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The Evaluation of Bone Regeneration Following Socket Preservation with Concentrated Growth Factor (CGF) and Poly Lactic-Co-Glycolic Acid (PLGA) Scaffold in Rabbits

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ABSTRACT

Various grafting materials are utilised to facilitate regeneration. There is currently a paradigm shift towards applying poly lactic-co-glycolic acid (PLGA), which is regarded as an excellent scaffold for tissue engineering. Concentrated growth factor (CGF) has also been reported to promote wound healing. Nevertheless, the role of PLGA microspheres as a substitute for bone graft material with CGF in bone regeneration remains unclear. This study was designed to evaluate the effect of CGF with PLGA on bone formation and the expression of alkaline phosphatase (ALP) following socket preservation. PLGA microspheres were prepared using double solvent evaporation method and observed under scanning electron microscopy (SEM). A 6 mL of rabbit's blood was collected from the marginal ear vein and centrifuged to obtain CGF. Blood was also collected for ALP assessment from 24 New Zealand White (NZW) male rabbits subjected to the first upper left premolar extraction. Sockets were filled with CGF, PLGA, CGF+PLGA or left empty and observed with microscopic computed tomography (micro-CT) at four weeks and eight weeks. The SEM image revealed a spherical shape with interconnected pores on the surface of the PLGA particles. Repeated measures ANOVA were used to evaluate the effect of time and treatment ($p < 0.05$) with significant differences in bone width, height, volume, volume fraction and expression of ALP was observed with CGF+PLGA. Both CGF and PLGA have the potential as the alternative grafting materials and this study could serve as an ideal benchmark for future investigations on the role of CGF+PLGA in bone regeneration enhancement.

Keywords: *Concentrated growth factor; poly lactic-co-glycolic acid; regeneration; socket preservation*

INTRODUCTION

Being a tooth-dependent structure, the alveolar bone requires continuous stimulation of the periodontal ligament through occlusal forces to maintain structural integrity (Dimova, 2014). However, the bone undergoes physiological resorption following tooth extraction which is accompanied by a rapid physiological bone resorption rate within the first six months of the extraction. This process continues throughout the individual's life, which may complicate implant and prosthesis replacement in the future (Fee, 2016). In an empty socket, osteogenesis occurs following the differentiation of osteoblasts by the osteoprogenitor cells in the ruptured and remnant periodontal ligament (Devlin & Sloan, 2002). The activity of osteoblasts results in the deposition of osteoid matrix and bone formation, which is reflected through a series of bone markers, including alkaline phosphatase (ALP) (Gundberg, 2000). Socket preservation is a clinical procedure aimed at reducing physiological alveolar bone resorption (Jung *et al.*, 2018). The procedure entails the application of various grafting materials immediately after tooth extraction. By reducing the amount of physiological bone resorption, socket grafting helps in the preservation of the bony architecture (Sbordone *et al.*, 2016; Aimetti *et al.*, 2018; Tomasi *et al.*, 2018). Over the past decades, various approaches are utilised to manage extraction sockets. Apparently, there is no evidence that highlights the type of material that is superior in facilitating bone formation in regeneration procedure, including socket grafting (Chen *et al.*, 2015).

Currently, the focus has shifted to the application of poly lactic-co-glycolic acid (PLGA). The use of PLGA as a synthetic biodegradable polymer is approved by the Food and Drug Administration (FDA) in regenerative medicine and dentistry (Makadia & Siegel, 2011; Martins *et al.*, 2018). The unique surface morphological characteristics demonstrated by porous PLGA microspheres enable them as excellent carriers for local drug delivery in treating periodontitis as the surface feature allows sustained release of drugs and growth factors (Ali *et al.*, 2019; Lecio *et al.*, 2020). In addition, PLGA has been acknowledged as a biodegradable scaffold that facilitates a conducive environment to support cell attachment and proliferation, thereby contributing to tissue growth and formation. The exclusive surface features of PLGA contribute to its porosity and osteoconductivity property and make it a prospective excellent scaffold for bone graft materials substitute used in new bone regeneration by facilitating cell behaviour and performance (Martins *et al.*, 2018; Qi *et al.*, 2019; Tao *et al.*, 2019; Zhao *et al.*, 2021).

On the other hand, concentrated growth factor (CGF), which is the latest generation of platelet concentrates introduced by Sacco in 2006, provides an alternative to the expensive recombinant human growth factors to be delivered to the respected sites besides functioning as scaffolds for cells (Chen & Jiang, 2020). It relies on the role of activated platelets as the natural source of growth factors. CGF provides concentrated suspension of growth factors such as platelet-derived growth factor (PDGF), transforming growth factor beta (TGF-β), insulin-like growth factor (IGF), epidermal growth factor (EGF) and vascular endothelial growth factor (VEGF) to induce better wound healing (Feigin & Shope, 2019). The application of CGF may also further reduce the cost of treatment and minimise cross-infection due to its autologous nature. It was found that CGF is a potential alternative to promote new bone formation as its sole use or in combination with other bone graft substitute which results in better new bone formation (Xu *et al.*, 2019; Fang *et al.*, 2020).

