Calcium Phosphate/Collagen Ratio in Bone Grafts Influences Bone Repair in a Rabbit Femoral Condyle Defect Model

Sivakumar Avanthika and Xiaoman Luo

Abstract Bone grafts are used in orthopaedics for treatment of conditions including tumours, trauma, and infection, and critical-sized defects that do not spontaneously heal without surgical intervention. While autografts harvested from the patient's body and allografts from donors are often used as graft material, a wide variety of synthetic bone graft substitutes are also available as alternatives. In this study, we aimed to determine how calcium phosphate/collagen ratio would influence the capacity of a biphasic bone graft to support bone repair. The in vitro degradation of scaffolds with three different calcium phosphate/collagen ratios were evaluated in PBS over 7 days. It was found that higher calcium phosphate/collagen ratios resulted in less pH decrease, and scaffolds with a lower calcium phosphate/collagen ratio plateaued at higher pH. The in vivo performance of the three implants were evaluated in a femoral condyle defect model in New Zealand White Rabbits over 12 weeks, and the results obtained revealed that calcium phosphate/collagen ratios have no significant impact on degradation rates of the calcium phosphate component of the biphasic scaffold. Percentage of bone growth, on the other hand, increased from 15.4 to 25.8% from the lowest to highest calcium phosphate/collagen ratios, potentially due to calcium phosphate's osteoconductive properties. Overall, higher proportions of calcium phosphate to collagen led to better outcomes in defect healing due to greater bone formation, and matching implant resorption and bone growth rates.

Keywords Bone graft substitutes · Tricalcium phosphate · Collagen · Bone repair

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1 Introduction

Bones possess an intrinsic ability to regenerate during the repair process in the healing of injuries such as fractures, where original tissue is completely regenerated and bone remodelling enhances the mechanical stability of newly grown bone as well [1]. However, the treatment of critical-sized bone defects, which do not heal completely without surgical intervention [2], together with other conditions such as tumours and certain types of trauma, often requires the usage of bone grafts. Autografts have been widely regarded as the 'gold standard' as they possess favourable osteoconductive, osteoinductive, and osteogenic properties, along with a low risk of immunoreactions [1]. They are, however, associated with limitations such as donor site discomfort and morbidity [3], restricted quantity, and considerable costs. Allografts from donors circumvent the aforementioned limitations of autografts but pose an increased likelihood of immunogenicity and infection transmission [1]. Bone graft substitutes have been developed as alternatives, composing a multitude of synthetic and natural biomaterials.

This study focuses on Calcium Phosphate (CaP)-collagen scaffolds as bone graft substitutes for defect healing. CaP, specifically β-Tricalcium Phosphate (β-TCP), has similar characteristics to that of bone mineral and is biodegradable. However, it cannot be used alone in bone implants due to its brittleness [4]. Similarly, collagen performs poorly as a standalone graft material, but significantly enhances graft incorporation when coupled with CaP [5]. There appears to be, however, insufficient data regarding the influence of CaP/collagen ratios in these bone grafts on their performance in defect healing, in terms of bone formation and implant resorption. The rate of scaffold degradation and resorption is critical in determining its biological performance, as where the persistence of graft material is linked to poor clinical outcomes, overly rapid resorption impairs the regenerative ability of natural bone by providing inadequate scaffold support for bone apposition [6].

Ideally, the degradation profile of the implant should match the rate of bone formation for optimum defect healing. CaP and collagen have differing degradation rates and result in the production of different end products, where CaP degradation releases calcium and phosphate ions which stimulate bone mineral formation and cell adhesion, and collagen is resorbed by macrophages and osteoblasts through the action of collagenase [7]. CaP/collagen ratio is hence significant when considering biological performance of the implants.

This study examined the in vitro degradation and in vivo performance of three implants of different CaP/collagen ratios in a critical-sized femoral condyle defect in New Zealand White Rabbits, where we aimed to identify the optimum ratio of CaP to collagen that most successfully promotes bone healing.

