and fillers with a zinc-based glass polyalkenoate cement, J. Mater. Sci., 2010, 45, 5244-5251

[25] Shapiro S.S., Wilk M.B., An analysis of variance test for normality (complete samples), Biometrika, 1965, 52, 591-611

[26] Mann H.B., Whitney D.R., On a test of whether one of two random variables is stochastically larger than the other, Ann. Math. Statist., 1947, 18, 50-60

[27] Morton D., Kemp R.K., Francke-Carroll S., Jensen K., McCartney J., Monticello T.M., et al., Best practices for reporting pathology interpretations with GLP toxicology studies, Toxicol. Pathol., 2006, 34, 806-809

[28] The International Organization for Standardization, ISO 10993-1:2009 Biological evaluation of medical devices – part 1: Evaluation and testing within a risk management process, 2009, https://www.iso.org/obp/ui/#iso:std:iso:10993:-1:ed-4:v1:en

[29] Wang Z., Lu B., Chen L., Chang J., Evaluation of an osteostimulative putty in the sheep spine, J. Mater. Sci.: Mater. Med., 2011, 22, 185-191

[30] Schallenberger M.A., Rossmeier K., Lovick H.M., Meyer T.R., Aberman H.M., Juda G.A., Comparison of the osteogenic potential of OsteoSelect demineralized bone matrix putty to NovaBone calcium-phosphosilicate synthetic putty in a cranial defect model, J. Craniofac. Surg., 2014, 25, 657-661

[31] Fredericks D., Petersen E.B., Watson N., Grosland N., Gibson-Corley K., Smucker J., Comparison of two synthetic bone graft products in a rabbit posterolateral fusion model, Iowa Orthop. J. 2016, 36, 167-173

[32] Wilson J., Low S.B., Bioactive ceramics for periodontal treatment: comparative studies in the patus monkey, J. Appl. Biomater., 1992, 3, 123-129

[33] Wilson J, Pigott G.H., Schoen F.J., Hench L.L., Toxicology and biocompatibility of bioglasses. J. Biomed. Mater. Res. 1981, 15, 805-817