Nevertheless, the effects of PLGA microspheres as the substitute for bone graft materials and in combinatory with CGF on bone regeneration are unclear and requires further research. In addition, most of the studies had investigated the expression of ALP following CGF application on cell

culture. To the best of our knowledge, there is no study that provide comparative effects of both CGF and PLGA in bone regeneration following socket preservation. Therefore, this animal experimental study was designed to provide a comparative effect and to investigate the influence of CGF with PLGA scaffold on bone regeneration outcome (i.e., at four weeks and eight weeks of study) and expression of ALP following tooth extraction using New Zealand White (NZW) rabbits as subjects.

The extraction socket bone width, bone height, bone volume and fraction of bone volume were observed and evaluated using microscopic computed tomography (micro-CT). These parameters are considered relevant and frequently reported upon observing bone regeneration in an extraction socket following socket preservation procedures (Yasunami *et al.*, 2015; Okada *et al.*, 2019). Overall, it was hypothesised that the application of CGF and PLGA will result in better clinical outcomes as manifested by greater mean bone width, mean bone height, mean bone volume and mean fraction of bone volume of newly formed bone occupying the extraction sockets as compared to the control group. This is in accordance with other animal and clinical studies that have highlighted the use of various bone grafting materials in socket preservation procedures to minimise the rate of physiological alveolar bone resorption and augment new bone formation (Manso *et al.*, 2011; Ramaglia *et al.*, 2018; Tomasi *et al.*, 2018). It was also hypothesised that the application of both materials will result in greater ALP expression as compared to the control.

MATERIALS AND METHODS

This study was reviewed and approved by the Committee on Animal Research and Ethics (UiTM CARE) (Ref. No.: 256/2018), and was conducted according to the Animal Research: Reporting of *In Vivo* Experiments (ARRIVE) Guidelines 2.0 (Percie du Sert *et al.*, 2020). In this study, 24 NZW healthy male rabbits, aged between 8 and 12 months, weighing between 2.5 and 4.5 kg and had no physical deformity were recruited as the study subjects. They were quarantined for one week in a quarantine room.

Thereafter, the rabbits were transferred to a procedure room for two weeks to allow for acclimatisation before the research procedure. Each rabbit was housed in a different cage with temperature-controlled rooms, fed with a standardised diet and had free access to tap water. Furthermore, the health, feeding and housing condition of each rabbit was examined daily by a researcher. Each rabbit was randomly assigned with an alphabet before being randomly assigned to four groups (six rabbits per group); (1) CGF, (2) PLGA, (3) CGF+PLGA and (4) empty socket (as the control).

Preparation of PLGA Particles

PLGA microspheres were prepared using double solvent evaporation method where 1 g PLGA was mixed with 5 mL dichloromethane. A 250 µL aliquot of phosphate-buffered saline was then added to the mixture and homogenised at 9,000 rpm to form the primary emulsion. Next, 200 mL of 0.3% polyvinyl alcohol solution was added to the mixture and homogenised at 4,000 rpm to form the secondary emulsion. The mixture was then stirred for 4 h at 300 rpm to allow dichloromethane to evaporate and the microspheres to precipitate. The microspheres were washed with distilled water by centrifugation and suspended in 10 mL ethanolic sodium hydroxide solution to enhance the porosity of the microspheres. The suspension was mixed and vortexed at 1,400 rpm in different periods, sieved for the size of 40 µm and rewashed using distilled water. The particles were then freeze-dried for 72 h to get the final PLGA microspheres (Fig. 1) (Qutachi *et al.*, 2013)

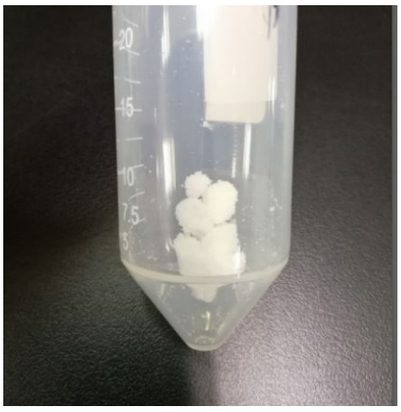

Fig. 1 The PLGA microspheres particles after being freeze-dried for 72 h.