Since the release of calcium and phosphate ions from CaP regulates the activation of osteoclasts and osteoblasts to facilitate bone regeneration [7], a larger proportion of CaP in the bone graft will increase osteoconductivity. As such, we hypothesised that the scaffold that contains the highest ratio of CaP in relation to collagen will most successfully promote bone defect healing.

Table 1 Components of each group of samples used for in vitro degradation study

Group	No. of replicates	Scaffold used	Treatment	Total volume/ml
1	3	Low-CaP	PBS + Collagenase (16 U/ml)	5
2			PBS	
3		Mid-CaP	PBS + Collagenase (16 U/ml)	
4			PBS	
5		High-CaP	PBS + Collagenase (16 U/ml)	
6			PBS	
7		–	PBS + Collagenase (16 U/ml)	
8			PBS	

2 Materials and Methods

Cylindrical scaffolds of three different CaP/collagen ratios with diameter of 6 mm and length of 8 mm were synthesised prior to the commencement of this study. As the exact composition and ratios of CaP to collagen utilised cannot be disclosed due to confidentiality rules of our host organisation, the scaffolds will be named 'Low-CaP', 'Mid-CaP', and 'High-CaP', corresponding to the increasing CaP/collagen ratio.

2.1 In vitro *Degradation Assay*

The in vitro degradation profiles of the CaP-collagen scaffolds were assessed by measuring pH changes over time. Eight groups of scaffolds with a diameter of 6 mm and length of 4 mm were immersed in phosphate-buffered saline (PBS) solution intended to mimic in vivo conditions with or without 16 U/ml of collagenase at 37 °C with gentle shaking (Table 1). Groups 1, 3, and 5 contained collagenase, while Groups 7 and 8 were used as controls to confirm that any changes in pH in groups 1 to 6 were solely due to scaffold degradation. pH was measured at 0, 0.5, 1, 2, 4, 6, 8, 24, 48, 72, and 168 h. The assays were performed in triplicates.

2.2 In vivo *Biological Evaluation*

The in vivo biological evaluation was done prior to the commencement of this study. A cylindrical defect measuring 6 mm in diameter and 8 mm in depth was created in the femoral condyle of New Zealand White Rabbits in both the left and right knees and was filled with one CaP-collagen scaffold of the same dimensions (Ø6mm × 8 mm). After a period of 12 weeks, the animals were euthanised via a lethal injection of pentobarbital and the implanted scaffolds were retrieved. The harvested implants

were fixed, dehydrated, and embedded in Poly(methyl methacrylate) (PMMA) prior to conducting this study. Each group included eight replicates and four replicates from each group were analysed in this project due to time constraints.

Micro-Computed Tomography (μ-CT) of Scaffolds. μ-CT scans were performed on Low-CaP, Mid-CaP, and High-CaP scaffolds prior to and at 12 weeks after implantation along the axial plane using the Bruker Skyscan 1176. The scans were processed using CtAn software with a standardised circular region of interest (ROI) of diameter 6 mm and threshold level of 55–255. The following parameters were determined: the total volume of the ROI (TV), total bone volume (BV), and bone volume relative to total volume (BV/TV%). Since CaP has almost identical x-ray attenuation to bone [8], it was not possible to distinguish bone from residual scaffold granules post-implantation; hence, the BV comprised both bone and scaffold material volume. Three replicates of unused scaffolds for each ratio were also scanned using μ-CT as 0-week controls and were analysed using the same parameters to find the TV, BV, and BV/TV%, where the BV/TV% would represent the starting percentage of CaP in the scaffold.