Observation of PLGA Microspheres under Scanning Electron Microscopy (SEM)

PLGA microspheres were observed and analysed for particle and pore size measurement under SEM. The freeze-dried PLGA microspheres were directly dropped onto its platform, air dried and covered by a 15–20 nm layer of gold. The pore characteristic of the microspheres was then examined with SEM (Tabletop TM3000, Hitachi, Tokyo, Japan) under 400× magnification (Qutachi *et al.*, 2013; Vysloužil *et al.*, 2016) EBs are dispersed to single cell suspensions with a subsequent monolayer culture. Moreover, where the 3D integrity of an EB is maintained, cytokines or drugs of interest to stimulate differentiation are often added directly to the culture medium at fixed concentrations and effects are usually limited to the outer layers of the EB. The aim of this study was to create an EB model with localised drug and or growth factor delivery directly within the EB. Using poly(DL-lactic acid-co-glycolic acid.

Preparation of CGF

A wooden box was used to restrain the rabbits and 6 mL blood was drawn from their marginal ear vein. The blood was placed into a vacutainer tube and immediately centrifuged to process the CGF (Tabletop Centrifuge Model 2420, Kubota, Tokyo, Japan) at different speeds to let the blood separate into fractions. The speeds were set according to the literature; 30 seconds acceleration, 2 min at 2,700 rpm, 4 min at 2,400 rpm, 4 min at 2,700 rpm, 3 min at 3,000 rpm, 36 seconds deceleration and stop (Kim *et al.*, 2014; Takeda *et al.*, 2015). As shown in Fig. 2, three layers were generated following centrifugation: an upper layer containing platelet poor plasma, a middle layer consisting of CGF and a lower layer made up of red blood cells. CGF was scraped off from the red blood cell layer using a sterilised surgical scissor once the layer has been removed from the vacutainer after centrifugation.

The combined treatment (CGF+PLGA) was fabricated by incorporating the PLGA microspheres and CGF together. This was achieved by the centrifugation of both materials at 1,000 rpm for 2 min (Table Top Micro Refrigerated Centrifuge Model 3500, Tokyo, Japan) at room temperature (Lee *et al.*, 2015).

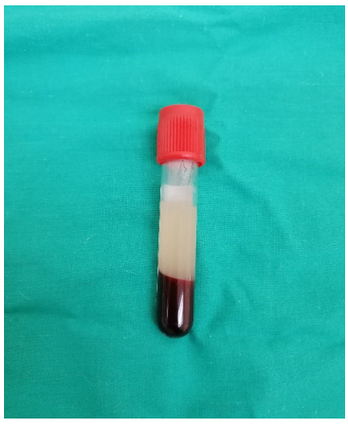

Fig. 2 Layers that formed after the centrifugation process.

Serum Sampling for ALP Analysis

Serum samples were collected from each rabbit by drawing 6 mL of venous blood from marginal ear vein. Thereafter, the whole blood was allowed to clot by placing the samples at room temperature for 2 h. After 2 h, the serum vacutainer was centrifuged using a centrifugation

machine (Table Top Centrifuge Model 2420, Kubota, Tokyo, Japan) for 20 min at 1,000 rpm. Serum samples were retrieved using a pipette and stored in the 1.5 mL Eppendorf tube (Eppendorf®, Sigma-Aldrich, USA) at –80°C. Blood was collected for serum preparation prior to the surgical procedure, and at four weeks and eight weeks post-surgery, and analysed in triplicate using rabbit ALP enzyme-linked immunosorbent assay (ELISA) kit (FineTest®, Wuhan, China).

Socket Preservation Procedure

The rabbits were weighed before the surgical procedure to determine the amount of anaesthetic needed. Each rabbit was sedated and anaesthetised following intramuscular injection of a cocktail of ketamine (35 mg/kg, Ilium Ketamil, Australia) and tiletamine/zolazepam (15 mg/kg, Zoletil®, Virbac, New Zealand). After the rabbit was fully anaesthetised, access into the oral cavity was facilitated by placing a rodent mouth gag (KRUUSE®, Denmark) on the upper and lower incisors. The surgical site on the upper left first premolar tooth, as shown in Fig. 3, was disinfected with 0.12% chlorhexidine digluconate (Oradex®, Malaysia). The buccal and palatal sides of the tooth were locally anaesthetised with mepivacaine hydrochloride 2% (Scandonest® 2% L, France).

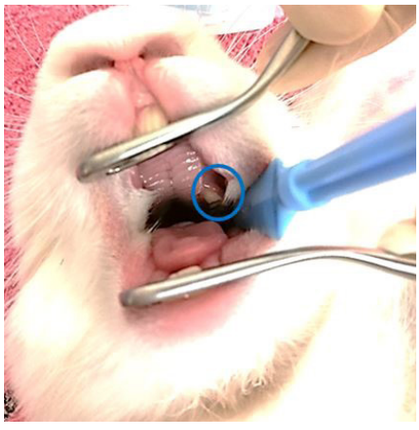

Fig. 3 The surgical site on the upper left first premolar tooth of the rabbit.

Periodontal ligaments surrounding the tooth were loosened using a periodontal probe and the tooth was luxated with an elevator. Next, rabbit molar teeth extraction forceps (KRUUSE®, Denmark) was used to extract the upper left first premolar (Chen *et al.*, 2015). The extraction socket was cleaned with normal saline and chlorhexidine digluconate 0.12% (Oradex®, Malaysia). The extraction sockets were filled with (1) CGF, (2) PLGA, (3) CGF+PLGA or (4) left empty (control) as previously described for treatment group allocation. The extraction socket was protected with simple interrupted sutures using 4/0 resorbable suture (Neosorb PGLA, Medipac, Greece).

Post-operative analgesia was administered by subcutaneous injection of meloxicam (0.5 mg/kg, Melonex®, India) immediately after the surgical procedure and every 24 h for five days. In addition to the food pellet, the rabbits were fed green leafy vegetables for one week. Behaviours such as lethargy, decreased appetite, strained facial expression, increased frequency of respiration and rapid shallow breathing were observed closely daily for two weeks. These behaviours were monitored as they may indicate signs of infection or discomfort experienced by the rabbits.

Three rabbits from each group were euthanised in the fourth week while the remaining three in the eighth week following the procedure with an anaesthetic overdose of sodium pentobarbital (0.7 mL/kg, Dolethal®, France) via intracardiac injection. Their heads were removed and the maxillary sections on the upper left first premolar region were retrieved for radiographic assessment.

Micro-CT Assessment

Specimens were scanned with SkyScan 1076 software (Bruker Corporation, USA) according to the manufacturer instructions using the setting as follows: 80 kV voltage, 0.5 mm aluminium filter and 18 µm resolution.

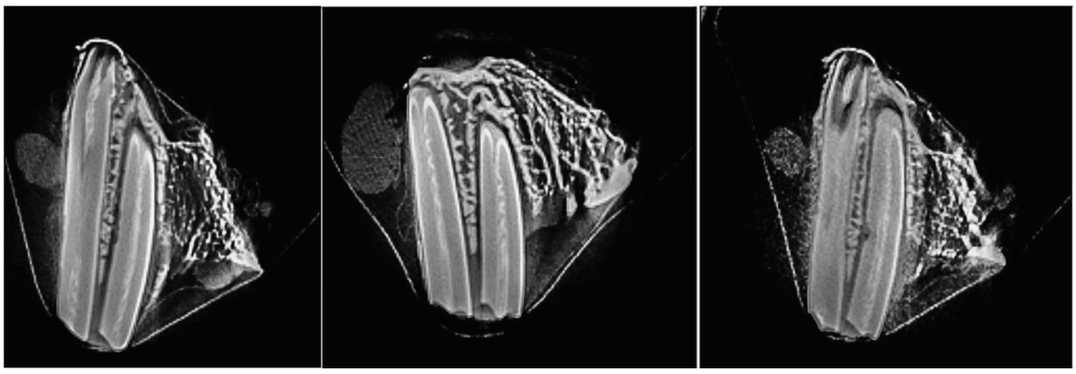

Fig. 4 Radiographic images of bone specimen from three different samples.

Fig. 4 illustrates the examples of the images. The images were reconstructed using GPUReconServer version 1.6.5.0 (Bruker Corporation, USA) and viewed using DataViewer version 1.5.1.2 (Bruker Corporation, USA). Horizontal bone width at the level of alveolar crest and the vertical bone height were measured at the centre of the extraction socket and bone volume and fraction were analysed using CTAn software version 1.14 (Bruker Corporation, USA).

Statistical Analysis

SPSS software version 26.0 (IBM, USA) was used to perform statistical analyses. Descriptive statistics were reported as mean and standard deviation. All data were subjected to data exploration and cleaning before analysis by checking any missing values through the SPPS software. Assumption of normality, homogeneity of variances and compound symmetry were checked and fulfilled. A repeated-measures ANOVA was applied to evaluate the time and treatment effects. Pairwise comparison with confidence interval adjustment with Bonferroni correction was performed for time-effect analysis. Meanwhile, post-hoc multiple comparisons were executed when a significant treatment between groups was found. A p-value < 0.05 was considered for significant effects and statistical difference between the treatments.

RESULTS

SEM Observation of PLGA Particles

The surface configuration of PLGA microspheres was observed using SEM, in which there is spherical shape with the interconnected pores on its surface. Fig. 5 shows the features of the microspheres under SEM with 400× magnification. The particle size ranged from 53.709 μm to 120.375 μm and an average of 40 μm pore size was observed based on the analysis using Image J software (Fiji 1.49, USA).