Histological and Histomorphometric Evaluation. The explants were processed through ethanol dehydration and embedded in PMMA following μ-CT analysis for histology and histomorphometric analysis. Acidic ethanol, basic fuchsin, and methylene blue dyes were prepared for histological staining using procedures detailed in the Appendix. The embedded scaffolds were sectioned with a thickness of 10–20 μm using a circular saw microtome and stained. The slides were sectioned parallel to the long axis of the inserted implant and at least 10 stained sections were obtained per sample. Scanned images of the prepared slides were imported into Adobe Photoshop and a standardised ROI was defined. Histomorphometry data was analysed using the histogram function. Material degradation rate was calculated by comparing material percentages at 0 weeks and 12 weeks using the formula:

$$\text{Material degradation rate} = \frac{\text{Initial material\%} - \text{Final material\%}}{\text{Initial material\%}} \times 100$$

2.3 Statistical Analysis

Statistical analysis was performed using Student's *T*-test, where $p < 0.05$ was considered statistically significant, with data presented in the form of mean ± standard deviation. $p < 0.05$ was represented with a single asterisk '*' on graphs.

3 Results

3.1 In vitro *Degradation Assay*

It can be observed that in the Low-CaP groups, there was a pH increase within the first 6 h, and the group lacking collagenase reached a higher peak pH of 8.75 ± 0.06 at 4 h than the group with collagenase, which reached a peak of 8.45 ± 0.03 at 6 h (Fig. 1a, $p < 0.05$). The effect of collagenase was apparent beyond the 6 h mark for Low-CaP, where without collagenase the pH plateaued at around 8.70 ± 0.02, while with collagenase the pH declined gradually to 7.89 ± 0.06 at the 168 h mark. For groups containing the Mid-CaP scaffold, there was an initial increase in pH regardless of the presence of collagenase until a peak pH of 8.25 ± 0.16 was reached at the 24 h mark (Fig. 1b). Beyond the 72 h mark, the pH of the Mid-CaP group with collagenase decreased slightly to 8.09 ± 0.11 at 168 h, while the pH for the Mid-CaP group without collagenase did not decrease ($p < 0.05$). For groups containing the High-CaP scaffolds, the pH remained constant at around 7.10 ± 0.03 throughout 7 days, with the presence of collagenase showing no effect on pH (Fig. 1c).

Across the three groups without collagenase, scaffolds with a lower CaP/collagen ratio plateaued at higher pH and at earlier time points. The Low-CaP and Mid-CaP scaffolds plateaued at pH 8.70 ± 0.02 and 8.25 ± 0.03 ($p < 0.05$), at time points 8 h and 24 h, respectively. Across the three groups with collagenase, Low-CaP and Mid-CaP scaffolds saw a peak pH of 8.45 ± 0.03 and 8.25 ± 0.16 at 4 h and 24 h, respectively. The final pH value at 168 h for groups containing the Low-CaP scaffold with collagenase was 7.89 ± 0.06, which was lower than that of the Mid-CaP

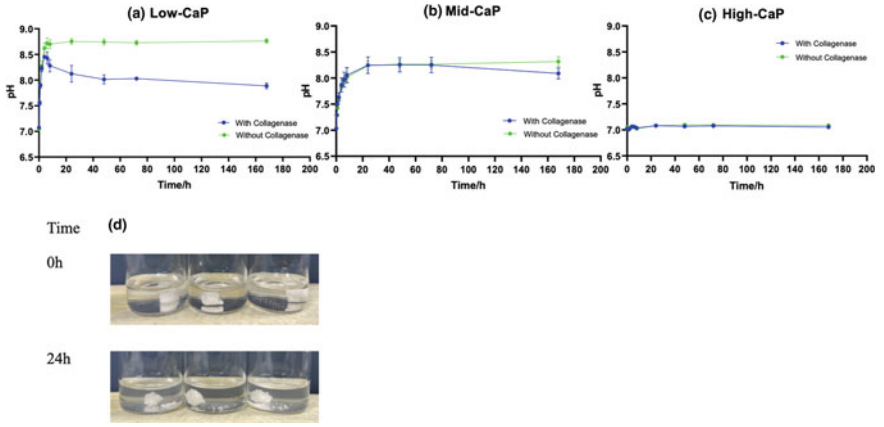

Fig. 1 **a** pH value of Low-CaP in PBS over time ± 16 U/mL collagenase at 37 °C. **b** pH value of Mid-CaP in PBS over time ± 16 U/mL collagenase at 37 °C. **c** pH value of High-CaP in PBS over time ± 16 U/mL collagenase at 37 °C. **d** Visual appearance of Low-CaP scaffold in PBS ± 16 U/mL collagenase at 0 and 24 h

scaffold with collagenase, which was 8.09 ± 0.11 ($p < 0.05$). The High-CaP scaffold maintained a constant pH of around 7.10 ± 0.03 throughout the 168 h regardless of presence of collagenase.