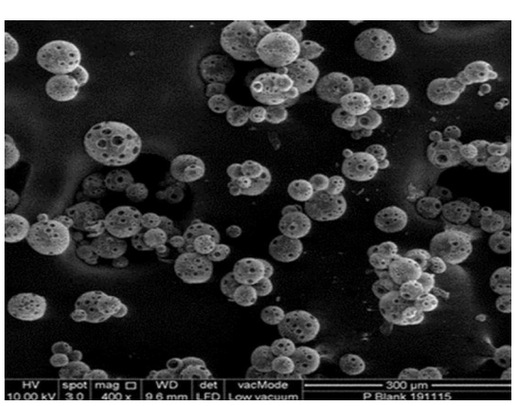

Fig. 5 SEM image observation of porous PLGA microspheres (400× magnification).

Radiographic Measurement of the Extraction Sockets

All extractions of the upper left first premolar healed uneventfully without any complications and all 24 rabbits were subjected to data analysis according to their time and allocation to treatment groups. Table 1 shows the average horizontal bone width and height of the extraction socket after the micro-CT assessment. There was a gradual increase in mean horizontal bone width for CGF and CGF+PLGA groups from four to eight weeks with the PLGA group showing the highest mean horizontal width of 3.50 mm and 3.53 mm at four weeks and eight weeks, respectively. There was a gradual increase in mean bone height for all treatment groups from four to eight weeks. Compared to other treatment groups, the CGF+PLGA group showed the highest mean bone height of 6.82 mm and 7.58 mm at four weeks and eight weeks, respectively.

Table 2 shows the average bone volume and fraction of bone volume with regard to treatment. There was an increase in mean bone volume for all treatment groups from four to eight weeks with a two-fold increase in CGF+PLGA group. CGF+PLGA showed the highest mean bone volume of 68.3 mm^3 and 135.30 mm^3 at four weeks and eight weeks, respectively. There was a gradual increase in the mean fraction of bone volume in all treatment groups from four to eight weeks. Compared to other treatment groups, the CGF+PLGA group showed the highest mean fraction of bone volume of 31.66% and 54.52% at four weeks and eight weeks, respectively.

Table 3 shows the average ALP concentration and all treatment groups showed a marked increase in ALP concentration from baseline to four weeks. At four weeks, CGF+PLGA showed the highest mean concentration of ALP, followed by the CGF and PLGA groups with 5.97 ng/mL, 5.37 ng/mL, and 4.14 ng/mL, respectively. At eight weeks, only a small increase in ALP concentration was observed in all the treatment groups with the CGF+PLGA group recording the highest mean concentration of ALP of 5.98 ng/mL.

Repeated measures ANOVA within group analysis was performed, showing that there was a significant difference in all the variables except the average horizontal bone width within each group based on time pair ($F = 2.077$, p-value = 0.158). Tables 4 and 5 show the results following the pairwise comparison with adjusted confidence interval. Repeated measures ANOVA between group analyses was applied, followed by post hoc multiple comparisons and the results are presented in Table 6.

Table 1 Mean horizontal bone width and bone height (mm)

Time	Control		CGF		PLGA		CGF+PLGA	
	Bone width	Bone height	Bone width	Bone height	Bone width	Bone height	Bone width	Bone height
4 weeks	2.80 (0.47)	4.54 (1.00)	2.83 (0.71)	6.07 (0.08)	3.50 (0.80)	6.38 (1.18)	3.05 (0.54)	6.82 (0.86)
8 weeks	2.83 (0.49)	7.08 (0.50)	3.40 (0.92)	7.10 (0.25)	3.53 (0.24)	7.26 (1.18)	3.42 (0.39)	7.58 (0.56)

Note: Treatment group (presented as mean [SD]).

Table 2 Mean bone volume (mm^3) and fraction of bone volume (%)

Time	Control		CGF		PLGA		CGF+PLGA	
	Bone volume	Fraction of bone volume	Bone volume	Fraction of bone volume	Bone volume	Fraction of bone volume	Bone volume	Fraction of bone volume
4 weeks	49.13 (6.29)	25.12 (2.49)	47.63 (20.96)	25.44 (1.57)	67.15 (7.51)	30.71 (12.92)	68.39 (8.43)	31.66 (1.56)
8 weeks	60.86 (21.60)	31.84 (0.75)	76.79 (14.62)	37.37 (1.49)	112.68 (14.26)	40.55 (0.41)	135.30 (27.60)	54.52 (4.29)

Note: Treatment group (presented as mean [SD]).

Table 3 Mean concentration of ALP (ng/mL)

Time	Control	CGF	PLGA	CGF+PLGA
Baseline	1.80 (0.17)	1.77 (0.23)	1.98 (0.29)	1.87 (0.15)
4 weeks	3.70 (0.52)	5.37 (2.09)	4.14 (1.09)	5.97 (2.24)
8 weeks	3.77 (0.66)	5.41 (1.64)	4.44 (0.57)	5.98 (0.88)

Note: Treatment group (presented as mean [SD]).

Table 4 Comparison within each treatment group based on time pairs (time effect)

Comparison 4 weeks – 8 weeks	Control *p*-value	CGF *p*-value	PLGA *p*-value	CGF+PLGA *p*-value
Horizontal bone width	0.914	0.319	0.944	0.019*
Bone height	<0.001*	<0.001*	0.034*	0.032*
Bone volume	0.391	0.025*	0.066	0.046*
Fraction of bone volume	0.034*	<0.001*	0.312	0.021*

Note: *Significant.

Table 5 Comparison of mean ALP concentration within each treatment group based on time (time effect)

Comparison	Control *p*-value	CGF *p*-value	PLGA *p*-value	CGF+PLGA *p*-value
Baseline – 4 weeks	<0.001*	<0.001*	<0.001*	<0.001*
Baseline – 8 weeks	<0.001*	<0.001*	<0.001*	<0.001*
4 weeks – 8 weeks	1.000	1.000	0.829	1.000

Note: *Significant.

Table 6 Mean difference of variables among four treatment groups

Variable	Comparison	Mean difference (95% CI)	*p*-value
Horizontal bone width	Control and CGF	–0.30 (–0.71, 0.11)	0.228
	Control and PLGA	–0.70 (–1.11, –0.29)	<0.001*
	Control and CGF+PLGA	–0.42 (–0.83, –0.01)	0.045*
	CGF and PLGA	–0.40 (–0.81, 0.01)	0.057
	CGF and CGF+PLGA	–0.12 (–0.53, 0.29)	0.862
	PLGA and CGF+PLGA	0.28 (–0.13, 0.69)	0.272
Bone height	Control and CGF	–0.77 (–1.68, 0.14)	0.122
	Control and PLGA	–1.01 (–1.92, –0.10)	0.024*
	Control and CGF+PLGA	–1.39 (–2.29, –0.48)	0.001*
	CGF and PLGA	–0.24 (–1.15, 0.67)	0.894
	CGF and CGF+PLGA	–0.62 (–1.53, 0.29)	0.276
	PLGA and CGF+PLGA	–0.38 (–1.29, 0.53)	0.680
Bone volume	Control and CGF	–7.21 (–46.11, 31.68)	0.933
	Control and PLGA	–34.92 (–73.81, 3.98)	0.080
	Control and CGF+PLGA	–46.85 (–85.75, –7.95)	0.020*
	CGF and PLGA	–27.71 (–66.60, 11.19)	0.184
	CGF and CGF+PLGA	–39.64 (–78.54, –0.74)	0.046*
	PLGA and CGF+PLGA	–11.93 (–50.83, 26.97)	0.769

(*Continued on next page*)

Table 6 (*Continued*)

Variable	Comparison	Mean difference (95% CI)	*p*-value
Fraction of bone volume	Control and CGF	–2.92 (–12.98, 7.13)	0.795
	Control and PLGA	–7.15 (–17.20, 2.91)	0.185
	Control and CGF+PLGA	14.61 (–24.66, –4.56)	0.007*
	CGF and PLGA	–4.22 (–14.28, 5.83)	0.569
	CGF and CGF+PLGA	–11.69 (–21.74, –1.63)	0.024*
	PLGA and CGF+PLGA	–7.46 (–17.52, 2.59)	0.161
ALP concentration	Control and CGF	–1.09 (–1.72, –0.47)	<0.001*
	Control and PLGA	–0.43 (–1.06, 0.19)	0.273
	Control and CGF+PLGA	–1.52 (–2.14, –0.89)	<0.001*
	CGF and PLGA	0.66 (0.04, 1.28)	0.034*
	CGF and CGF+PLGA	–0.42 (–1.05, 0.20)	0.293
	PLGA and CGF+PLGA	–1.08 (–1.71, –0.46)	<0.001*

Notes: *Significant; CI = confidence interval.

DISCUSSION

Histomorphometric, radiographic and clinical investigations have shown that marked physiological bone resorption occurs within the first three to six months post-tooth extraction (Schropp *et al.*, 2003; Araújo & Lindhe, 2005; Discepoli *et al.*, 2013). Such event may complicate future prosthetic planning due to lack of amount of available bone; thus, the socket grafting or socket preservation procedure is performed to minimise the amount of bone resorption (Fee, 2016; Jung *et al.*, 2018). In addition, the procedure helps to facilitate the augmentation of new bone formation in the extraction sockets. A suitable environment for cellular attachment and proliferation, adequate supply of blood and nutrients and complete removal of by-products are required for bone regeneration to take place (Abbasi *et al.*, 2020). These events are promoted when the surface material employed as a scaffold possess interconnected pores and adequate porosity, which is manifested by PLGA microspheres in this study.