White CaP granules collapsed from the cylindrical scaffolds in all three replicates of Low-CaP scaffolds in the presence of collagenase at 24 h, with part of the structure no longer being maintained (Fig. 1d). Mid-CaP and High-CaP scaffolds, on the other hand, showed no visible structural changes throughout.

3.2 In vivo *Biological Evaluation*

μ-CT of Scaffolds Before and After Implantation. μ-CT scans only visualised high-density tissue, that is hard bone tissue and CaP, due to the threshold of 55–255 used for analysis. High-density bone and CaP cannot be differentiated due to their similar x-ray attenuation values [8]. Visual observation on the unused scaffolds (Fig. 2a) portrayed differences in the starting amount of CaP in the Low-CaP, Mid-CaP, and High-CaP scaffolds. The packing of CaP granules also differed amongst the scaffolds, where scaffolds with lower CaP content contained larger spaces between granules. From the scans taken at 12 weeks, it can be observed trabecular bone had formed for all three scaffolds, indicating that bone regeneration had occurred. Comparison of the high-density tissue volume percentages revealed that there were no significant differences between 0 and 12 weeks ($p > 0.05$) regardless of CaP/collagen ratio (Fig. 2b). This demonstrated that while there was no change in total high-density tissue volume, the composition of the high-density tissue had changed, such that CaP had been replaced partly by new bone.

Histomorphometric Evaluation. In the histological slides depicted in Fig. 3a, trabecular bone tissue was stained pink by basic fuchsin, whereas CaP granules appeared as black granules. The CaP granules appeared to be rounded after 12 weeks of implantation, indicating degradation of CaP in vivo. Fewer granules were present for the Low-CaP scaffold than the Mid-CaP and High-CaP scaffolds. Bone growth appeared around and incorporated CaP granules. Large empty spaces devoid of both bone and material were observed in the slides for Low-CaP and Mid-CaP scaffolds.

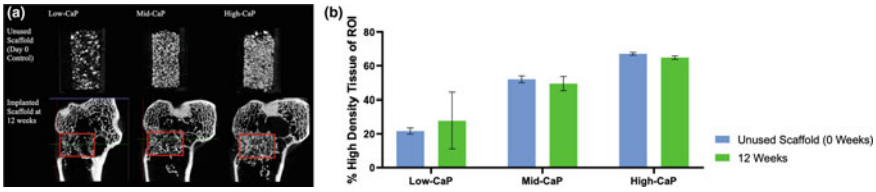

Fig. 2 **a** μ-CT scans of unused and harvested scaffolds where for unused scaffolds, dimensions are Ø6 × 8 mm, and for scaffold at 12 weeks, dimensions of ROI as defined by red frame are Ø6 × 8 mm. **b** % High-density tissue volume in the ROI before and after implantation

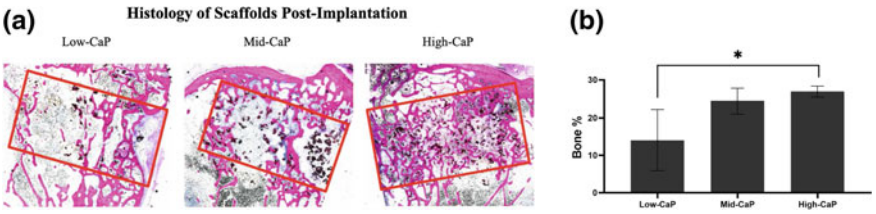

Fig. 3 **a** Representative histology of scaffolds post-implantation. **b** Bone % in ROI after 12 weeks based on histomorphometry