In addition to surface characteristics of the PLGA particles with interconnected pores that enable it to act as a scaffold for cellular invasion and attachment, adequate particle and pore size is required to allow for a sustained and controlled release of an active agent, which in this study was the growth factors from CGF. Based on the SEM imaging, these features were also manifested by the PLGA microspheres in this study as the porous particle size ranged between 53.709 μm and 120.375 μm. Furthermore, it was also reported that the particle size of 10 μm to 200 μm precipitates an optimum active agent release (i.e., drugs or growth factor) from the PLGA (Lemperle *et al.*, 2004; Han *et al.*, 2016). In terms of pore size, the resulting microspheres in this present study was in average 40 μm as pore size measuring between 10 μm and 50 μm allows for zero order release of an agent from the PLGA (Molavi *et al.*, 2020). Besides, this pore size is acceptable to favour scaffold vascularisation and the growth of osteoblasts (Sicchieri *et al.*, 2012; Abbasi *et al.*, 2020).

The extraction socket bone width, bone height, bone volume and fraction of bone volume were observed and evaluated using micro-CT. Interestingly, mean bone width was shown to be the highest when extraction sockets were treated with PLGA at four weeks and eight weeks, followed by CGF+PLGA group. However, no significant difference was observed between four weeks and eight weeks among all the treatment

groups, except for the CGF+PLGA group. Furthermore, there was a significant difference between treatment groups using CGF+PLGA and PLGA alone compared to the control. This finding corroborates the result from a previous study when PLGA was added with beta-tricalcium phosphate in the extraction socket of maxillary first premolar in beagle dogs (Okada *et al.*, 2019).

In addition, it was hypothesised that CGF+PLGA would augment the amount of newly formed bone height in the extraction socket. An overall significant difference of mean bone height with each treatment group was observed at four weeks and eight weeks. This could be due to the onset of new bone formation within the first week and second week of healing post-tooth extraction as reported in previous animal studies (Araújo & Lindhe, 2005; Discepoli *et al.*, 2013). In these studies, new bone formation was observed histologically following distal root extraction of premolars in the beagle dogs within the first week, whereas a woven bone was noticed at four weeks before the onset of remodelling. The present study showed that the highest mean bone height was observed at four weeks and eight weeks following treatment with CGF+PLGA. Similarly, there was a significant difference in the bone height in the extraction sockets treated with CGF+PLGA and PLGA alone compared with the control group. This finding is consistent with other studies reporting the use of PLGA with other materials (Brown *et al.*, 2015; Yasunami *et al.*, 2015; Okada *et al.*, 2019). For instance, the role of PLGA as a carrier for CGF can be acknowledged as there was a significant difference observed between control and CGF+PLGA groups. This concurs with a previous study whereby the incorporation of PLGA with active agents such as fluvastatin resulted in greater vertical bone height at 28 days after maxillary first molar extraction in Wistar rats than with PLGA alone (Yasunami *et al.*, 2015). The present study reflects that PLGA is able to act as an excellent carrier for other active components and the application of PLGA alone as scaffold in facilitating bone formation. It is evidenced that the application of PLGA particles alone could be an alternative to bone grafting materials due to its potentiality to augment the rate of bone formation, which is interpreted as bone width, bone height, bone volume and the fraction of bone volume in the present animal study.

It was also hypothesised that a combination of CGF and PLGA would result in a better bone volume of newly formed bone in the extraction sockets. Overall, the mean bone volume was significantly different in each treatment group at four weeks and eight weeks. These findings were observed in the group treated with CGF and CGF+PLGA. Nevertheless, the highest mean bone volume was detected in groups treated with CGF+PLGA, followed by PLGA and CGF groups at four weeks and eight weeks. Although no significant difference was noticed between the control and PLGA groups, the mean bone volume increased in all treatment groups. The outcome could be due to the deposition of new bone as early as the first week of healing following tooth extraction, which continues until the fourth week of healing (Araújo & Lindhe, 2005; Discepoli *et al.*, 2013). However, a significant difference was observed in treatment comparisons between the control and CGF+PLGA, and between CGF and CGF+PLGA groups. This finding agrees with previous studies that demonstrated the role of PLGA as scaffolds, carrier of active agents, and enhancing a greater bone volume (Yasunami *et al.*, 2015; Okada *et al.*, 2019).

Additionally, it was postulated that CGF+PLGA would yield a greater fraction of bone volume of newly formed bone in the extraction sockets. Similar findings were observed as the highest fraction of bone volume was noticed in CGF+PLGA group at four weeks and eight weeks of treatment. The overall mean fraction of bone volume was significantly different at four weeks and eight weeks in each treatment group. These findings were observed in the group treated with CGF and CGF+PLGA. Likewise, the

treatment comparison between the control and CGF+PLGA, and between CGF and CGF+PLGA were significantly different. These findings are similar to that of Chen *et al.* (2015) whereby the addition of osteogenic inducers such as dexamethasone, vitamin C and beta sodium glycerophosphate resulted in a greater fraction of bone volume.

CGF also had an osteogenic inducing effect based on the outcomes of bone volume and fraction of bone volume when compared to the CGF+PLGA group. This reinstates that CGF has the potential to augment the amount of bone volume and corroborates the reports from previous studies. These studies employed CGF in various defects of the parietal, calvarial and femur bones, as well as peri-implant defect and maxillary sinus augmentation (Kim *et al.*, 2014; Takeda *et al.*, 2015). CGF also contributes similarly based on its denser structure, presence of naturally formed fibrin clot, and abundant growth factors, fibrinogen and CD34 cells (Yu *et al.*, 2019; Fang *et al.*, 2020). For instance, CD34 cells are required for angiogenesis and tissue regeneration (Rodella *et al.*, 2011; Fang *et al.*, 2020), while the structure is protective against early degradation, and promotes a higher and sustained release of growth factors (Lee *et al.*, 2015; Qiao *et al.*, 2016). Furthermore, due to the alternate speed used in its fabrication, it resulted in a superior fibrin structure with interwoven cross-linked network that provides an attachment for cytokine and recruitment of cells towards the CGF scaffold (Qiao *et al.*, 2016; Fang *et al.*, 2020). Therefore, this present study also showed that CGF itself is able to promote greater bone formation compared to the control group.

In this study, the application of CGF+PLGA resulted in a significant and greater mean bone volume, the fraction of bone volume, bone width and bone height compared to the other groups. This could be due to the combined unique features of CGF and PLGA. Previous studies also found that the combination of CGF with other grafting materials resulted in better regeneration outcome (Qiao *et al.*, 2016; Xu *et al.*, 2019; Fang *et al.*, 2020).

In this study, the combination of CGF+PLGA indicated the highest mean concentration of ALP at four weeks and eight weeks, followed by the groups treated with only CGF and PLGA. The mean concentration of ALP increased significantly from the baseline and four weeks, whereas the measurement was comparable between four weeks and eight weeks in all the treatment groups. The overall mean concentration of ALP was significantly different between baseline and four weeks, and between baseline and eight weeks in each treatment group. However, there is no significant difference observed between four weeks and eight weeks.

One of the reasons for these results is the role of ALP in facilitating the deposition of bone matrix and maturation, which is crucial during the early stage of bone formation. This might be responsible for the significant increase in ALP from the baseline to four weeks and eight weeks as observed in this study. Besides, a gradual release of ALP is expected after the initial phase of healing (Golub & Boesze-Battaglia, 2007; Vimalraj, 2020). Similar findings were reported in a study that evaluated the expression of genes that encode for ALP in bone samples following tooth extraction in Wistar rats (Rodrigues *et al.*, 2016). Likewise, ALP expression increased from 7 to 28 days and no significant difference was noticed between 14 and 28 days (Rodrigues *et al.*, 2016). This shows that the ALP expression occurs in a greater amount during the initial stage of bone formation and mineralisation and expressed gradually throughout the healing period as found in the present study.

ALP expression was also significantly different between the treatment groups; control and CGF, control and CGF+PLGA, CGF and PLGA, and PLGA and CGF+PLGA. The current result is consistent with previous studies where the application of CGF promoted a greater release of ALP from osteoblasts. CGF enhanced the release of

the osteogenic markers, and the increment occurred gradually up to 14 and 21 days compared to the control group without CGF (Takeda *et al.*, 2015; Sahin *et al.*, 2018; Zhang & Ai, 2019; Rochira *et al.*, 2020). When there is a need of new bone formation, various signals will be sent to facilitate the process. Since CGF is enriched with various growth factors, these growth factors will activate various signalling pathways in differentiation and proliferation of osteoblast cells. Signalling pathways such as BMP/RUNX2 (CBAf1, AML3)/Osterix network and the WNT signalling cascade will be activated. Besides, PI3K/Akt pathway is also activated by IGF, which are abundant in CGF (Vimalraj, 2020). In addition, it was postulated that the signalling pathways are enhanced through the application of PLGA as there was a significant difference noted and the average expression of ALP is the highest in the CGF+PLGA group. This signifies that there is more active bone regeneration activity by osteoblasts with application of grafting materials as compared to the control.

Due to limitation, the collected serum of the rabbits was investigated for bone-specific ALP only in the fourth and eighth weeks. Even though this study provides radiographic and ALP analysis following the application of material of interest, which is the combination of CGF+PLGA, the results can be accompanied with histological and histomorphometric analysis and other osteogenic markers in the future studies.

CONCLUSION

In a nutshell, this study could serve as a standard and ideal benchmark for future investigations on the role CGF+PLGA microspheres to improve bone regeneration rate. Both CGF and PLGA also act as a scaffold to promote cell behaviour, thus, making them a great alternative potential regenerative material.

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