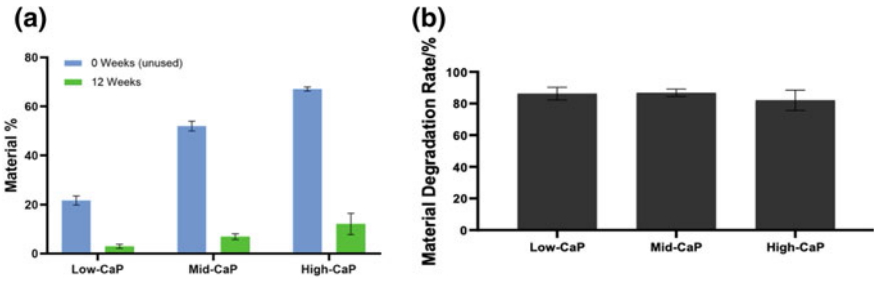

Fig. 4 Material degradation analysis based on histomorphometry. **a** Material % in ROI at 0 and 12 weeks. **b** Material degradation rate after 12 weeks

The percentage of bone present in the ROI at 12 weeks was the lowest for Low-CaP scaffolds (14.0 ± 8.5%), followed by Mid-CaP scaffolds (24.4 ± 3.39%), and was the highest for High-CaP scaffolds (26.9 ± 1.5%) (Fig. 3b). There was a significant difference between Low-CaP and High-CaP scaffolds, with nearly twice the amount of bone in High-CaP scaffolds in comparison to Low-CaP scaffolds ($p < 0.05$). Bone percentages were observed to be inconsistent for Low-CaP scaffolds, with a relatively larger standard deviation value than Mid-CaP and High-CaP scaffolds.

Material % in the ROI clearly decreased from 0 (blue bars) to 12 weeks (green bars) for all three scaffold ratios (Fig. 4a). Surprisingly, the material degradation rates were similar across the three scaffolds (Fig. 4b, $p > 0.05$), illustrating that the influence of CaP/collagen ratio on the degradation rate of CaP was not significant. Overall, CaP/collagen ratio appeared to have little impact on the CaP degradation rate but influenced the amount of bone formation and residual granules.

4 Discussion

The in vitro degradation assays demonstrated that higher CaP/collagen ratios resulted in reduced change in pH. The constant pH observed for the High-CaP scaffold-containing groups indicated that CaP is not the main contributing factor to pH

changes. The pH decrease observed for groups containing Low-CaP and Mid-CaP scaffolds but not High-CaP revealed that collagen was the component of the composite scaffold that caused both the initial increase in pH, and subsequent decrease in pH for groups with collagenase. The initial increase in pH may have occurred due to the presence of residual NaOH used for the neutralisation of collagen during scaffold preparation. The separation of CaP granules from the cylindrical scaffold for Low-CaP group treated with collagenase may indicate the collagen fibrils that held the granules in place have broken down. Therefore, the subsequent decrease in pH in the presence of collagenase is most likely due to the collagen breakdown products' acidic nature. However, literature shows that amino acids that form collagen such as glycine and hydroxyproline are not acidic [9]. The specific causes of pH decrease upon the addition of collagenase hence cannot be definitively explained because the in vitro assay conducted in this study was not designed to investigate so, in addition to differing information found in existing literature. The separation of CaP granules from the cylindrical scaffold could also potentially lead to inadequate mechanical support [4] in the ROI. It is worth noting that though the environment became more acidic, the overall pH continued to remain basic, possibly due to the buffer capabilities of PBS, which mimicked the pH, osmolarity, and ion concentrations of the human body [10].

μ-CT scans suggested that CaP resorption and new bone formation had occurred in the ROI. This could potentially explain the similar percentage of high-density tissue volume between 0 and 12 weeks, respectively, observed for all three scaffold ratios, where CaP granules have been partly degraded and replaced by newly formed bone. Bone percentages post-implantation demonstrate that bone formation was greater in groups containing more CaP. This can be attributed to the osteoconductive properties of CaP, where its molecular interactions with surrounding tissues can cause the formation of an interfacial, apatite layer on the CaP granule surface, leading to the incorporation of biological molecules and attachment of cells to induce bone formation [11]. Bone formation occurring directly around the CaP granules is in line with literature which states that osteoid tissue forms directly on the CaP surface, without intervening soft tissue [12]. Similar material degradation rates across scaffolds highlight that the CaP/collagen ratio did not impact CaP resorption significantly. This may be because CaP degradation appears to be influenced by its solubility and chemical stability, which have been kept constant in this experiment as the same type of CaP was used amongst all three scaffold ratios [13]. The large standard deviation of bone formation in Low-CaP scaffold suggested inconsistencies in in vivo performance, reducing its reliability as a bone graft material.

When viewed together, the in vivo μ-CT scans and histomorphometry evidence suggested that High-CaP scaffolds may be the most successful in promoting defect healing, where defect healing is evaluated based on two criteria: one being the extent to which the material degradation profile matched bone growth rates, and the other being the amount of bone formation. μ-CT analysis revealed that for all three scaffolds, total high-density tissue volume remained relatively unchanged, indicating a CaP degradation profile complementary to bone growth rates, while histomorphometry results indicated greater bone growth at higher CaP/collagen ratios. This

demonstrates that scaffolds with higher CaP/collagen ratios are able to both provide mechanical support during initial stages of bone formation, after which it degrades to provide sufficient space for new bone tissue [14].

One limitation of this project was the lack of scaffolds consisting of pure collagen and pure CaP to serve as controls for the in vitro assay. Including such scaffolds will allow us to confirm that pH change was solely due to collagen and not CaP. Moreover, harvesting some implants over shorter time intervals such as 3 weeks and 6 weeks during the in vivo study could provide information on the direction and speed of bone remodelling processes.

5 Conclusion

Ultimately, the results of our study generally confirm the hypothesis that higher ratios of CaP to collagen better support defect healing in terms of scaffold resorption and bone formation. While the conditions for an 'ideal' bone graft substitute have not yet been established, identifying the impact of CaP/collagen ratio on the biological performance of the biphasic scaffold may prove useful when preparing composite implant materials for future experimentation. Future research may include investigating the effects of varying collagen and CaP types on biological performance, and the influence of additional factors such as bone morphogenetic protein (BMP) and human mesenchymal stem cells (hMSC) in providing osteoinductive properties to the scaffolds.

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Appendix

Preparation of Dyes for Staining of Slides. Chemicals and dyes to be prepared: acidic ethanol, methylene blue, basic fuchsin.

96% Ethanol

1. Using a pipette, measure 96 ml 100% ethanol using a pipette and transfer into a glass bottle.
2. Using a pipette, add 4 ml of water to the glass bottle.
3. Mix well.

Acidic Ethanol

1. Using a pipette, measure 98 ml of 96% ethanol.
2. Add 2 ml of concentrated hydrochloric acid (37%).
3. Mix well and close bottle to prevent the solution from evaporating.

Methylene blue 1% in 0.1 M Borax pH 8.5

1. Using a pipette, measure 160 ml distilled water and add to a beaker.
2. Using an electronic weighing scale, weigh 7.62 g borax and add to water.
3. Stir until borax is completely dissolved.
4. Measure the pH, using a pH metre and adjust the pH to 8.5 with either hydrochloric acid (if overly basic) or sodium hydroxide (if overly acidic).
5. Using a pipette, add distilled water to make up the volume to 200 ml.
6. Using an electronic weighing scale, measure 2.0 g Methylene blue and add to the Borax solution.
7. Stir until the Methylene blue powder is dissolved.
8. Using a funnel and paper filter, filter the solution into a glass bottle.

0.3% Basic fuchsin in distilled water—prepare dye in a fume hood

1. Using a pipette, measure 200 ml distilled water and add to a beaker.
2. Using an electronic weighing scale, weigh 0.6 g basic fuchsin and add to the distilled water.
3. Place the beaker on a heating plate and heat the solution while stirring using a magnetic stirrer until boiling.
4. Let the solution cool down to room temperature.
5. Using a funnel and paper filter, filter the solution into a glass bottle.

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